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Chlorella, glucose uptake in the soleus was enhanced in normal mice ($p < 0.01$) and restored in STZ mice compared to their respective controls ($p < 0.01$).

Non-esterified fatty acid (NEFA) levels in normal and STZ mice

Fast serum NEFA levels in normal mice were not significantly affected by *Chlorella* treatment (see Fig. 3). Glibenclamide treatment produced a slightly but not significantly higher level in normal mice. However, NEFA levels were significantly higher in H₂O-treated STZ mice ($p < 0.01$). This elevated phenomenon was ameliorated after *Chlorella* treatment in STZ mice ($p < 0.005$).

Discussion

Previous studies have found that *Chlorella* alleviated hyperglycemic status in STZ mice (Cherng and Shih, 2005b) and in alloxan-induced diabetic rats (Rodriguez-Lopez and Lopez-Quijada, 1971). This is the first time the hypoglycemic mechanisms of *Chlorella* have been investigated.

Since no previous investigators have suggested any other possible mechanisms for *Chlorella*, the next question is how *Chlorella* works to produce its effects in hyperglycemic STZ mice. Blood glucose can be utilized by the liver and skeletal muscles to synthesize glycogen or to store in adipose tissue as triglycerides through the process of lipogenesis. Increased lipogenesis in brown adipose tissue can be represented as an increased fuel for thermogenesis, whereas the increase in white adipose tissue will tend to increase adiposity. Insulin-stimulated lipogenic rates in either brown adipose tissue or white adipose tissue of normal or STZ mice were not affected significantly by *Chlorella* treatment compared to their respective controls at the time they were killed for lipid extraction. Thus, acute changes in lipogenic rates in adipose tissues cannot account for the hypoglycemic effects of *Chlorella* observed in STZ mice. Chronic *Chlorella* administration did not increase body mass significantly in high fat diet-fed rats (Shih and Shih, 2002; Cherng and Shih, 2005a), therefore, lipogenic rate in white adipose tissues may not be induced after long-term administration of *Chlorella*. Since one of the side effects of insulin and sulphonylureas after long-term treatment is to increase body weight and exacerbate insulin resistance in diabetes, thus *Chlorella* may provide a preferable alternative for the control of blood glucose in diabetes.

Although the hypoglycemic effects of *Chlorella* are not due to an enhancement of lipogenesis in adipose tissues, its actions may be through increased glucose uptake. Glucose uptake is increased after *Chlorella* administration in normal mice (Fig. 2a). This may be the explanation for positive glucose tolerance test in normal mice observed in the previous study (Cherng and Shih, 2005b). Glucose uptake in the liver is lower in H₂O-treated STZ mice, which may account for the hyperglycemic state of diabetic mice. Impaired insulin action on the liver results in impaired modulation of glucose production and output, which

accounts for fasting hyperglycemia and contributes to postprandial hyperglycemia (Del Prato and Tiengo, 2001). Glucose uptakes showed no difference in soleus muscles between normal and STZ mice, however, the uptakes were enhanced after *Chlorella* administration. Therefore, increasing glucose uptake in the liver and soleus muscles after *Chlorella* treatment may provide part of the explanation for its ameliorating action in hyperglycemia.

Enhancement of the hypoglycemic effects of exogenous insulin by *Chlorella* treatment (Cherng and Shih, 2005b) may be also mediated by the lowering of fasting serum non-esterified fatty acid levels, given that metabolic signals are crucial for insulin secretion and fatty acids are a major part of B-cell fuel consumption (Berne, 1975; Malaisse et al., 1985). In support of this view, it has been shown that long-term (6–24 h) exposure to elevated fatty acids produces a time-dependent inhibition of glucose-induced insulin secretion (Grill and Ovigstad, 2000). Therefore, elevated portal free fatty acids might account for overproduction of liver glucose output with visceral adiposity. In addition, elevated plasma NEFA concentration has been increasingly recognized as a systemic mediator of insulin resistance in type 2 diabetes (Roden et al., 1996; Boden et al., 1998; McGarry, 1992). Excess plasma NEFA can inhibit insulin-stimulated glucose utilization in muscle (Roden et al., 1996; McGarry, 1992) and promote hepatic production of glucose (Roden et al., 1996; Boden et al., 1998) and VLDL triglyceride (TG) (Byrne et al., 1991; Laws, 1996). Whereas, reduction of plasma NEFA concentration improves glucose utilization (Reaven et al., 1988; Balasse and Neef, 1973), enhances the suppression of hepatic glucose production by insulin (Saloranta et al., 1991), and reduces hyperinsulinemia in patients with type 2 diabetes (Boden et al., 1998). Our data showed that glucose uptakes in the liver of H₂O-treated STZ mice were much lower than that in normal controls. This is supposed by recent study in which acute elevation of free fatty acids impairs hepatic glucose uptake subsequently inducing insulin resistance (Nakahara et al., 2004). *Chlorella* administration restores the suppressed glucose uptake, therefore the non-esterified fatty acid lowering effect of *Chlorella* may provide an additional benefit in preventing diabetes-associated complications other than its hypoglycemic effects in diabetes. Since the care of diabetes is long term, our future work will investigate the chronic effects of *Chlorella* on blood glucose homeostasis and lipid metabolism in STZ mice.

Insulin resistance has been related to high levels of Interleukin (IL)-2, which is the first step in the cascade of events leading to insulin resistance (Penttinen, 1995). Obesity and insulin resistance are also associated with adipose tissue secretion of IL-6 and tumor necrosis factor (TNF)- α (Kern et al., 2001). TNF- α is expressed not only in adipose tissue but also in human muscle, which is the major site of insulin-mediated glucose uptake. The degree of TNF- α expression is higher in muscle tissue from insulin-resistant and diabetic subjects than from normal subjects (Saghizadeh et al., 1996). TNF- α has also been shown to impair insulin action in peripheral tissues (Lang et al., 1992). In an earlier section we have mentioned that *Chlorella* has been shown to increase

immune function (Halperin et al., 2003; Pugh et al., 2001; Tanaka et al., 1984; Singh et al., 1998). This effect of *Chlorella* may be due to an increase in certain cytokines, e.g. Interferon (INF)- γ , IL-2, IL-12, IL- β and TNF- α , in infected animals but not in normal controls (Hasegawa et al., 1997; Queiroz et al., 2002). *Chlorella*-derived Polysaccharides were shown to increase mRNA expression of IL-1 β and to activate monocytes in vitro (Pugh et al., 2001). Although increases of cytokines are known to be detrimental to insulin sensitivity, *Chlorella* seems to increase these cytokines in particular types of cells, e.g. leukocytes. Our results show that *Chlorella* improved insulin sensitivity (Cherng and Shih, 2005b) and this is possibly due to the modification of glucose uptake in the liver (Fig. 2a), in the muscle (Fig. 2b), and NEFA levels (see Fig. 3). Increased cytokine levels are not always related to impaired insulin effects, for example; short term infusion of IL-6 does not induce insulin resistance in vivo (Sopasakis et al., 2004) nor does a pharmacological TNF- α blocker affect insulin sensitivity (Ofei et al., 1996; Rocco et al., 2004). In addition, higher TNF- α expression in diabetic subjects is not always related to higher TNF- α release compared to normal subjects (Blüher et al., 2001; Febbraio et al., 2003). Therefore, affecting cytokine production by *Chlorella* in infected animals, or by its derivatives in monocytes, does not directly imply that *Chlorella* will cause insulin resistance in long-term use. Since *Chlorella* improves insulin sensitivity and enhances the release of proinflammatory cytokines from inflammatory cells, it would be interesting to investigate, in future work, whether *Chlorella* administration can modify cytokine production from leukocytes as well as other cells, e.g. adipocytes, in hyperglycemic animals.

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Materials and methods

Chlorella material

Commercially available spray-dried preparations of *Chlorella* paranooids cultured in the outdoor cultivation pool at GONG BIH Enterprise Co., Ltd (Doo-Liu City, Taiwan) were suspended in distilled water prior to use. The quality of *Chlorella* powder is described and provided by the company.

Animals

Male ICR mice were purchased from the National Science Council Animal Center in Taiwan. The number of experimental animals per group was kept to a minimum and they were used only once. Mice ($n=8-10$, age 3 weeks) received 60 mg/kg (i.p.) of Streptozocin (STZ) in 10 mM, pH 4.8 citrate buffer (Dresner et al., 1997), and designated as STZ mice, or buffer only as control mice.

All animals were fed with Lab rodent diet (containing 64% carbohydrate, 23% protein 4.5% fat, and 6 % fiber (LabDiet, Nutrition International Inc. USA) and provided ad libitum access to tap water. Mice were used at age between 6 and 8 weeks. Their housing was maintained at a temperature of 20–22 °C, relative humidity of 50–80%, and a 12 h light/dark cycle of 07.00 to 19.00 h with no twilight. All animals were anaesthetized briefly prior to being killed. All experimental procedures followed the principle of laboratory animal care and were carried out according to a protocol approved by the local animal ethics committee.

Drugs

Glibenclamide (G 0639, Sigma) was dissolved in distilled water to give a dose volume of 0.1 ml/10 g body weight orally. The insulin used was human Actrapid (Novo Nordisk 100 IU/ml). *Chlorella* powder was provided by GONG BIH Enterprise Co., Ltd (Taiwan) and prepared as suspension. Control mice received the equivalent volume of distilled water (oral) or saline (i.p.). Glibenclamide and *Chlorella* suspension were given orally 60 min prior to assessments. Mice were lightly anesthetized with diethyl ether prior to drug administration by oral gavage. The test dose for glibenclamide (as positive control) was 2.5 mg/kg according to previous work (Williams et al., 1999a; Cherng and Shih, 2005b) and insulin was 2.5 IU/kg (Williams et al., 1999b; Cherng and Shih, 2005b).

Adipose tissue lipogenic rate measurement

Fatty acid synthesis was measured in vivo by following the incorporation of [^3H]- H_2O into adipose tissue fatty acids according to the previously described procedure (Williams et al., 1999b). Briefly, fed mice were given *Chlorella* or water 1 h prior to the dose of insulin (2.5 IU/

kg body weight i.p.) and 30 min later received 0.5 μCi [^3H]- H_2O i.p. (Amersham Life Science). Animals were killed 60 min after receiving the tritiated water and blood samples were collected into heparinized capillary tubes. Blood was centrifuged and plasma prepared. The interscapular brown adipose tissue and a sample of epididymal white adipose tissue (<500 mg) were cleaned, weighed and the lipid extracted into petroleum ether (Stansbie et al., 1976) then dried and the residue weighed. Total extracted lipids and triplicated samples of 10/ μl plasma were solubilized in Emulsifier-safe LSC cocktail (Packard, Groningen) prior to scintillation counting. Lipogenic rate was calculated as microgram H incorporated per hour per milligram wet tissue.

Glucose uptake assay

Glucose uptake was estimated in vivo by measuring the incorporation of 2-deoxy-D-[1,2- ^3H] glucose (Amersham Life Science) into glycogen of the liver and soleus muscles (Meszaros et al., 1987). Following an overnight fast, *Chlorella* or water was administered by oral gavage 60 min prior to a bolus injection of 3 μCi of 2-deoxy-D-[1,2- ^3H] glucose/kg body weight. Animals were killed 40 min after receiving the 2-deoxy-D-[1,2- ^3H] glucose. Blood was centrifuged and plasma prepared. Desired tissues were dissected, cleaned, and weighed. After that, the tissues were immersed immediately into ice cold 0.5 M perchloric acid, homogenized, and centrifuged at 3000 $\times g$ for 15 min. The supernatant was neutralized with 5 M KOH and assayed for total radioactivity. The free phosphorylated 2-deoxy-D-[1,2- ^3H] glucose metabolite radioactivity was prepared by treating the neutralized supernatant with Somogyi reagent. The phosphorylated metabolite concentration of the tissues was calculated as the difference between the total radioactivity of the neutral extract and the radioactivity remaining after treatment with Somogyi reagent.

Non-esterified fatty acid (NEFA) assay

Following an overnight fast, *Chlorella* or water was administered by oral gavage 60 min prior to blood sampling. Serum was prepared for assay, using commercial assay kits (Randox Laboratories Ltd. USA). Briefly, the non-esterified fatty acids are determined after enzymatic synthesis and oxidation with Acyl CoA synthase and Acyl CoA oxidase, respectively. The end reaction purple adduct (measured at 550 nm) is formed from hydrogen-peroxide (H_2O_2), *N*-ethyl-*N*-(2-hydroxy-3-sulphopropyl) *m*-toluidine and 4-aminoantipyrine under the catalytic influence of peroxidase.

Statistical analysis

Data from each group of 8 mice were combined from at least two different experimental days. A two-tailed student's unpaired test was used to compare the mean values of two populations of continuous data, which were part of a normal distribution.

Results

Lipogenic rate in normal and STZ mice

Chlorella had been found to enhance the hypoglycemic effects of exogenous insulin in previous study (Cherng and Shih, 2005b). Therefore it was considered appropriate to investigate whether insulin-stimulated lipogenic rates were also increased after *Chlorella* treatment. Insulin-stimulated lipogenic rate in brown adipose tissue showed no difference between normal and H_2O -treated STZ mice (Fig. 1a). Nor did *Chlorella* administration alter the rate in the brown adipose tissue in either normal or STZ mice. Although lipogenic rate was slightly, but not significantly, higher in white adipose tissue in normal mice, there were no changes between H_2O -treated and *Chlorella*-treated STZ mice after *Chlorella* treatment (Fig. 2b).

Glucose uptake in normal and STZ mice

Glucose uptake in the liver was significantly lower in H_2O -treated STZ mice compared to the normal controls ($p < 0.05$). The uptake was increased after *Chlorella*

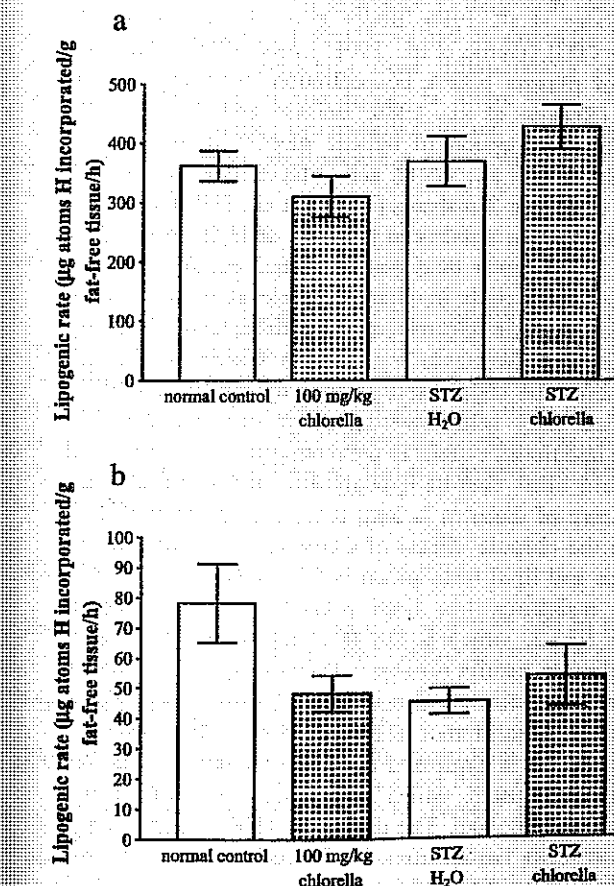


Fig. 1. Effects of *Chlorella* on lipogenic synthesis in brown (a) and white (b) adipose tissues in STZ mice. Mice received H_2O or 100 mg/kg of *Chlorella*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

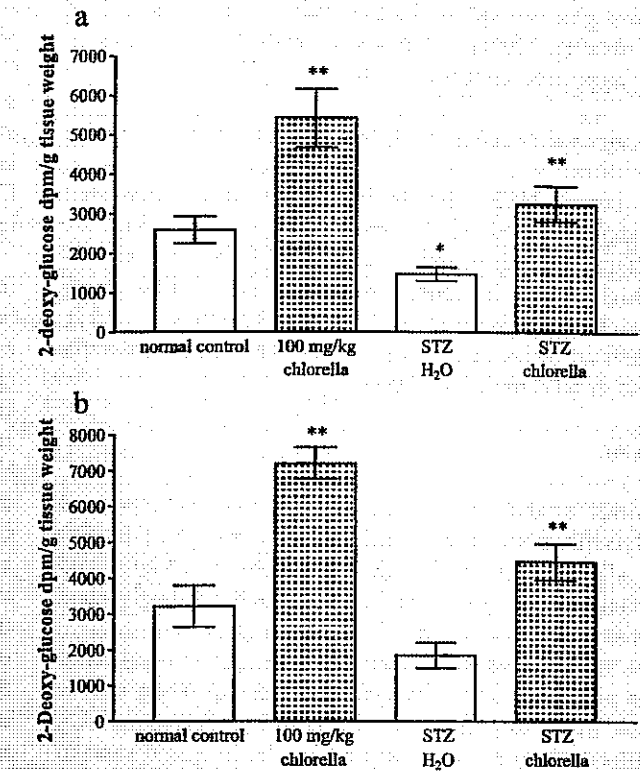


Fig. 2. (a) Effects of *Chlorella* on 2-deoxyglucose uptake in the liver in STZ mice. Mice received H_2O or 100 mg/kg of *Chlorella*. Statistics are shown for 100 mg/kg of *Chlorella* in normal and in STZ mice, $**p < 0.01$ compared to normal control and H_2O -treated STZ mice, respectively, and H_2O -treated STZ mice, $*p < 0.05$ compared to normal controls. (b) Effects of *Chlorella* on 2-deoxyglucose uptake in soleus muscle in STZ mice. Mice received H_2O or 100 mg/kg of *Chlorella*. Statistics are shown for 100 mg/kg of *Chlorella* in normal and in STZ mice, $**p < 0.01$ compared to normal control and H_2O -treated STZ mice, respectively.

treatment both in normal mice ($p < 0.01$) and in STZ mice ($p < 0.01$, see Fig. 2a). The glucose uptake in the soleus in H_2O -treated STZ mice was only slightly lower than that in normal controls (Fig. 2b). After receiving 100 mg/kg of

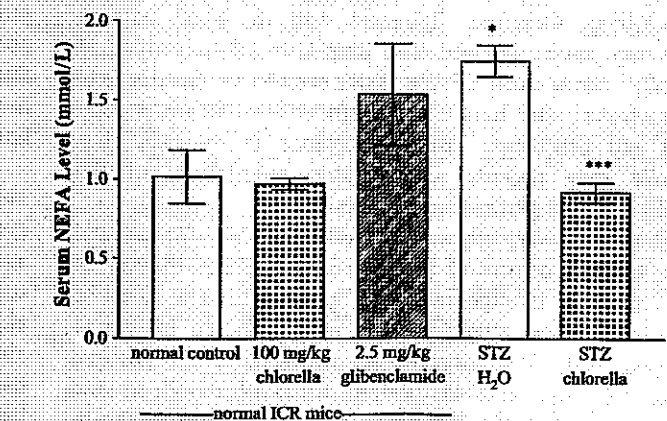


Fig. 3. Effects of *Chlorella* on serum non-esterified fatty acid (NEFA) level. Normal mice received H_2O , 2.5 mg/kg of glibenclamide, or 100 mg/kg of *Chlorella*. STZ mice received H_2O or 100 mg/kg of *Chlorella*. Statistics are shown for H_2O -treated STZ mice, $*p < 0.01$ compared to normal control and 100 mg/kg of *Chlorella* in STZ mice, $***p < 0.005$ compared to H_2O -treated STZ mice.



Improving glycogenesis in Streptozocin (STZ) diabetic mice after administration of green algae *Chlorella*

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Abstract

Chlorella, a type of unicellular fresh water algae, has been a popular foodstuff in Japan and Taiwan. Studies have shown the hypoglycemic effects of *Chlorella* in alloxan-induced and Streptozocin (STZ)-induced diabetic animals. However, the mechanisms by which *Chlorella* treatment affects blood glucose homeostasis have not been studied. Diabetes in ICR mice was induced by injection of STZ. Lipogenesis in vivo was measured by incorporating ³H-H₂O into lipids in brown and white adipose tissues. Glucose uptake in the liver and soleus muscles was measured by assaying 2-deoxy-D-[1,2-³H] glucose levels. The effects of *Chlorella* on serum non-esterified fatty acids (NEFA) were measured with commercial assay kits. Insulin-stimulated lipogenic rates in brown and white adipose tissues were unaffected by *Chlorella*. However, *Chlorella* increased 2-deoxyglucose uptake in the livers and soleus muscles in normal and STZ mice compared to that in their respective controls ($p < 0.01$). In addition, fasting NEFA levels were lower in *Chlorella*-treated STZ mice compared to H₂O-treated STZ mice ($p < 0.005$). The current results suggest that the hypoglycemic effects of *Chlorella* are due to an enhancement of glucose uptake in the liver and in soleus muscles. The improved insulin sensitivity after *Chlorella* treatment could be also due to lower NEFA levels, since insulin sensitivity is usually blunted by elevated NEFA in diabetes.

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Keywords: STZ diabetic mice; *Chlorella*; Lipogenesis; Glucose uptake; Insulin sensitivity

Introduction

Our previous study has shown that acute administration of *Chlorella* produced a significant hypoglycemic effect in STZ mice (Shih, 2001; Cherng and Shih, 2005b). *Chlorella*, a unicellular green alga, has long been a popular foodstuff in Japan and Taiwan. It provides a good source of protein (Morimura and Tamiya, 1954), lipid soluble vitamins, choline, and essential minerals in an available form (Shimo et al., 1967). Administration of *Chlorella* has also been shown to affect some biochemical and physiological functions, such as promoting the growth rate of animals (Shibashi, 1972), boosting immune function (Halperin et al., 2003; Pugh et al., 2001; Tanaka et al., 1984; Singh et al., 1998), preventing stress-induced ulcers (Tanaka et al.,

1997) and preventing high fat-diet induced dyslipidemia (Cherng and Shih, 2005a; Singh et al., 1998; Sano et al., 1988; Okuda et al., 1975). Although, the hypoglycemic effects of *Chlorella* have also been demonstrated in alloxanized rats (Rodriguez-Lopez and Lopez-Quijada, 1971), the possible mechanisms of its hypoglycemic effects have not been investigated.

Our previous study showed that administration of *Chlorella* improved glucose challenge in both normal and STZ mice, however this was not due to an increase of insulin secretion (Cherng and Shih, 2005b). Blood glucose is taken up and utilized by tissues under the influence of insulin, or stored as glycogen in the liver and muscles. Excessive glucose can also be stored as triglyceride via a process of lipogenesis in adipose tissues. Thus the aim of this study is to investigate the primary mechanisms of the hypoglycemic effects of *Chlorella* by measuring blood glucose utility pathways.

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