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Influence of *Chlorella* powder intake during swimming stress in mice

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ABSTRACT

We used the forced swimming test to investigate the influence of *Chlorella* powder intake during muscle stress training in mice. After day 14, swimming time was about 2-fold longer for *Chlorella* intake mice than for control swimming mice. Microarray analysis revealed that the global gene expression profile of muscle from the *Chlorella* intake mice was similar to that of muscle from the intact (non-swimming) mice, and the profile of these two groups differed from that of the control (swimming) mice. Gene ontology and pathway analyses of gene expression data showed that oxidoreductase activity and the leukotriene synthesis pathway were repressed in the *Chlorella* intake mice following the swimming test. In addition, measurements of free fatty acids, glucose, triglycerides, and lactic acid in the blood of *Chlorella* intake mice were higher than that of control mice. These findings suggest that metabolism in tissues is altered by *Chlorella* intake.

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1. Introduction

Muscle stress and fatigue cause an inflammatory response in tissues [1]. Activities associated with physical fitness or aerobic exercise cause consumption of glycogen in muscle tissues, exhaustion of creatine, and rapid production of lactic acid [1–4]. During vigorous exercise, glycogen reserves rapidly deplete and cells are damaged. This is followed by the occurrence of immune responses such as enhanced macrophage phagocytosis, activation of natural killer cells, and inducible responses of cytokines [5–7]. Although there is a close relationship between exercise-induced muscle stress and inflammatory response, the molecular mechanism of this relationship remains unknown.

Several supplements have been suggested to relieve physical fatigue. Vitamin complexes and some amino acids effectively repress the upregulation of lactic acid in tissues during vigorous exercise [8,9]. In addition, some foods containing various vitamins and amino acids have been shown to have anti-fatigue effects in the forced swimming test [10].

Chlorella, a unicellular microalga, is made into a powder or extract and is used as a supplement in tablets, drinks, and foods. It has effects on immune function, hypertension, hypercholesterolemia, and diabetes [11,12]. In addition, *Chlorella* induces apoptosis in rats with induced hepatocarcinoma [13].

Here we investigated the effect of *Chlorella* powder intake on inflammatory responses induced by muscle damage during a forced swimming test. In addition, we analyzed global gene expression after *Chlorella* powder intake in order to investigate the molecular mechanism of the effect of *Chlorella*.

2. Materials and methods

2.1. Animals

Six-week-old BALB/c male mice (Charles River Laboratories, Atsugi, Japan) were kept in polysulfone cages under the following conditions: temperature, 23 ± 3 °C; humidity, 55% ± 20%; a 12 h light/dark cycle (light from 8:00 to 20:00); and free access to water and food. Mice weighed 19–21 g at the beginning of the experiment. Three groups of animals (n = 10 for each group) were subjected to the following diets and tests: the intact group was fed a normal diet with no *Chlorella* and not subjected to a swimming test; the control group was fed a normal diet with no *Chlorella* and subjected to a swimming test; and the *Chlorella* intake group was fed a diet with *Chlorella* and subjected to a swimming test.

The intact and control groups were fed only AIN-93G food (Research Diet Inc., New Brunswick, NJ, USA) and the *Chlorella* intake group was fed the same food containing 0.5% *Chlorella* powder. The *Chlorella* powder intake was equivalent to 1 mg kg^{-1} day^{-1}. All animal care and procedures were conducted in accordance with the Animal Protection Law of Japan and Guidelines of Japan on the care and the pain relief of laboratory animals.

2.2. Forced swimming test

We used a water pool with an adjustable current system to evaluate the effect of *Chlorella* powder intake on physical fatigue

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in mice, as described previously [10,14]. Briefly, we used an acrylic plastic pool (Anitec Co., Otsu, Japan) filled with water and measured the maximum swimming time of the mice under the following conditions: flow rate, 6 L/min; average water current, 17–18 cm/s; and water temperature, 34 °C. The mice capable of swimming for the same amount of time in a preliminary swim test were randomly assigned to the three groups.

The animal swimming test was performed on days 0, 7, and 14 after the start of the feeding protocol. A mouse was judged to be immobile when it floated passively in water for 5 s. Each swimming time was calculated relative to the swimming time measured on day 0, which was assumed to be 100%.

2.3. Biochemical tests

Blood was collected from the tail vein immediately after the forced swimming test on day 14, and used, without separation, for assaying the glucose level using hexokinase. Using separated sera, triglyceride (TG) and free fatty acids (FFAs) levels were measured using the Hitachi 7070 Automatic Serum Analyzer (Hitachi Co., Ltd., Japan) and lactic acid (LA) levels were measured by ELISA (BioVision Inc., Mountain View, CA, USA).

The liver and muscle tissue were removed after neutralization with potassium hydroxide proteins by perchloric acid. After centrifugation, the supernatant was used to measure LA using an ELISA kit, and glucose and glycogen were measured by hexokinase methods [15].

2.4. RNA extraction and microarray analysis

After blood sampling, we collected hind limb muscle tissue and divided it into several parts immediately after dissection. Subsequently, a portion of the muscle tissue was placed in RNAlater RNA Stabilization Reagent (Qiagen Inc., Valencia, CA, USA), kept overnight at 4 °C, and subsequently stored at −20 °C. RNA was isolated using an RNeasy Mini Kit (Qiagen) and was purified according to the manufacturer's instructions. RNA purity and concentration were evaluated using a spectrophotometer with an absorbance ratio of 260/280 nm.

The Whole Mouse Genome Oligo Microarray kit (Agilent Technologies, Palo Alto, CA, USA) was used for gene expression analysis. Total RNA from each group was pooled ($n = 10$ for each group) and labeled with Cyanine 3 (Perkin Elmer, Boston, MA, USA) using 500 ng total RNA, according to the manufacturer's instructions, which generated single color-labeled cRNAs. After hybridization, intensities were measured using Agilent Feature Extraction software 9.5 (Agilent Technologies). Data were deposited in NCBI's Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo) under the accession number GSE15798.

2.5. Gene expression data handling

Global normalization was performed for inter-chip normalization. To define the expression level of the gene, the signal intensity of each gene on a chip was divided by the median value of the signal intensities of all genes on the chip. The signal intensity values were filtered using the Agilent Feature Extraction software. In this case, we incorporated the flag denoted as "*p* (present)" (the gene was judged to be greater than the background with no error of height) and subjected the gene set to further analyses. In addition, when two or more Agilent probe IDs corresponded to an Entrez Gene ID, the median value of those probes was defined as the expression value of the gene. Expression data for 13,683 genes were selected by these procedures. Subsequently, gene and sample clustering was performed using multiple experiment viewer (MeV) v.4.1 (The Institute for Genomic Research, Boston, MA, USA) [16].

To investigate gene expression differences and the relevant annotation among the 3 groups, we selected genes that were over- or under-expressed by setting a threshold of 1.5-fold. To summarize the biological aspects of the selected genes, we employed Gene Ontology (GO) terms [17] and pathway analysis, using the mouse gene information data (Mm-Std_20060628.gdb) and mouse pathway data (Mm_Contributed_20070917) provided by the Gene Map Annotator and Pathway Profile (GenMAPP) and MAPPFinder (http://www.genmapp.org), respectively [18]. The MAPPFinder analysis was performed by focusing on the level of expression: an increase (log ratio > 0.5, shown in red) or a decrease (log ratio < 0.5, shown in green).

2.6. Statistical analysis

Statistical significance was determined by one-way ANOVA followed by a Student's *t*-test. Each value in the text is presented as mean ± SE.

3. Results

3.1. Chlorella powder intake and the length of the swimming period

After 14 days, a significant difference was observed among the three groups; the intact mice weighed 22.8 ± 0.2 g, control mice weighed 21.8 ± 0.4 g, and *Chlorella* intake mice weighed 23.5 ± 0.4 g ($p < 0.01$; one-way ANOVA). Student's *t*-test showed that the difference between the intact and control groups was significant ($p < 0.05$), while that between the intact and *Chlorella* intake groups was not significant.

On day 14, the average maximum swimming period of the *Chlorella* intake group was significantly ($p < 0.005$) longer than that of the control group (Fig. 1). Although the swimming period of the *Chlorella* intake group was extended on day 14, no significant differences were observed in the swimming period between the *Chlorella* intake group and the control group before or on day 7. These results suggest that the anti-fatigue effects of *Chlorella* are seen in the animal swimming test after 14 days of its intake. Because the length of the forced swimming period is dependent on fatigue and stress in the tissues, there may have been some differences in the physiology of the muscle tissue between the *Chlorella* intake and control mice.

We measured the relative organ weight of the liver and hind limb muscle in each group after the forced swimming test (Supple-

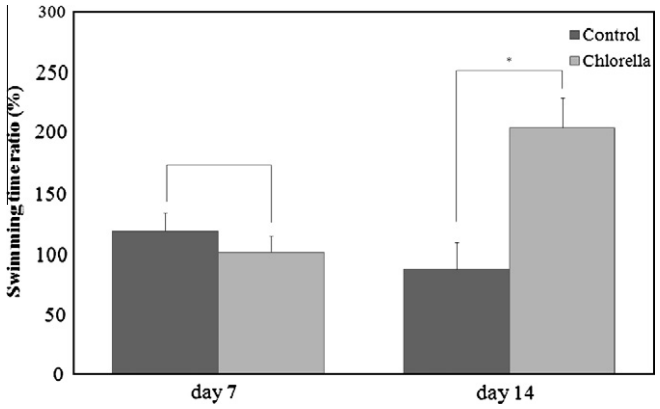

Fig. 1. Change in swimming time ratio after *Chlorella* intake. The maximum swimming time of the *Chlorella* intake group and the control group was measured. At day 14, *Chlorella* intake mice had significantly longer swimming times than control mice. Error bars represent mean ± SE of 10 mice. $^{*}p < 0.005$ by Student's *t*-test.

Table 1
Effects of *Chlorella* for biochemical items in BALB/c mice with forced swimming.

Group	Test sample	Dose (mg/kg/day)	Swimming	No. of animals	LA		Glucose		Glycogen	
					In liver	In muscle	In liver	In muscle	In liver	In muscle
Intact	None	0	None	10	2.30 ± 0.08	2.67 ± 0.04	0.99 ± 0.01	0.90 ± 0.01	1.63 ± 0.18	1.34 ± 0.12
Control	None	0	Forced	10	3.50 ± 0.04	3.54 ± 0.04	0.97 ± 0.01	0.91 ± 0.02	0.63 ± 0.12	1.13 ± 0.07
Chlorella	*Chlorella*	1000	Forced	10	3.05 ± 0.04	3.21 ± 0.05	1.00 ± 0.01	0.92 ± 0.01	2.15 ± 0.49	0.88 ± 0.08
p-value					$$	$$	$		$$	$$

LA: nmol/g tissue; Glucose and Glycogen: ng/dL.
LA, Glucose, and Glycogen were measured in liver and muscle.
Each value represents mean ± SE.
$$p < 0.05$$, $$$p < 0.01$$, significantly different by one-way ANOVA.

mentary Table 1). Notably, there were significant differences ($p < 0.01$), with the weight of the hind limb muscle of the *Chlorella* intake mice being significantly heavier than that of the intact mice and slightly heavier than that of the control mice. These results suggest that intake of the *Chlorella* powder by these mice helped induce greater strength and increased the thickness of their muscles.

3.2. Effectiveness of metabolism in Chlorella intake mice

To investigate stress in the liver and muscle, the concentration of LA, glucose, and glycogen was determined as a measure of the anti-fatigue effectiveness of sugar metabolism in the tissue after the animal swimming test. The concentration of glycogen in the liver of control mice was significantly lower ($p < 0.005$; Student's *t*-test. Table 1) compared with that in intact mice. Although the glucose concentration remained unchanged in the muscle tissue among the groups, the glycogen concentration was slightly lower in the muscle tissue of control and *Chlorella* intake mice compared with the intact mice. Moreover, the LA concentration in both liver and muscle was significantly different among the groups with the control mice having higher LA levels compared with both the intact and *Chlorella* intake mice ($p < 0.01$ for both liver and muscle). These findings suggest that fatigue was severe in both the muscle and the liver. However, the *Chlorella* intake mice appeared to have a similar metabolism as the intact mice. These results show that the energy stores were depleted because of the high energy consumption in the liver tissue and loss of energy that was stored in the muscles of the control mice.

The effects of *Chlorella* intake were also evaluated by studying the blood chemistry of the animals. The blood glucose concentration in the *Chlorella* intake mice was significantly increased compared with that in the control mice (Table 2). This difference may have been due to stress resulting from the increase in demand for energy because of the extended swimming period of the *Chlorella* intake mice. Both TG and FFA concentrations in the blood were significantly higher in the control mice compared with the intact mice (Table 2). Notably, although TG was lower in the *Chlorella* intake mice compared with the control mice, FFA concentrations were higher. These results suggest that *Chlorella* intake increases FFA secretion by white adipose tissue but does not increase TG intake (i.e., an FFA increase in the blood indicates TG consumption).

Table 2
Effects of *Chlorella* for blood chemistry in BALB/c mice with forced swimming.

Group	Test sample	Dose (mg/kg/day)	Swimming	No. of animals	Glucose (mg/dL)	TG (mg/dL)	FFA (mEq/L)	LA (mmol/L)
Intact	None	0	None	10	114.80 ± 3.25	74.30 ± 8.53	384.20 ± 25.29	7.24 ± 0.17
Control	None	0	Forced	10	104.40 ± 19.02	170.50 ± 20.73	637.00 ± 106.96	9.68 ± 0.05
Chlorella	*Chlorella*	1000	Forced	10	194.30 ± 25.44	86.20 ± 9.55	809.40 ± 48.50	9.01 ± 0.13
p-value					$	$$	$$	$$

Glucose, TG, FFA, and LA were measured in blood.
All blood samples were collected from postcava without fasting.
Each value represents mean ± SE.
$$p < 0.05$$, $$$p < 0.01$$, significantly different by one-way ANOVA.

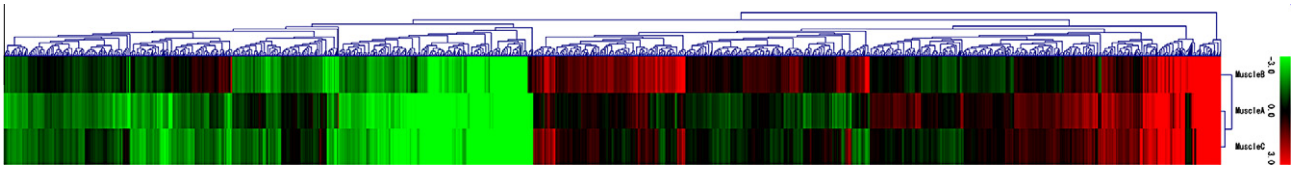

Fig. 2. Hierarchical clustering of 1759 genes with differences between intact and control mice. The three groups (rows) and 1759 genes (columns) are clustered by Euclidean Distance (linkage method is average linkage). Expression levels are depicted as above (in red) or below (in green) the mean. The control and *Chlorella* intake groups were grouped to the same cluster. The MuscleA row shows gene expression levels from the intact group, the MuscleB row shows gene expression levels from the control group, and the MuscleC row shows gene expression levels from the *Chlorella* intake group.

The LA concentration in the blood exhibited a similar pattern as in the liver and muscle tissue. Compared with the intact mice, the LA concentration was higher in the control and *Chlorella* intake mice, but the *Chlorella* intake mice had slightly lower levels compared with the control mice (Table 2).

3.3. Gene expression analysis of muscle tissues

In the swimming test, on day 14, the *chlorella* intake mice could swim for twice as long as the control mice. This may have been due to the differences in the metabolism of the muscle tissue of these mice. To investigate the effect of *Chlorella* intake on gene expression, a microarray analysis was performed on muscle tissue.

A cluster analysis of global gene expression showed that the *Chlorella* intake mice had similar levels of gene expression as the intact mice, but these groups differed from the control mice (Fig. 2). Of 13,683 expressed genes, 1759 were identified as being differently expressed between the intact and control mice. Of these differently expressed genes, 1135 genes were upregulated and 624 genes were downregulated in the control mice. Of these

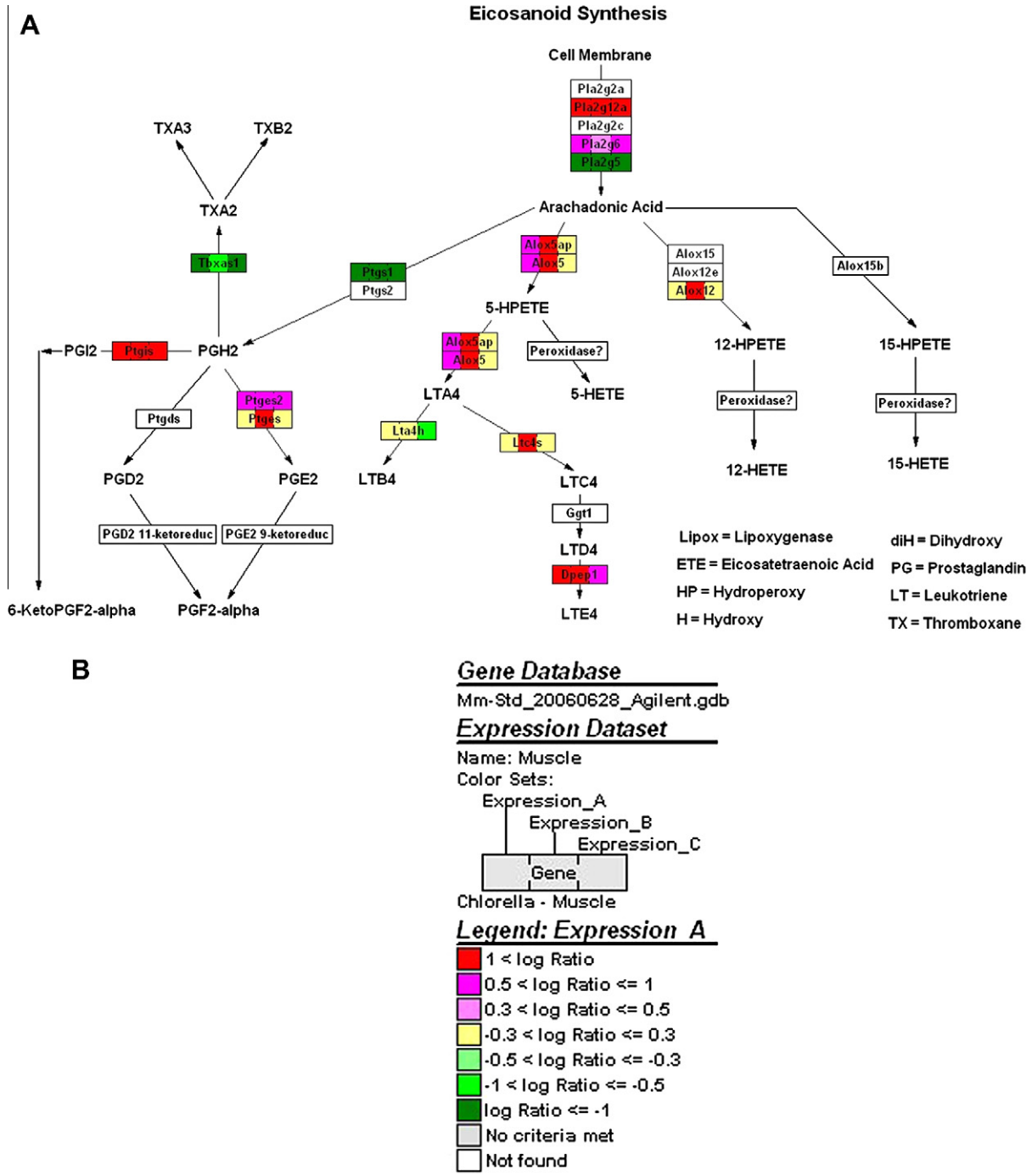

Fig. 3. Illustration of GenMAPP pathways. (A) GenMAPP pathways of gene expression in the Eicosanoid Synthesis pathway. (B) Colored histogram showing different levels of gene expression. The individual expression levels of each gene from three microarray analyses and their median expression levels (the extreme right box in histogram) in a given pathway are indicated by a multiple color histogram. The leukotriene synthesis pathway was repressed in *Chlorella* intake mice.

upregulated genes, we identified 774 genes that were downregulated in the *Chlorella* intake mice. In addition, of the 624 differently expressed downregulated genes, 220 genes were upregulated in the *Chlorella* intake mice. Therefore, the intake of *Chlorella* influenced these expression patterns. GO term analysis [17,19] was performed for these genes (corrected $p < 0.05$, Supplementary Tables 2 and 3). For the 774 genes, GO term analysis results indicated that the "immune response"- and "cell cycle"-related terms were identified as biological processes significantly associated with *Chlorella* intake, "oxidoreductase activity" was identified as a molecular function term, and "chromosome part" was identified as a cellular component term (Supplementary Table 2). For the 220 genes, GO term analysis results indicated that the "blood vessel"-related and "angiogenesis" terms were identified as biological processes significantly associated with *Chlorella* intake, "transcription regulator activity" was identified as a molecular function term, and "sarcomere" was identified as a cellular component term (Supplementary Table 3).

The upregulation of "immune response," "lipid metabolic process," and "oxidoreductase activity" due to swimming stress could be repressed by the intake of *Chlorella*, resulting in a profile that is similar to that of the intact mice (Supplementary Tables 4–6). On the other hand, "angiogenesis activities" were downregulated in the control mice and recovered in the *Chlorella* intake mice (Supplementary Table 7). We identified a similar pattern in the gene regulatory network in muscle tissues between the intact and *Chlorella* intake mice during swimming. These results indicate that the effects on gene expression during severe exercise were regulated by the intake of *Chlorella*.

Subsequently, to identify the effect of *Chlorella* intake compared with control mice, we performed a pathway analysis of these microarray data using GenMAPP software [18]. Among various pathways, 12 pathways were identified as being more than 2-fold different in the average levels of gene expression in control mice compared with the median of all samples (Supplementary Table 8). In particular, we found that the Eicosanoid Synthesis (leukotriene synthesis) pathway, which is an inflammatory response, was significantly increased ($p < 0.002$) by Student's *t*-test in control mice compared with the intact and *Chlorella* intake mice (Fig. 3, Supplementary Table 9). Leukotrienes are important mediators in the inflammatory response. Leukotriene C4 affects bronchial and vascular smooth muscle via the cysteinyl leukotriene (CysLT) 1 and CysLT2 receptors that are present on the cell surface of mast cells and eosinophil granulocytes [20]. They act to recruit leukocytes to sites of inflammation. Consequently, it may be possible that *Chlorella* intake affected the anti-inflammatory response in those mice that consumed *Chlorella*.

4. Discussion

Gene network analysis by global expression profiling is a powerful tool for studying the mechanism of physical fatigue at a molecular level. We found significant effects of *Chlorella* intake on the forced swimming test in mice. Pathway analysis showed that *Chlorella* affected the leukotriene synthesis pathway, which is involved in asthmatic and allergic reactions and acts to sustain inflammatory reactions. In this pathway, CysLT C4 synthesis, which has been implicated as a mediator of anaphylaxis and inflammatory conditions such as bronchial asthma [21,22], may respond to the intake of *Chlorella*. GO term analysis of the differences in gene expression also indicated that oxidoreductase activity, such as that of the cytochrome family and hydroxysteroid dehydrogenase family, was upregulated in the control mice compared with the intact mice and was downregulated in the *Chlorella* intake mice. Although muscle damage usually causes increased oxidoreductase activity [23,24], this action would be repressed by the effect of *Chlorella* intake on the immune system.

The physical fatigue increases not only the inflammatory response but also the stress response because of the levels of swimming time required in this experiment. Thus, cytokines may be activated and appear to be upregulated in the leukotriene synthesis pathway and by oxidoreductase activity. *Chlorella* intake repressed those activities at the gene expression level, and therefore, they might be more affected with the longer required swimming times in the forced swimming test.

It is important to identify the mechanism by which *Chlorella* intake maintains the inflammatory response. It was reported that the intake of *Chlorella* can improve anti-oxidative capacities in oxidatively-stressed tissue [25]. In that study, *Chlorella* intake affected the generation of serum and liver superoxide radicals after 10 weeks. However, this may have been an indirect effect because of the time needed to show an effect to intake. *Chlorella* is a single-cell alga that contains approximately 60% protein, chlorophyll, dietary fiber, various amino acids, minerals, and vitamins. Two polysaccharides with major components contributing to immunity, rhamnose and mannose, were isolated from liquid extracts of *Chlorella pyrenoidosa* [26]. The polysaccharides could affect the intestine and result in an immune response [27]; however, the detailed mechanism of this remains to be elucidated.

In the forced swimming test, the upregulation of cytokine activities indicated stress on various tissues [28]. When swimming causes such stress, the autonomic nervous system may shift from the parasympathetic to the sympathetic nervous system, and consequently, the production and secretion of adrenalin would be increased [29–32]. In addition, the level of glucose in the serum would be high because of the upregulation of glucagon in spleen α-cells. Because the mice experienced both physical and mental fatigue as a result of forced swimming, the level of glucose was a result of the balance of those factors. The amount of glycogen, which reflects the source of the energy, would be more suitable as a marker of physical fatigue.

In general, glycogen is consumed in both muscle and liver during physical fatigue. Our results showed that the glycogen of liver was higher in *Chlorella* intake mice than in control mice, but there were similar levels of glycogen in the muscle of both *Chlorella* intake and control mice. In addition, the glucose level in the blood was higher in the *Chlorella* intake mice than in the control mice. These results indicate that exercise causes a reduction of glycogen in liver tissues but *Chlorella* intake preserves these stores. On the other hand, TG levels in the blood were degraded in *Chlorella* intake mice. These results indicate that while the glycogen stores were conserved in the liver by *Chlorella* intake, the good performance during the swimming test was due to the energy supplied by fatty acid degradation in the *Chlorella* intake mice.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.078.

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