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Effects of L-Malate on Physical Stamina and Activities of Enzymes Related to the Malate-Aspartate Shuttle in Liver of Mice

J. L. WU[1,3,4], Q. P. WU[1], J. M. HUANG[2], R. CHEN[2], M. CAI[2], J. B. TAN[2]

[1]Guangdong Institute of Microbiology, Guangzhou 510070, [2]Department of Toxicology of the Center for Disease Control and Prevention of Guangdong Province, Guangzhou 510020, [3]South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, [4]Graduate School of the Chinese Academy of Sciences, Beijing 100049, China

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Summary

L-malate, a tricarboxylic acid cycle (TCA) intermediate, plays an important role in transporting NADH from cytosol to mitochondria for energy production and may be involved in the beneficial effects of improving physical stamina. In the present study, we investigated the effects of L-malate on the performance of forced swimming time and blood biochemical parameters related to fatigue – blood urea nitrogen (BUN), glucose (Glc), creatine kinase (CK), total protein (TP) and lactic acid (LA). To investigate the effects of L-malate on the malate-aspartate shuttle and energy metabolism in mice, the activities of enzymes related to the malate-aspartate shuttle were measured. L-malate was orally administered to mice continuously for 30 days using a feeding atraumatic needle. The swimming time was increased by 26.1 % and 28.5 %, respectively, in the 0.210 g/kg and 0.630 g/kg L-malate-treated group compared with the control group. There were no differences in the concentrations of Glc, BUN and TP between the L-malate-treated groups and the control groups. However, the levels of CK were significantly decreased in the L-malate-treated groups. The results predict a potential benefit of L-malate for improving physical stamina and minimizing muscle damage during swimming exercise. The activities of cytosolic and mitochondrial malate dehydrogenase were significantly elevated in the L-malate-treated group compared with the control group. These enzymatic activities may be useful indicators for evaluating changes affecting the malate-aspartate shuttle and energy metabolism in the liver of mice.

Key words

L-malate • Tricarboxylic acid cycle • Malate-aspartate shuttle • Malate dehydrogenase • Aspartate dehydrogenase • Mitochondria

Introduction

L-malate is a tricarboxylic acid cycle (TCA) intermediate. Citrulline L-malate is usually prescribed as an antiasthenic treatment. The antiasthenic effect, widely reported in humans and animals, may be mediated by either citrulline or L-malate or both (Bendahan *et al.* 2002). L-malate may be involved in the beneficial effects

Fax +420 241 062 164
E-mail: physres@biomed.cas.cz http://www.biomed.cas.cz/physiolres

on improving physical stamina. We hypothesized that the antiasthenic effect of L-malate may be due to the changes affecting the malate-aspartate shuttle and energy metabolism.

A weight-loaded swimming test was considered to be a model for evaluating of the extent of behavioral despair and fatigue. Recently, the weight-loaded swimming test has also been used as a reference for measuring the fatigue level objectively and quantitatively (Moriura *et al*. 1996, Tanaka *et al.* 2003, Kim *et al*. 2005). Blood urea nitrogen (BUN), glucose (Glc), creatine kinase (CK), total protein (TP) and lactic acid (LA) are blood biochemical parameters related to fatigue. The BUN test is a routine test used primarily for evaluating renal function. Serum CK is known to be an important indicator of muscle damage (Connor *et al*. 1998, Sotiriadou *et al*. 2003). TP is a rough measure of serum protein. Protein measurements can reflect the nutritional state, kidney disease, liver disease, and many other conditions (Dorchy 2002). As is commonly known, the decrease of Glc provides information about consumption of energy and the increases of BUN, LA and CK indicate a fatigue.

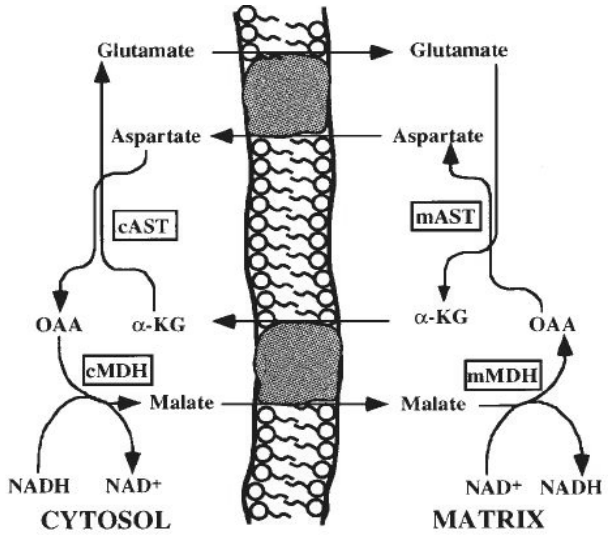

Fig. 1. Schematic illustration of the malate aspartate shuttle. A section of inner mitochondria membrane is shown. Abbreviations: cAST and mAST, cytosolic and mitochondrial aspartate aminotransferase; cMDH and mMDH, cytosolic and mitochondrial malate dehydrogenase; α-KG, α-ketoglutarate.

L-malate is easy to be absorbed and enters mitochondria through the cell membrane and promotes production of energy in mitochondria. It has been reported that oral treatment with citrulline L-malate increases the resistance to fatigue in infected rats with bacterial endotoxins. However, this beneficial effect has been hypothetically linked to nitric oxide synthesis through the production of citrulline (Goubel *et al*. 1997). Citrulline L-malate supplementation promotes a greater contribution of aerobic ATP production. It has been suggested that enhancing malate supplementation may activate ATP production from the tricarboxylic acid cycle through anaplerotic reactions (Bendahan *et al*. 2002). However, this mechanism is still not clear. As is shown in Figure 1 (Scholz *et al*. 2000), L-malate plays a central role in the transfer from cytosolic nicotinamide adenine dinucleotide (NADH) to mitochondrial NADH, a possible proton exchange (Eto *et al*. 1999). Glycolysis and other cytosolic reactions produce NADH and $FADH_2$, which are oxidized by the electron transport chain (ETC) to produce ATP in the mitochondria. Since mitochondria are impermeable to NADH and $FADH_2$, other shuttle systems are necessary for transferring the cytosolic reducing potential into mitochondria. The cytosolic NADH is transferred into the mitochondria through two NADH shuttles, the glycerol phosphate shuttle and the malate-aspartate shuttle (MacDonald 1981, 1982, Hedeskov *et al.* 1987), and activates mitochondrial energy metabolism in the tissues (Eto *et al*. 1999). The malate-aspartate shuttle is the dominant shuttle in liver and cardiac mitochondria (Scholz *et al*. 2000). Activities of some kinds of enzymes in the malate-aspartate shuttle are considered to reflect the metabolic state in the whole body of mammals (Arai *et al*. 1998). We supposed that L-malate could increase the efficiency of the NADH shuttle and energy synthesis in mice by enhancing the activities of enzymes related to the malate-aspartate shuttle.

In this study, we evaluated the antistress and antifatigue effects of L-malate on the stamina and endurance response in mice employing the swimming test. BUN, LA, CK, TP and Glc are parameters related to fatigue. To investigate the effects of L-malate on fatigue, we measured these parameters. Cytosolic malate dehydrogenase (cMDH), mitochondrial malate dehydrogenase (mMDH), cytosolic aspartate aminotransferase (cAST), mitochondrial aspartate aminotransferase (mAST) and lactate dehydrogenase (LDH) are related to the malate-aspartate shuttle and energy metabolism. To investigate the effects of L-malate on the malate-aspartate shuttle and energy metabolism in mice, activities of these enzymes were also measured in the liver from mice supplemented with L-malate.

Methods

Preparation of chemicals

L-malate, enzymes, bovine serum albumin and all substrates were purchased from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Animal preparation

Male mice of NIH strain (SPF grade; 18 to 22 g body weight; n=144) were used in this study. The mice were housed in large spacious cages and were given food and water *ad libitum*. The animal room was well ventilated with a 12 h light: 12 h dark cycle, throughout the experimental period.

Grouping of animals

For the swimming test and determination of biochemical parameters, the mice were divided into three major groups (one for determination of swimming time, one for determination of BUN, CK, TP and Glc and one for determination of LA). Each of these groups was further subdivided into four groups (n=12).

Group I: control mice administered physiological saline alone, Group II: administered L-malate 0.105 g/kg body weight, Group III: administered L-malate 0.210 g/kg body weight, Group IV: administered L-malate 0.630 g/kg body weight administrated *via* intragastric canula for 30 days.

Swimming test

Weight-loaded forced swimming test was used as described previously with some modifications (Moriura *et al.* 1996). The test was induced by forcing animals to swim until exhaustion. The mice were loaded with lead rings that weighed 5 % of their body weight to the tail, and were then placed in the swimming tank filled with fresh water (approximately 30 cm deep water). Water temperature was maintained at 25±0.5 ºC. Exhaustion was determined by observing loss of coordinated movements and failure to return to the surface within 10 s. This 10 s criterion was considered to correlate with exhaustion, and was used as an indication of the maximum swimming capacity of the animal. Mice were removed at this point, before drowning.

Determination of biochemical parameters

In a separate group of mice, the concentrations of blood urea nitrogen, creatine kinase, glucose and total protein were determined by an autoanalyzer (Hitachi 747, Hitachi Japan). The mice were placed in the swimming tank filled with fresh water (approximately 30 cm deep water). Water temperature was maintained at 30±0.5 ºC. After the mice swam freely for 90 min without any load, blood was collected by removing the eyeball from the socket under general anesthesia with a pair of tissue forceps. Blood serum was prepared by centrifugation at 800 x *g* at 4 ºC for 10 min. Animals were sacrificed by rapid neck disarticulation. The liver was excised immediately and immersed in physiological saline and used for further analysis.

In another separate group of mice, the concentration of blood LA was determined by an autoanalyzer (Hitachi 747, Hitachi Japan). The blood samples were withdrawn from orbital sinus by a capillary before swimming exercise, and then the mice were placed in the swimming tank, which contained water (approximately 30 cm deep water). Water temperature was kept at 30±0.5 ºC. When the mice had swum for 90 min, second blood samples were withdrawn from the canthus. The third blood samples were extracted at the time of 20 min after swimming. Blood serum was prepared by centrifugation at 800 x *g* at 4 ºC for 10 min.

Isolation of mitochondria and determination of enzyme activities

Liver mitochondria were isolated by a modification of the technique described by Scholz *et al.* (2000). All isolation steps were performed at 0-4 ºC. Briefly, tissues were minced and homogenized in eight vol (w/v) of MSE solution (220 mM mannitol, 70 mM sucrose, 5 mM potassium Hepes and 2 mM EDTA buffer, pH 7.4). The homogenate was centrifuged at 1200 x *g* for 10 min and the pellet of nuclei and cell debris was discarded. The supernatant fraction was centrifuged at 12 000 x *g* for 10 min. The new supernatant was further centrifuged at 48 000 x *g* for 20 min and the final resulting supernatant was saved as the cytosolic fraction for assay of LDH, cMDH and cAST. The mitochondrial pellet centrifuged at 12 000 x *g* was washed 4 times with MSE solution by centrifugation at 12 000 x *g* for 10 min. A portion of mitochondria was resuspended in 1 ml of 50 mM potassium phosphate buffer (pH 7.2) with 0.5 % Triton X-100. The suspension was incubated for 30 min at 0 ºC and then sonicated for 5 s at 140 W with an ultrasonic processor (VP-5, Taitek, Koshigaya, Japan). The sample was then centrifuged at 48 000 x *g* for 20 min, and the supernatant was used for mitochondrial

enzyme and protein assays. Both cytosolic and mitochondrial fractions were stored at –80 ºC until assayed.

The activities of MDH in the cytosolic and mitochondrial fractions were measured by previous reported methods (Wise *et al.* 1997). The activities of LDH and AST were determined by an autoanalyzer (Hitachi 747, Hitachi, Japan). MDH assays were performed using the spectrophotometric absorbance of solution in cuvettes at 340 nm. Changes of NADH to NAD^+ were used to calculate enzyme activities. All enzymatic activities were determined at 24-26 ºC and expressed as nmol of substrate degraded per min per mg of protein. The protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as the standard.

Statistical analysis

The original data were tested with SPSS software. All the results are presented as means ± standard deviation (S.D.). One-way analysis of variance (ANOVA) was used for statistical analysis, and all tests were considered to be statistically significant at * $P<0.05$ or very significant at ** $P<0.01$.

Results

Swimming tests

The mice loaded with 5 % of their body weight were placed in the water at room temperature (25±0.5 ºC) to swim. The time to exhaustion of the control group and treated groups was 491.31±145.25 s, 557.54±109.70 s, 619.69±141.33 s and 631.38±134.43 s, respectively (Fig. 2). There were no differences in the time to exhaustion between the control group and treated Group II ($P=0.212$). The time to exhaustion of the treatment Group III and Group IV was longer than that of the control group ($p<0.05$). The increase was 26.1 % for 0.210 g/kg body weight group and 28.51 % for 0.630 g/kg body weight group.

Effects of L-malate on blood biochemical parameters

Blood biochemical parameters of control and treated mice are clearly depicted in Table 1. There were no significant differences in the concentrations of BUN, Glc and TP between the groups. The CK activities of the supplementation groups were significantly lower than those of the control groups ($P<0.01$). The serum CK activities were decreased by 31.7 %, 64.2 % and 68.7 %, respectively, in the L-malate supplemented groups compared with the control group.

Effects of L-malate on blood lactic acid levels

The concentrations of blood LA in mice with and without supplementation of L-malate are represented in Table 2. There was no significant change observed in the concentrations of blood LA at the time immediately after swimming. The concentrations of blood LA after 20 min rest were significantly decreased in the 0.210 g/kg body weight group ($p<0.01$) and the 0.630 g/kg body weight group ($p<0.05$). The decrease was 33 % in Group III and 26 % in Group IV compared with the control group.

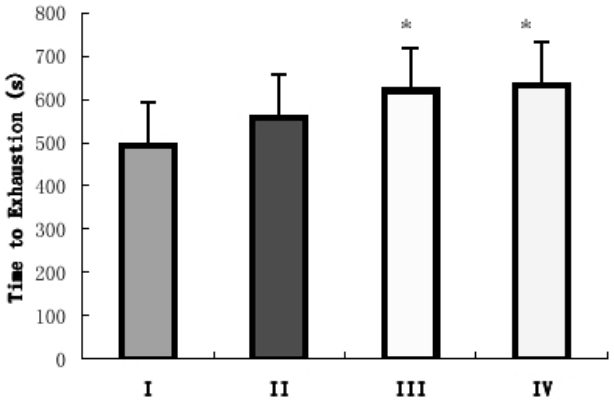

Fig. 2. Time to exhaustion in swimming tests (n=12, respectively). Group I: control mice administered physiological saline alone, Group II: administered L-malate 0.105 g/kg body weight, Group III: administered L-malate 0.210 g/kg body weight, Group IV: administered L-malate 0.630 g/kg body weight. Values are the means ± S.D. *$P<0.05$ indicates significant difference from the control group.

Effects of L-malate on cytosolic and mitochondrial enzyme activities

The activities of the enzymes related to the malate-aspartate shuttle in the liver are shown in Table 3. There were no significant differences between the control group and treated groups in the activities of mAST, whereas the cAST activities in the liver from the control group were significantly lower than those in the liver from the treatment groups ($p<0.01$). The activities of cMDH and mMDH were significantly increased in the latter treated groups ($p<0.01$), while the cytosolic LDH activities in the liver mice were significantly decreased in Group III ($p<0.01$) and Group IV ($p<0.01$).

Table 1. Effects of L-malate on blood biochemical parameters in mice.

	Glucose (mmol/l)	BUN (mmol/l)	TP (g/l)	CK (U/l)
Group I	9.78±1.94	10.54±1.01	58.16±3.17	591.30±184.89
Group II	10.18±2.09	10.20±1.08	59.00±2.72	403.61±110.93**
Group III	10.25±2.14	10.27±0.91	59.15±2.74	211.69±74.65**
Group IV	10.47±2.47	10.28±0.86	57.15±2.31	185.03±62.56**
F	0.213	0.239	1.145	30.71
P	0.887	0.868	0.342	<0.01

Values are the means ± S.D. (n=12, respectively). **P<0.01 indicates very significant difference from the control group.

Table 2. Effects of L-malate on content of blood lactic acid in mice (mmol/l).

	0 min before swimming	0 min after swimming	20 min after swimming
Group I	1.64±0.53	5.03±1.19	1.90±0.57
Group II	1.37±0.31	4.68±1.14	1.53±0.49
Group III	1.45±0.31	4.93±1.20	1.28±0.39*
Group IV	1.59±0.33	4.87±0.98	1.40±0.48*
F	1.275	0.209	3.680
P	0.295	0.889	0.019

Values are the means ± S.D. (n=12, respectively). *P<0.05 indicates significant difference from the control group.

Table 3. Activities of enzymes in liver of mice before and after supplementation of L-malate

	Cytosol			Mitochondria	
	LDH	MDH	AST	MDH	AST
Group I	1501.35±126.69	18.53±2.77	923.93±87.40	15.72±2.38	220.80±39.78
Group II	1475.11±119.82	25.67±2.78**	855.42±66.83**	21.60±2.80**	236.93±40.59
Group III	1325.83±116.10**	50.02±3.30**	786.56±54.07**	24.25±3.53**	241.56±56.30
Group IV	1275.33±108.84**	64.35±6.29**	676.27±34.10**	26.44±3.69**	252.23±68.67
F	7.74	275.30	27.56	21.65	0.614
P	<0.01	<0.01	<0.01	<0.01	0.610

Values are the means ± S.D. (n=10, respectively). **P<0.01 indicates very significant difference from the control group. Each enzyme activity was measured on the same day for all mice. The activities are expressed as nmol/min per mg protein.

Discussion

It is commonly accepted that swimming is an experimental exercise model. It has been demonstrated that swimming can also be used effectively as a stress factor since mice are not normally aquatic animals (Hilakivi-Clarke and Dickson 1995). Thus, it appeared that the mouse swimming model used in this study can be employed to evaluate exercise durability and stress effects. The data of the present study showed that mice treated with L-malate swam for a significantly longer time until exhaustion. This result indicated that L-malate-treated mice adjusted to stressful environments by increasing their stamina and endurance that may be caused by changes of certain energy metabolites in the system. In order to clarify its mechanisms, blood biochemical parameters and activities of enzyme related to malate-aspartate shuttle were firstly investigated in this

study.

Serum CK is widely accepted as an indicator of muscle damage after endurance exercise. Many investigators have used the enzyme to indicate the injury damage of muscles (Clarkson *et al*. 1994, Volfinger *et al*. 1994, Li *et al*. 2005). The main findings of our study were that L-malate supplementation decreased the indicator of muscle damage after swimming exercise. The levels of serum CK were significantly lower in L-malate-treated groups than in the control group. These results indicated that supplementation of L-malate will decrease serum CK after endurance exercise. It was likely that muscle damage in the treated groups, as reflected by the serum CK levels, was minimized by L-malate. It could be considered that this minimizing muscle damage contributes to improving the physical stamina and endurance of mice treated with L-malate. We speculated that these decreased levels of serum CK were due to reduced muscle damage and less leakage of CK occurring during the swimming exercise. It was reported that the CK isozymes contained CK-BB, CK-MB, CK-MM and SMtCK. Different CK isozymes have a specific site of intracellular localization (Dzeja and Terzic 2003). The relative distribution of the four major CK isozymes in the myocyte should be studied to identify the mechanisms of muscle damage and the benefical effects of L-malate. It is supposed that L-malate supplementation increases anabolic processes and decreases catabolic processes of proteins in the muscle. These could lead to a reduction of damage to the proteins associated with membranes that could reduce the amount of disruption of the ultrastructure of the muscle. Therefore, less damage to membrane-bound proteins could enhance membrane integrity and explain the decrease in muscle leakage suggested by our results. In this study, we did not investigate the CK activity in skeletal and cardiac muscles, but we can expect similar changes in these muscles as in the serum.

It has been clearly demonstrated that a 1-h period of exercise in both fed and fasted rats results in increased concentration of malate in the liver and its mitochondria. Because mitochondrial malate is known to facilitate mitochondrial uptake of other carboxylic substrates, the increased mitochondrial malate is responsible for the increased rate of oxidation (Bobyleva-Guarriero and Lardy 1986). This is in agreement with our results showing that the concentrations of blood LA after 20-min rest were significantly decreased in Group III and Group IV compared with the control group. Lactate serves as an energy source in highly oxidative tissues (Dorchy 2002). It can thus be considered that the liver of L-malate-treated group utilized lactate more effectively than those of the control group during the recovery period. This might be a consequence of increased enzyme activities of the malate-aspartate shuttle, and hence mitochondria shuttles and energy metabolism would work effectively for supplementation of L-malate.

The LDH activities can increase in muscle after exercise in rodents and humans (Lawler *et al*. 1993). The LDH activities of rugby players during a summer training camp significantly increased (Mashiko *et al*. 2004). The unexpected outcome of this study was that the LDH activities were decreased by the administration of L-malate. The reaction that converts pyruvate to lactate, catalyzed by LDH, utilizes cytosolic NADH. Therefore, decreased LDH activities appear to reduce the depletion of cytosolic NADH, resulting in more ATP production in mitochondria. These decreases in LDH activities indicate high energy production in the liver of mice.

Among the TCA intermediates, malate shows a significant change during exercise, typically accounting for 350 % of the net increase in total pool size. This increase in TCA intermediates would increase the overall rate of TCA cycle flux by substrate activation of other near equilibrium reactions (Gibala *et al*. 1998). Supplementation of L-malate accelerated the flux rate of tricarboxylic acid cycle that would enhance glycolysis and resulting reactions, including the NADH shuttle systems. Glycolytic NADH is transferred into the mitochondria to be oxidized by ETC for ATP production. The NADH shuttle system is essential for coupling glycolytic metabolism with activation of mitochondrial generation of energy (Matschinsky 1996). As MDH is a rate-limiting enzyme for the malate-aspartate shuttle (MacDonald 1982), an increase in the activity of MDH will result in a higher efficiency of the malate-aspartate shuttle and the production of more ATP in the mitochondria. In our present study, elevations of the cytoslic and mitochondrial MDH activities in liver were shown in mice with supplemented L-malate. These increases in the activities of liver MDH might be concerned with yielding oxaloacetate by dehydrogenating L-malate to increase overall rates of the TCA flux and malate-aspartate shuttle. Therefore, increases in cytosolic and mitochondrial MDH activities are considered to reflect higher energy production in livers of mice. The implications of the difference in the activities of MDH and AST between the cytosolic and mitochondrial

fractions are not clear from this study. The skeletal and cardiac muscles are aerobic tissues and the malate-aspartate shuttle plays an important role in these tissues, so it may be expected that the beneficial effects of malate also concerns the skeletal and cardiac muscles. Further studies on L-malate in these tissues should be performed. Moreover, there are no data in the literature about the potential role of L-malate on the activities of electron transport complexes. The changes of the activities of electron transport complexes and the enzymes associated with the malate-aspartate shuttle systems should be studied at the cellular and molecular levels to further elucidate the exact mechanisms by which L-malate exerts these effects.

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Corresponding author

Qingping Wu, Guangdong Institute of Microbiology, Guangzhou 510070, China. Fax: 0086-20-8768-8131. E-mail: microwjl@yahoo.com