Effects of Chitooligosaccharide Lactate Salt on Sleep Deprivation-Induced Fatigue in Mice

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Chitooligosaccharides (COS), oligosaccharides composed of two to seven glucosamine residues, are known to exhibit various biological activities. In this study, we investigated the effects of COS in an *in vivo* mouse sleep deprivation-induced fatigue model in an effort to develop a functional food with anti-fatigue efficacy. Male Balb/c mice were orally administered 500 mg (kg d)$^{-1}$ of COS lactate or COS HCl for 2 weeks, and severe fatigue was induced by sleep deprivation. To evaluate the extent of fatigue, the swimming time, representing the immobility time, was measured in a forced swim test. As a result, oral intake of COS lactate-manifested anti-fatigue effects could be observed by the attenuation of fatigue-induced body weight loss and shorter immobility period. In addition, COS lactate was shown to alleviate the fatigue-induced increase in cortisol and lipid peroxidation and a decrease in superoxide dismutase (SOD) activity. Of particular note, the oral administration of COS lactate increased the mitochondrial membrane potential and the mitochondrial number significantly, indicating that COS lactate may enhance mitochondrial function. In support of this, COS lactate increased the expression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and cytochrome *c* (Cyt C) mRNA, indicating that it may increase mitochondrial biogenesis. These results suggest that COS lactate can be an effective anti-fatigue functional food, and this anti-fatigue effect may result from, at least in part, the enhancement of mitochondrial biogenesis and the inhibition of free radical generation.

Key words chitooligosaccharide lactate salt; fatigue; sleep-disturbance; mitochondrial function; anti-oxidation

Fatigue is defined as difficulty in initiating or sustaining voluntary activity.[1)] Despite the prevalence of fatigue in developed countries, the pathophysiology and etiology of fatigue are unknown. Several underlying mechanisms of chronic fatigue syndrome (CFS) have been suggested: for example, allergic and psychological factors,[2)] heavy exercise, viral infection,[3)] muscle damage,[4,5)] CNS disorders and immunological factors.[6)] Recent studies have demonstrated that energy metabolism is involved in the pathophysiology of fatigue.[7)] At the biochemical level, fatigue is represented as the depletion of metabolic energy available to the cells that perform a myriad of functions vital to the survival of an organism.[7)] Metabolic energy is generated in the form of ATP through oxidative metabolism in the mitochondria. During energy generation, however, reactive oxygen species (ROS) are produced which have the potential to cause mitochondrial damage. The accumulation of mitochondrial dysfunction induced by ROS is proposed as "the mitochondrial theory of aging."[8)] In addition, in CFS, impairment of mitochondrial function is frequently observed as represented by reduced oxidative metabolism,[9)] increased anaerobic metabolism and subsequently increased lactate production.[10,11)] Mitochondrial abnormalities and degeneration are also frequently found in patients with CFS,[12)] reflecting the crucial role of mitochondria in the development of fatigue syndrome. In line with this, a wide variety of natural health products are used or are being developed to replenish the production of cellular energy in the mitochondria.[13)]

Chitooligosaccharides (COS) is an oligosaccharide made from chitin or chitosan by chemical or enzymatic decomposition.[14,15)] COS has attracted huge attention as a new biomedical material owing to its water-soluble character and versatile therapeutic activities. Those activities include anti-diabetic,[16)] anti-cancer,[17)] anti-bacterial,[18)] anti-fungal,[19)] anti-oxidant,[20)] and anti-mutagenic features.[21)] As a health food, it restores healthy blood pressure, reduces cholesterol,[22,23)] prevents alcoholic liver disease,[24)] and increases immunity.[25)] COS is also used as a health food for the treatment of fatigue. It has been suggested that the anti-fatigue effect of COS is related to its improvement of peripheral circulation, anti-oxidation, immunomodulation and nutritional effects. However, the exact mechanism underlying the anti-fatigue effect of COS remains to be elucidated.

Sleep disturbance causes excessive energy consumption which leads to daytime fatigue and mental enervation.[26,27)] Sleep deprivation increases the levels of the stress hormone cortisol,[28)] and elevated cortisol levels induce the development of depression.[29)] Researchers from the Karolinska Institute recently reported that decreased production of ATP in mitochondria may aggravate depressive disorders through the induction of high levels of somatization (physical symptoms).[30)]

In this study, we investigated the effect of COS in a sleep deprivation-induced fatigue model[31)] in an effort to explore the utility of COS as a novel functional food with anti-fatigue effects, and examined its efficacy in terms of mitochondrial function and antioxidant activities. We discovered that COS lactate markedly decreased the severity of fatigue, as demonstrated by the decreased duration of immobility and oxidative stress and enhanced mitochondrial function. Therefore, we believe this provides an important line of evidence was provided for the potential of COS lactate as a novel functional food for the recuperation of energy and the prevention of fatigue syndrome.

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MATERIALS AND METHODS

Preparation of COS COS lactate and COS HCl were purchased from Biolandkorea Co. (Chungnam, Korea). COS lactate was prepared from chitosan dissolved with 1% lactic acid, and COS HCl was prepared from chitosan dissolved with 1% hydrochloric acid. The COS lactate and COS HCl have a low molecular weight; they contain molecules with a molecular weight of less than 1000.

Animals Model The experiments were performed in accordance with The AmorePacific Institutional Animal Care and Use Committee and the OECD guidelines. Twelve-week-old male BALB/c mice, with an initial weight range of 23—25 g, were obtained from Samtako (Osan, Korea), and were allowed to adapt to their surroundings for at least 1 week before being used in the experiments. The mice were housed in cages under controlled temperature (23±2 °C) and humidity (50±10%), with a 12/12-light–dark cycle. They were also given free access to the laboratory diet (Purina Co., Seongnam, Korea) and ion sterilized tap water.

The mice were weight-matched and divided into the following four groups, with ten mice in each group: normal sleep group, fatigue group (sleep deprivation group fed the vehicle), COS lactate group (sleep deprivation group fed COS lactate), COS HCl group (sleep deprivation group fed COS HCl). The COS lactate or COS HCl was administered orally in a dose of 500 mg/kg once daily for 2 weeks before the induction of fatigue. For the induction of fatigue, mice were placed on a slow rotating disc (150 rpm, Red rotor, Hoefer Pharmacia Biotech, Inc., CA, U.S.A.) for 24 h to prevent sleep.[31] The normal sleep group was concurrently placed on the same apparatus but with the disc locked so that sleep was allowed. The mice were then given 0.6% (w/v) agarose (Cambrex Bio Science Rockland, Inc., Rockland, ME, U.S.A.) instead of food and water for 24 h.

Forced Swim Test (Measurement of the Period of Immobility) The mice were forced to swim individually in a glass jar (25×12×25 cm) containing room temperature water. The water depth was kept at 15 cm through the experiments. After an initial period (2 min) of vigorous activity, each animal assumed a typical immobile posture. The duration of immobility was measured over a total period of 6 min. The mice were judged to be immobile when they ceased any struggling movements of their limbs to keep their head above water. Prolongation of the immobility period was considered a situation similar to fatigue.[32,33] One hour after the forced swim test, blood and muscle tissue samples were collected from the mice.

Preparation of Platelets The blood sample was anticoagulated with acid-citrate–dextrose (ACD, 85 mM sodium citrate, 71 mM citric acid, 111 mM dextrose, pH 6.5), and was centrifuged at 150×g for 10 min in order to achieve platelet-rich plasma. The platelet-rich plasma was centrifuged twice at 300×g for 10 min to isolate the platelets. The platelets of the precipitate were suspended with a Tyrode buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 2 mM KCl, 1 mM $MgCl_2$, 0.3 mM Na_2HPO_4, pH 7.4).

Measurement of the Mitochondrial Membrane Potential ($\Delta\Psi_m$) Fifty microliters of platelets were incubated at 37 °C for 20 min with 2 μg/ml JC1 (Molecular Probes, Invitrogen, Eugene, OR, U.S.A.) and analyzed by flow cytometry using an Epics XL cytometer (Beckman Coulter, Fullerton, CA, U.S.A.).[34] $\Delta\Psi_m$ was determined by the ratio-metric analysis of orange fluorescence emitted by JC1 aggregates (FL2) and that emitted by the free probe (FL1).

Determination of Mitochondrial Mass To measure the mitochondrial mass, 50 μl of platelets were incubated at 37 °C for 30 min with 25 nM MitoTracker Red (Molecular Probes, Invitrogen, Eugene, OR, U.S.A.) and analyzed by means of flow cytometry.

Blood Sample Analyses Blood samples were centrifuged at 1700×g for 10 min and the supernatant was collected. Serum creatinine and lactate dehydrogenase (LDH) were analyzed with a VITALAB Selectra E analyzer (Vital Scientific, Dieren, The Netherlands). The cortisol and blood glucose levels were assayed with a Cortisol ELISA kit (R&D Systems, MN, U.S.A.) and an Accu-Check Active kit (Roche-diagnostics, Seoul, Korea), respectively. Superoxide dismutase (SOD) activity was measured with an SOD Assay Kit-WST (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

Lipid Peroxidation Assay Frozen muscle tissue was thawed and homogenized on ice with the aid of a Physcotron homogenizer (Microtech, Chiba, Japan) in phosphate buffered saline. Subcellular debris was removed by centrifugation at 10000×g for 5 min, and the supernatant was used for the assay. The muscle malondialdehyde (MDA) levels were determined using a NWLSS™ MDA Assay kit (NWLSS, WA, U.S.A.). The protein concentrations were determined with a BCA protein assay kit (Pierce, Rockford, IL, U.S.A.).

Real-Time Quantitative Polymerase Chain Reaction (RT-PCR) Total RNA was extracted from frozen muscle tissue with TRIzol (Gibco-BRL, Invitrogen Corp., Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. The predesigned primers and probe sets specific for peroxisome proliferator-activated receptor coactivator-1α (PGC-1α), cytochrome *c* (Cyt C) and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were obtained from Applied Biosystems (Assay ID: Mm00447181_m1, Mm01621048_s1, and Mm99999915_q1). The reaction mixture was prepared with the aid of a Quantitect Probe PCR kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. The reaction and analysis were performed with a Rotor-Gene 3000 system (Corbett Research, Sydney, Australia). All reactions were performed in triplicate. The amount of mRNA was calculated by means of a comparative CT method.

Statistical Analysis All analyses were performed using the program SPSS 11.0 (SPSS, Chicago, IL, U.S.A.). The data were reported as a mean±standard error mean (S.E.M.) and were analyzed by one-way ANOVA with Dunnett's *post hoc* test. Values of $p<0.05$ were considered significant.

RESULTS

Effect of COS Administration on the Immobility Period in a Forced Swimming Test In an effort to explore the anti-fatigue effect of COS, mice were orally administered the vehicle, COS lactate or COS HCl once daily for 2 weeks and induced to a fatigue state by sleep deprivation for 24 h.[28] To evaluate the extent of fatigue, a forced swimming test was conducted. The reduced locomotor activity and resultant in-

creased immobility time in a forced swimming test reflects the symptoms of fatigue, and the measurement of the immobility time can indirectly quantitate the severity of fatigue of mice.[35,36] Mice fatigued as a result of sleep deprivation showed body weight loss, reduced locomotor activity and increased immobility time ($p<0.05$, Figs. 1, 2). In contrast, the fatigued mice pretreated with COS lactate showed attenuated body weight loss along with significant reduction in the immobility time ($p<0.05$), indicating that COS lactate may prevent the development of fatigue. Meanwhile, there was no statistically significant difference in the COS HCl treated group, indicating that certain discriminating factors might exist between COS lactate and COS HCl.

Effect of COS on Cortisol and LDH Levels Cortisol is usually referred to as a "stress hormone" because it is involved in the response to stress and anxiety. Sustained high cortisol levels can induce the destruction of healthy muscle and bone, and retard healing and normal cell regeneration.[36] The cortisol levels in fatigued mice were elevated significantly compared to normal sleep mice. In contrast, consistent with the reduced immobility time, the oral administration of COS lactate significantly attenuated the increase in cortisol level, while there was no significant decrease accomplished by COS HCl. We also found that the LDH activity was higher in fatigued mice than in normal sleep mice. An increased LDH level indicates the tissue damage, such as muscle and liver, *etc.*, suggesting that high cortisol levels might induce the destruction of muscle. Meanwhile, the oral administration of COS lactate effectively inhibited the increases in LDH levels (Table 1), further supporting the anti-fatigue effect of COS lactate.

Effect of COS on SOD and MDA An increase in MDA reflects lipid peroxidation by free radicals, indirectly indicating oxidative stress-induced cellular membrane damage. Decreased SOD activity indicates that the body's anti-oxidant system was depleted, whereas an increase indicates that the anti-oxidant system is very active and strong enough to effectively remove superoxide anions.[37] As shown in Table 1, fatigued animals have increased MDA and reduced SOD activity as compared to normal animals, indicating that fatigue impairs the body's antioxidant system and increases susceptibility to oxidative stress. In constrast, the pretreatment of COS lactate attenuated these changes significantly, indicating that COS lactate could protect animals from fatigue-induced tissue damage. However, COS HCl did not significantly restore the SOD activity and lipid peroxidation level.

Effect of COS on the Mitochondrial Mass and Membrane Potential Recent research has revealed that patients

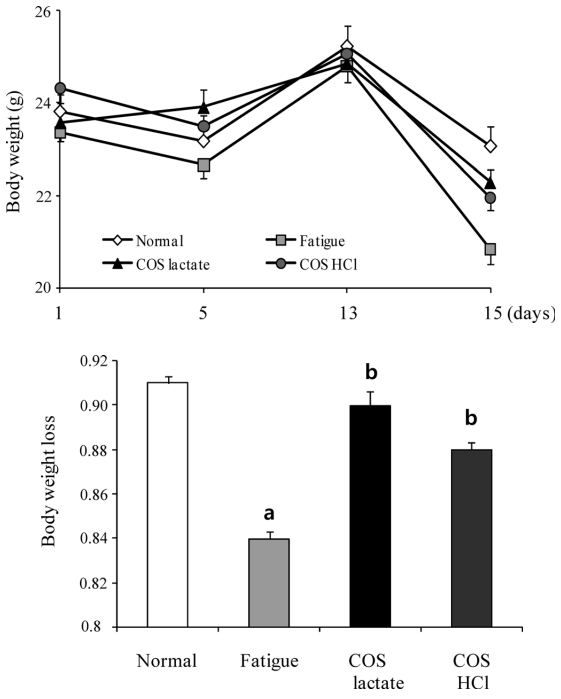

Fig. 1. Effect of COS Administration for 2 Weeks on Body Weight

Male Balb/c mice were orally administered 500 mg $(kg\,d)^{-1}$ of COS lactate, COS HCl or vehicle for 2 weeks and were induced to fatigue by sleep deprivation. (A) Normal sleep group, fatigue (sleep deprivation group fed with vehicle) group and COS administrated groups (sleep deprivation groups fed with either COS lactate or COS HCl) were weighed during a 2-week period. (B) Body weight loss is calculated as follows: (the weight before sleep-deprivation−the weight after sleep-deprivation)/the weight before sleep-deprivation. The values are the mean values±S.E.M. of 10 mice. "a" and "b" indicates significant difference ($p<0.05$) when compared to the normal sleep group and fatigue group (vehicle-treated sleep deprived mice), respectively.

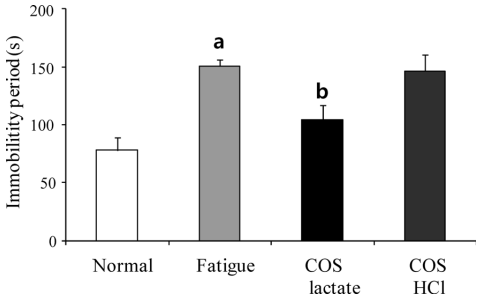

Fig. 2. Effect of COS Administration for 2 Weeks on the Immobility Period

COS lactate, COS HCl or a vehicle was administered for 2 weeks before sleep deprivation. After the mice were subjected to 24 h of sleep deprivation, their immobility period was measured in a forced swim test. The values are the mean values±S.E.M. of 10 mice. "a" and "b" indicates significant difference ($p<0.05$) when compared to normal sleep group and fatigue group (vehicle-treated sleep deprived mice), respectively.

Table 1. Effect of COS Administration on Various Parameters

Parameter	Normal	Fatigue	COS lactate	COS HCl
Cortisol (ng/ml)	6.61±0.60	8.06±0.27[a]	7.02±0.39[b]	7.08±0.69
Creatinine (mg/dl)	0.49±0.01	0.47±0.01	0.49±0.01	0.49±0.01
LDH (IU/l)	704.31±98.85	872.98±42.19	762.69±67.63	744.75±51.17
SOD (inhibition %)	82.05±1.36	72.52±2.21[a]	78.02±1.09[b]	74.52±2.11
MDA (μM/mg protein)	0.56±0.02	1.07±0.09[a]	0.81±0.04[b]	0.82±0.09
Glucose (mmol/l)	82.67±4.22	61.89±5.68[a]	54.60±2.48	57.10±3.40

Normal sleep group, fatigue (sleep deprivation group fed with a vehicle), COS lactate (sleep deprivation group fed with COS lactate), COS HCl (sleep deprivation group fed with COS HCl). The values represent the mean±S.E.M., $n=10$. "a" and "b" indicates significant difference ($p<0.05$) when compared to the normal sleep group and fatigue group (vehicle-treated sleep deprived mice), respectively.

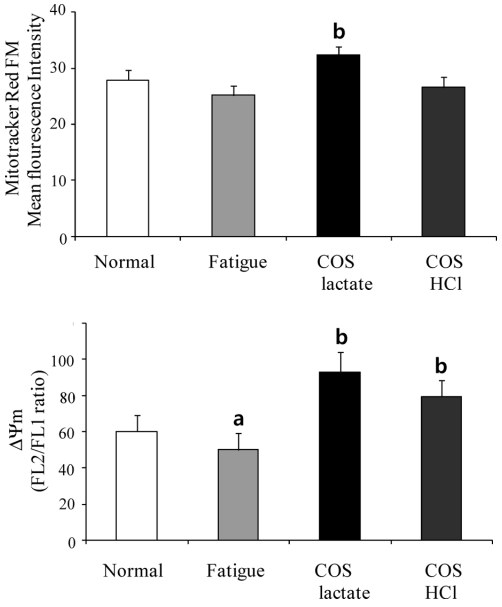

Fig. 3. Effect of COS on the Mitochondrial Mass and Membrane Potential

(A) The mitochondrial mass in the platelets was determined for sleep deprivation mice fed with COS lactate, COS HCl, and vehicle, as well as for normal sleep mice (n=10). This value was determined by flow cytometry with a MitoTracker as indicated in the Materials and Methods. (B) The mitochondrial membrane potential in the platelets was determined for sleep deprivation mice fed with COS lactate, COS HCl, and vehicle, as well as for normal sleep mice. This value was determined using JC1, as indicated in the Materials and Methods. "a" and "b" indicates significant difference (p<0.05) when compared to the normal sleep group and fatigue group (vehicle-treated sleep deprived mice), respectively.

with CFS have a reduced ability for mitochondrial energy production during times of exertion.[38] Fewer mitochondria indicate that maximum energy output cannot be achieved. Accordingly, treatment that restores mitochondrial function or number is considered an effective remedy for fatigue development.[39] Mitochondrial membrane potential ($\Delta\Psi_m$) is an important parameter of mitochondrial function, and is used as an indicator of respiratory rate, ATP synthesis and the generation of ROS.[40] The mitochondria from healthy young cells show a higher $\Delta\Psi_m$ and generate more ATP production than unhealthy old cells.[41] Furthermore, decreased mitochondrial membrane potential has been found in a variety of aging cell types from several mammalian species.[42] Exercise and reduced caloric intake increase mitochondrial number.[43] The increased mitochondrial number increases the number of electron transport chains, thereby reducing the rate of electron entry per electron transport chain. These effects may reduce the production of ROS and hence mitigate cellular damage.[44]

To study the effect of COS on the function of mitochondria, we measured the mitochondrial membrane potential and numbers in platelets. As shown in Fig. 3, the mitochondrial potential and numbers were significantly greater in mice given COS lactate pretreatment than in the vehicle-treated fatigued mice, indicating that COS lactate might produce manifested anti-fatigue through the improvement of mitochondrial function.

Effect of COS on the Expression of Mitochondrial Biogenesis Related Genes To confirm whether COS could induce an actual increase in the number of mitochondria, we examined the expression levels of PGC-1α and Cyt C, which are closely involved in mitochondria biogenesis and viability.[45] As a result, the expression of PGC-1α and Cyt C

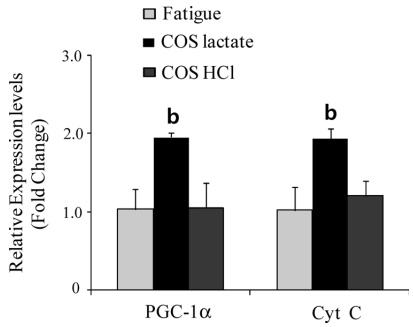

Fig. 4. Effect of COS on the Expression of PGC-1α and Cyt C Gene

Total RNAs were prepared from soleus muscle of the sleep-deprivation mice fed COS lactate, COS HCl, or vehicle (n=7). PGC-1α and Cyt C gene expression were assessed with a real-time RT-PCR. The data are represented as a mean±S.E.M. of the relative fatigue group calculated with GAPDH as standard. "b" indicates significant difference (p<0.05) when compared to the fatigue group (vehicle-treated sleep deprived mice).

was significantly increased in the mice given with COS lactate, but not in mice given COS HCl (Fig. 4), which was well in line with the findings above.

DISCUSSION

In our study, we demonstrated that COS lactate can protect mice from developing fatigue, as shown by reduced body weight loss, increased vigor in forced swimming tests and the alleviation of fatigue-associated biomarker changes. A sleep deprivation-induced fatigue model[28,46] induced severe fatigue signs such as a longer immobility period in a forced swimming test and significant elevation of cortisol levels and LDH activity, which are the typical biomarkers of fatigue.

Meanwhile, the pretreatment with COS lactate for 2 weeks attenuated the fatigue-induced body weight loss and shortened immobility time, demonstrating the anti-fatigue effects of COS lactate. These anti-fatigue effects of COS lactate can be further substantiated by decreased cortisol level and improved mitochondrial function.

Energy deficiency is considered one of the key reasons for fatigue development. Mitochondria are the most important source of cellular energy in our bodies. The decline of energy production with fatigue or aging may be due, in part, to mitochondrial damage by ROS.[47] Therefore, certain natural dietary products and supplements, such as antioxidants, NADH, coenzyme Q10, and vitamin B12, which can reduce oxidative damage and replace high-energy molecules or restore mitochondrial function, have been considered effective remedies for physical and mental fatigue.[13,48—50]

In the present study, COS lactate increased mitochondrial biogenesis and reduced the production of ROS, which was demonstrated by increased SOD activity, reduction of lipid peroxidation, increased mitochondrial membrane potential and the number of mitochondria, along with the increased expression of PGC-1α and Cyt C. Importantly, recent reports showed that calorie restriction or resveratrol upregulated mitochondrial biogenesis suppressed electron stalling, reduced the ROS, and hence conferred an anti-aging effects on cells.[44] Thus, we hypothesize that COS lactate exerts its anti-fatigue effects by upregulating mitochondrial biogenesis, reducing the oxidative damage and hence providing cellular energy. Although the exact molecular mechanism of COS

lactate on mitochondrial biogenesis was not determined, our preliminary experiment showed that COS lactate increased the silent information regulator two ortholog 1 (SIRT1) activity. SIRT1 is the human ortholog of the yeast sir2 protein, one of the most important regulators of life span extension by caloric restriction.[51] It possesses a NAD^+-dependent deacetylase activity. Activated SIRT1 deacetylates, activates several proteins such as PGC-1α and Cyt C, and increases mitochondrial biogenesis.[50] However, further studies are needed to determine whether COS lactate is an authentic SIRT1 activator.

In contrast to COS lactate, COS HCl showed no statistically significant anti-fatigue effects. These differences in activity between COS lactate and COS HCl might be explained in part by the difference in the extent of absorption of COS salt. To be absorbed through the gastrointestinal (GI) tract, COS salts must be a free acid form or non-ionized form and, therefore, the dissociation of salts form substantially affects the absorption through the GI tract. Based on pH partition theory, weak acid drugs have higher lipid solubility at physiological pH values and hence are better absorbed than strong acid drugs. COS lactate is a weak acid, and substantial portion exists in a non-ionized form at physiological pH values, whereas COS HCl is a strong acid and, therefore, exists preferentially in an ionized form. In this context, COS lactate may be better absorbed in the GI tract than COS HCl. Previously, Chen *et al.* showed that among various COS HCl, only chitobiose and chitotriose can be appreciably absorbed from the GI tract, while most COS HCl was not absorbable.[52] In addition, it can be presumed that the effects of COS lactate may result from the combined effect of COS and lactate, rather than COS alone. More studies are required to clarify these possibilities.

In conclusion, this study demonstrated that the oral administration of COS lactate (500 mg/kg) improved fatigue symptoms after sleep deprivation; the anti-fatigue effect of COS lactate might be related, at least in part, to the enhancement of mitochondrial function and anti-oxidation effects. These findings suggest that dietary supplements containing COS lactate might enhance mitochondrial function and facilitate the generation of ATP, providing an effective remedy for fatigue and fatigue-associated energy depletion. As the results described in this paper have been obtained using mice under controlled experimental conditions, the effects of COS on human remain unclear and further studies are necessary.

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