

transient fall in the basal blood glucose level. Thus, we chose this dose as the standard dose for subsequent studies. The same dose of *Chlorella* also increased exogenous insulin sensitivity in both normal and STZ mice. However, this was not due to a change in endogenous plasma insulin secretion. Other previous studies have shown that *Chlorella* alleviated the hyperglycemia in alloxan-induced diabetic rats (Rodriguez-Lopez, 1964; Rodriguez-Lopez and Lopez-Quijada, 1971). In these studies, alloxan-induced diabetic animals, which represent a model of IDDM, were used to study the hypoglycemic effects of *Chlorella*. In the present study, similar hypoglycemic effects of *Chlorella* were also found in the STZ diabetic mouse model for the first time. The results from the basal blood glucose experiments, glucose tolerance tests, and comparative insulin sensitivity tests in normal (non-diabetic) mice suggest that *Chlorella* has the potential to lower blood glucose levels. The effects of *Chlorella* on basal glucose and insulin levels in STZ mice are consistent with the earlier findings by Rodriguez-Lopez and Lopez-Quijada (1971).

Glibenclamide is a sulphonylurea hypoglycemic drug which is known to stimulate insulin secretion from the pancreas. In contrast, *Chlorella* did not alter insulin levels after acute administration. Thus, *Chlorella* seems to influence the blood glucose via mechanisms different to conventional sulphonylurea hypoglycemic drugs. The improvement of glucose tolerance produced by *Chlorella*, at the same time as an increased response to injected insulin, in the absence of any change in circulating insulin levels, is more similar to the action of metformin, which acts by increasing the insulin sensitivity of target tissues. We have shown previously that the fall in blood glucose following acute insulin in maximal at 60 min, and that the rate of return to normal is dose-dependent (Williams et al., 1999b). The present results from the comparative insulin sensitive tests indicate that the hypoglycemic effect of insulin is prolonged, which could result from increased tissue sensitivity or delayed insulin clearance. If tissue sensitivity to insulin is increased, *Chlorella* could be acting either to increase glucose uptake without stimulating insulin secretion, or by suppressing hepatic gluconeogenesis and glycogenolysis. The first possibility is supported by the measurement of plasma insulin levels as part of the assessment of the comparative sensitivity to insulin. The latter hypothesis is currently being investigated. Alternatively, it is possible that *Chlorella* is acting to disrupt the normal physiological counter-regulatory responses to rises in circulating insulin levels; for example, somatostatin or glucagon release. Further experiments including measurement of lipogenesis, lipolysis, and glucose uptake, will help to clarify the possible physiological basis for the hypoglycaemic and insulin-sensitizing actions of *Chlorella*.

Acknowledgement

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Plasma insulin level

Blood samples were taken 20 min after the drugs or saline (for injection experiments) or water (for oral experiments) as controls. Plasma was stored at -20°C for further assay. Plasma insulin was determined by using EIA system assay kits (Amersham Life Science Cat no. RPN 2567).

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

Effects of *Chlorella* on blood glucose level in normal ICR mice

In the acute glucose tolerance test the maximum rise in blood glucose occurred 30 min after the i.p. glucose challenge. A dose of 125 mg/kg of acute *Chlorella* significantly suppressed the rise in blood glucose at 30 and 60 min ($p < 0.05$). The lower doses did not produce any significant changes compared to the controls (see Fig. 1). The conventional oral hypoglycemic drug (glibenclamide 2.5 mg/kg, used as a positive control) also prevented the rise in blood glucose at 30 and 60 min after the glucose challenge.

Basal glucose levels and insulin sensitivity test in normal ICR mice

Glibenclamide produced a significant decrease in basal blood glucose levels 90 min after the treatment which was then sustained for a further 60 min in the controls ($p < 0.005$). In contrast, *Chlorella* only produced a transient decrease in basal blood glucose at 90 min after the dose of 100 mg/kg in normal mice (see Fig. 2). In order to investigate whether *Chlorella* would further increase the hypoglycemic action of glibenclamide, *Chlorella* (at doses of 75 and 100 mg/kg) was administered 60 min prior to administration of glibenclamide and the basal blood glucose level monitored for 120 min thereafter. This additive administration of *Chlorella* and glibenclamide produced a greater fall in blood glucose compared to glibenclamide or *Chlorella* alone ($p < 0.005$, Fig. 3). Glibenclamide and the 100 mg/kg dose of *Chlorella* prolonged the hypoglycemic effects of exogenous insulin for further 120 min ($p < 0.05$) and 60 min ($p < 0.01$), respectively (see Fig. 4). On the basis of these findings 100 mg/kg of *Chlorella* was chosen as the standard dose in the subsequent experiments.

Fig. 6. (A) Effects of *Chlorella* on basal glucose test in STZ mice. Normal mice and STZ mice received H₂O or 100 mg/kg of *Chlorella* at time -60 min. Statistics are shown for STZ mice+H₂O had significantly higher basal blood glucose level than controls, ### $p < 0.005$ and for 100 mg/kg of *Chlorella*, *** $p < 0.005$, compared to the control mice at the same time points. (B) Effects of *Chlorella* on comparative insulin sensitive test in STZ mice. Normal mice and STZ mice received H₂O or 100 mg/kg of *Chlorella* at time -60 min. Insulin (2.5 IU/kg) was given at time zero indicated. Statistics are shown for STZ mice+*Chlorella*, # $p < 0.05$, ### $p < 0.005$, compared to STZ mice+H₂O at the same time points; *** $p < 0.005$ compared to the time *Chlorella* was administered.

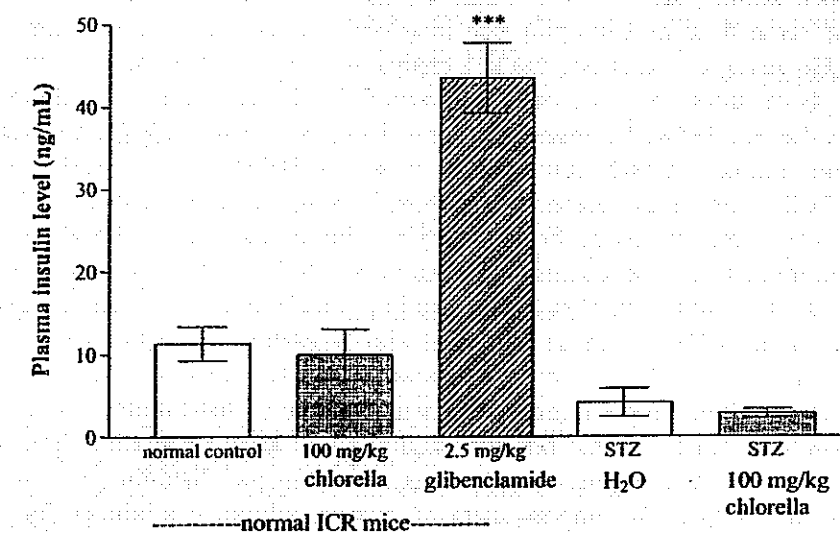


Fig. 7. Effects of *Chlorella* on plasma insulin level in STZ mice. Normal mice and STZ mice received H₂O, or 100 mg/kg of *Chlorella* and an additional group of normal mice received 2.5 mg/kg of glibenclamide 20 min prior to sample collection. Insulin levels were then determined subsequently. Statistic is shown for normal mice received 2.5 mg/kg of glibenclamide *** $p < 0.005$ compared to controls.

Effects of *Chlorella* on blood glucose level in STZ mice

Chlorella and glibenclamide did not significantly suppress the response to the acute glucose challenge (0.5 g/kg) in STZ mice (see Fig. 5). This lower dose of glucose was used because of the presence of hyperglycemia in STZ mice. The basal glucose levels in STZ mice were, as expected, significantly higher than in normal mice ($p < 0.005$, Fig. 6A). Although *Chlorella* (100 mg/kg) produced a transient decrease in blood glucose level in normal mice, the same dose did not affect the basal blood glucose level in STZ mice. Exogenous insulin treatment alone did not produce significant hypoglycemic effects in H₂O-treated STZ mice, however there was a dramatic decrease in blood glucose in the *Chlorella*-treated STZ mice 60 min after the administration of exogenous insulin ($p < 0.005$, see Fig. 6B). This enhancement of the hypoglycemic effects of insulin by *Chlorella* was then maintained for further 60 min.

Insulin levels in normal and STZ mice

Slightly lower insulin levels were observed in H₂O-treated STZ mice compared to the controls. Administration of *Chlorella* did not affect plasma insulin levels either in normal or in STZ mice (see Fig. 7). However, there was a dramatic increase, as expected, in the plasma insulin level in glibenclamide-treated mice ($p < 0.005$).

Discussion

The results from the dose response to *Chlorella* in normal mice indicated that a dose of 100 mg/kg was significant to suppress the hyperglycaemic response to an acute glucose challenge and produced a

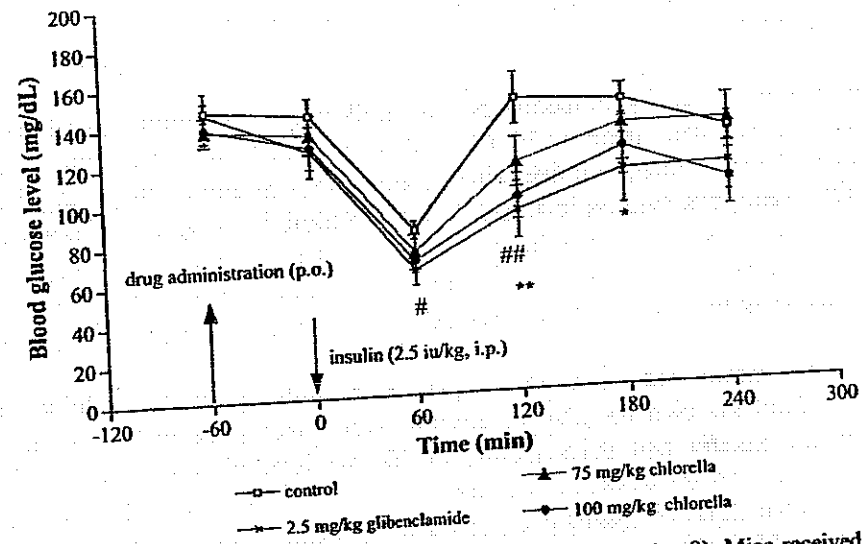


Fig. 4. Effects of *Chlorella* on comparative insulin sensitive test in normal mice (n=8). Mice received H₂O, 2.5 mg/kg of glibenclamide, 75, or 100 mg/kg of *Chlorella* at time -60 min. Insulin (2.5 IU/kg) was given at time zero. Statistics are shown for glibenclamide, # p<0.05 and ## p<0.01, and for 100 mg/kg of *Chlorella*, *p<0.05, **p<0.01 and compared to controls at the same time points.

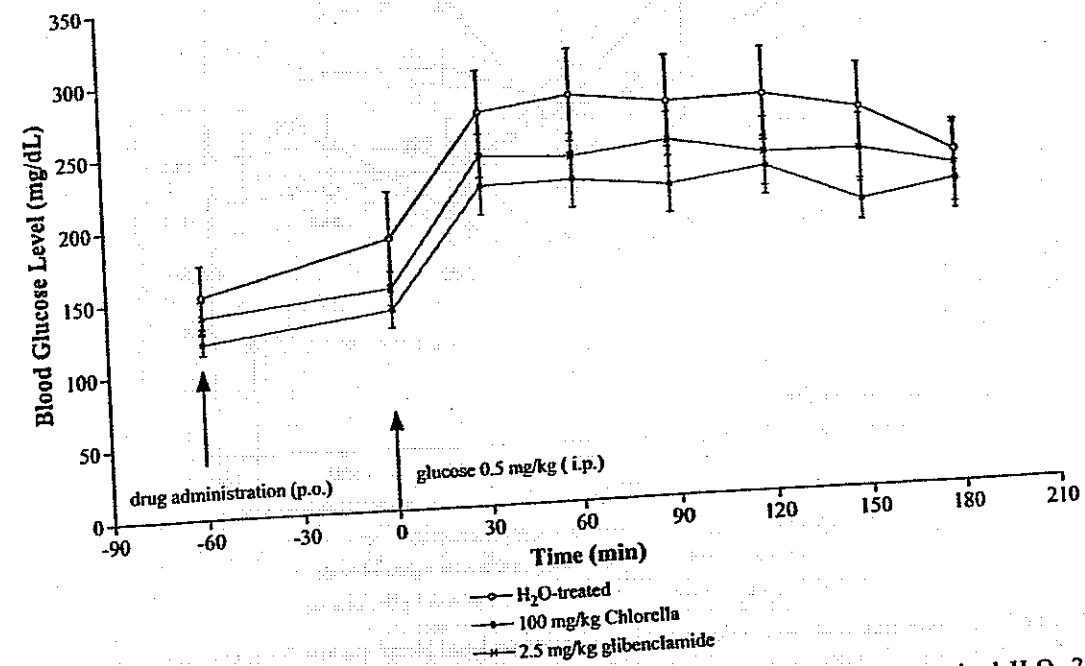
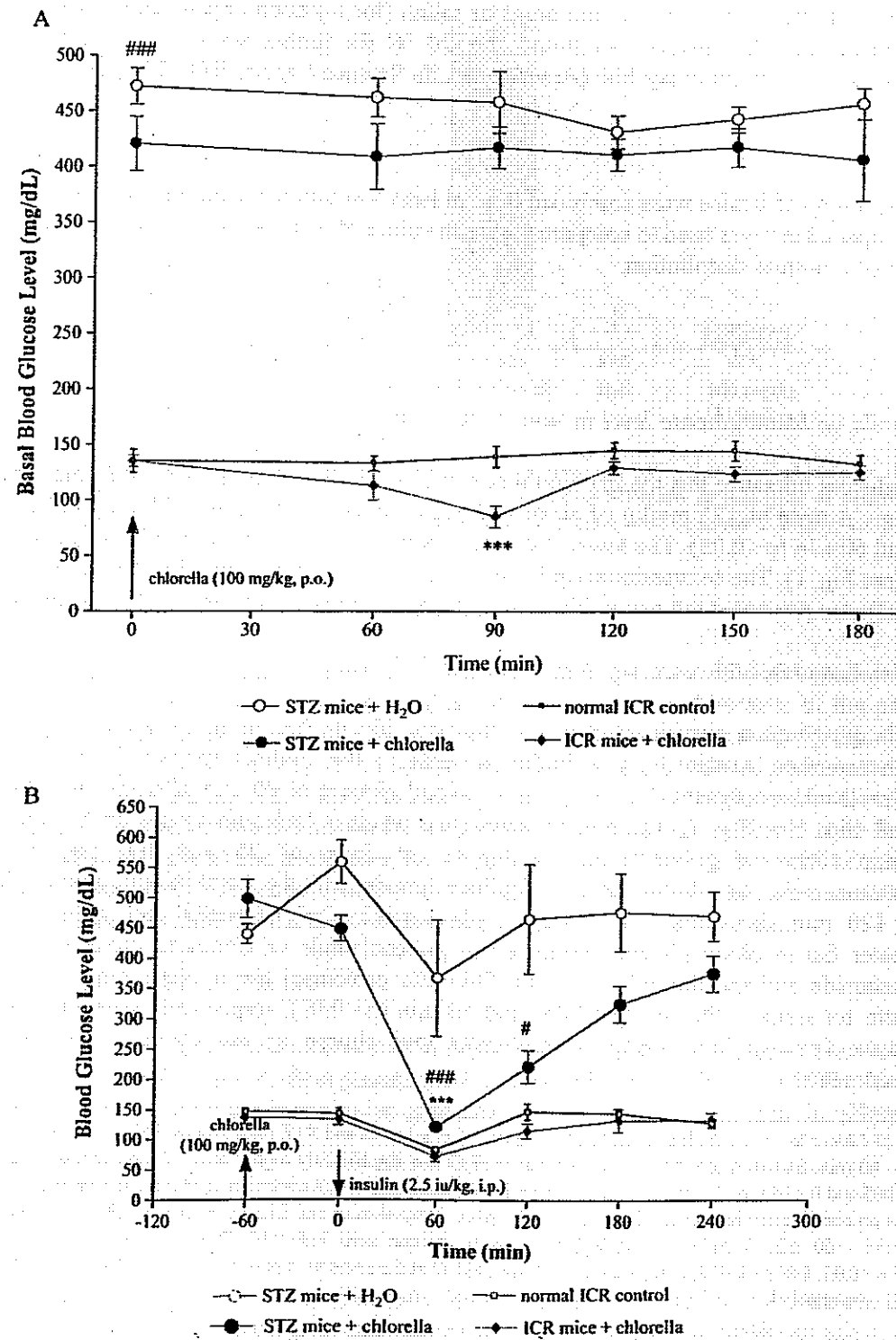


Fig. 5. Acute effects of *Chlorella* on glucose tolerance test in fasting STZ mice. STZ mice received H₂O, 2.5 mg/kg glibenclamide or 100 mg/kg of *Chlorella* at time -60 min. Glucose of 0.5 mg/kg, was i.p. injected to STZ mice at time 0 min. Blood glucose levels were subsequently measured at 30 min intervals.



mg/kg according to previous work (Williams et al., 1999a) and insulin was 2.5 IU/kg (Williams et al., 1999b).

Procedure

Acute experiments commenced between 09.00 and 10.00h using mice which had been housed in groups of eight in the same cage for at least 2 weeks. Standard diet and water were available ad libitum, except during the glucose tolerance test. Blood samples, 20 µl, were taken by venesection of the tail vein following light ether anaesthesia. To establish a minimal effective dose of *Chlorella* on glucose tolerance test in normal mice, doses of between 50-125 mg/kg were administered orally 60 min prior to glucose tolerance test (see below).

Glucose tolerance test

Following an overnight fast, *Chlorella* or water was administered by oral gavage 60 min prior to the challenge dose of 1 g/kg body weight glucose i.p. (zero time). In STZ mice the dose of glucose was reduced to 0.5 g/kg. Mice were anaesthetized with ether and 20 µl blood samples taken by distal venesection of the tail vein. Blood samples were taken immediately prior to administration of the *Chlorella* or water and the glucose then subsequently at 30 min intervals for a period of 150 min. Measurement of blood glucose was carried out using a glucocheck strip (Accutrend mini, Boehringer Ltd.).

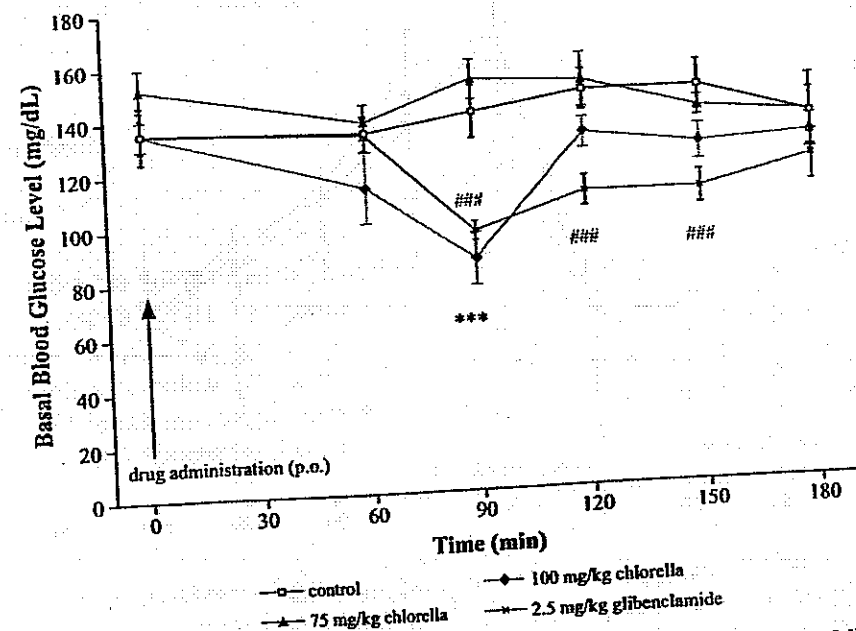


Fig. 2. Effects of *Chlorella* and Glibenclamide on basal blood glucose levels in normal mice (n=8). Mice received H₂O, 2.5 mg/kg of glibenclamide, 75, or 100 mg/kg of *Chlorella* at time -60 min. Statistics are shown for 100 mg/kg of *Chlorella* *** p<0.005 and for 2.5 mg/kg of glibenclamide ### p<0.005 compared to controls.

Basal glucose measurements

Chlorella or water was given orally immediately after collection of an initial blood sample (-60 min), subsequent blood samples were taken at 30, 60, 120, 180, and 240 min after drug administration for blood glucose analysis. In order to examine whether there were additive effects of *Chlorella* and glibenclamide, *Chlorella* was given 60 min after glibenclamide administration and blood samples were taken for assay as above.

Comparative insulin sensitivity test

Chlorella or water was administered orally 60 min prior to a single injection of soluble insulin, 2.5 IU/kg body weight i.p. Blood samples for glucose analysis were taken prior to the administration of *Chlorella* or water and the insulin and subsequently at 60 min intervals for a period of 240 min (Williams et al., 1999b).

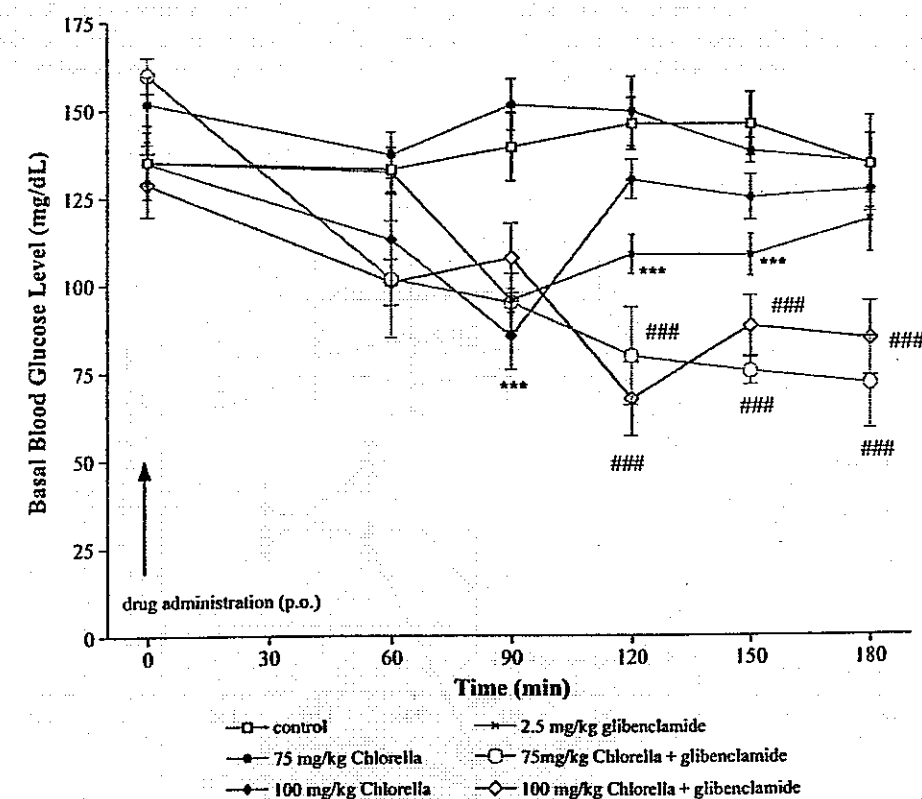


Fig. 3. Effects of additive administration of *Chlorella* and Glibenclamide on basal blood glucose level in normal mice (n=8). Mice received H₂O, 2.5 mg/kg of glibenclamide, 75, or 100 mg/kg of *Chlorella* only, or additive administration of glibenclamide with 75 or with 100 mg/kg of *Chlorella* at time -60 min. Statistics are shown for additive administration of 75 mg/kg *Chlorella* + glibenclamide ### p<0.005, compared to their glibenclamide or 75 mg/kg *Chlorella* and for additive administration of 100 mg/kg *Chlorella* + glibenclamide ### p<0.005 compared to their glibenclamide or 100 mg/kg *Chlorella*. Statistics are shown for 100 mg/kg of *Chlorella* and for 2.5 mg/kg of glibenclamide *** p<0.005 compared to controls.

Introduction

Diabetes mellitus, both of the insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM) type, is a common and serious disorder throughout the world (Keen, 1986; Harris et al., 1987). This metabolic disorder often leads to physical disability arising from the vascular complications of coronary artery disease and cerebrovascular disease, resulting in renal failure, blindness, and limb amputation in addition to neurological complications and premature death (Goldstein and Massry, 1978; Weidmann et al., 1993; Strippoli et al., 2003; He and King, 2004). Treatment of diabetes mellitus by insulin and oral hypoglycemic drugs fails to prevent these complications in many patients, indicating that additional alternative treatment could be helpful.

Chlorella, a type of unicellular green algae, 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 (Shino et al., 1967). Administration of *Chlorella* has been shown to affect some biochemical and physiological functions, such as promoting the growth rate of animals (Ishibashi, 1972), boosting immune function (Tanaka et al., 1984; Singh et al., 1998; Pugh et al., 2001), preventing stress-induced ulcers (Tanaka et al., 1997). *Chlorella* also influences the lipid content of the liver and serum in ethionine treated rats (Wang et al., 1980) and in cholesterol containing diet-treated rabbits (Sano and Tanaka, 1987; Sano et al., 1988). In addition, acute administration of *Chlorella* produced a significant hypoglycemic effect in alloxanized rats (Rodriguez-Lopez, 1964; Rodriguez-Lopez and Lopez-Quijada, 1971). However, there have been no subsequent studies on the mechanism of the hypoglycemic effect of *Chlorella*. Here we have used a different diabetic animal model (streptozotocin (STZ)-induced) to study the effects of *Chlorella* on blood glucose homeostasis.

Materials and methods

Chlorella material

Commercially available spray-dried preparations of *Chlorella pyrenoidosa* 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 spray-dried preparations of *Chlorella pyrenoidosa* contain 3-5% moisture with particle size below 50 mesh (~300 μm), bulk density of 1.8-2.3 ml/g, tapped density of 1.5-2.0 ml/g, and intact microalgae cell wall through microscopic examination (The information provided by GONG BIH Enterprise Co., Ltd.).

Animals

Male ICR mice were purchased from the National Science Council Animal Center of 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 Streptozotocin (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 6-8 weeks. Their housing was maintained at a temperature of 20-22 $^{\circ}\text{C}$, relative humidity of 50-80%, and a 12 hours light/dark cycle of 07.00 hr to 19.00 hr with no twilight. All animals were anaesthetised 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 (Cat. No 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* was given as a gift from 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

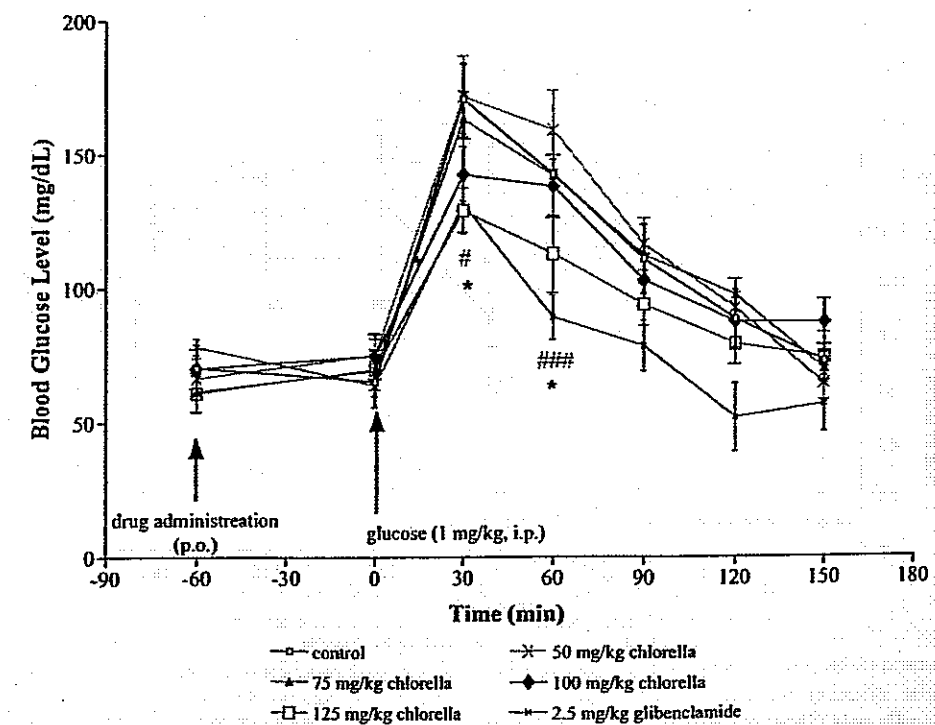


Fig. 1. Dose-response effect of *Chlorella* on glucose tolerance test in fasting normal mice (n=8). Mice received H₂O, 2.5 mg/kg of glibenclamide, 50, 75, 100, or 125 mg/kg of *Chlorella* at time -60 min. Statistics are shown for 125 mg/kg of *Chlorella*, * p<0.05, and 2.5 mg/kg of glibenclamide # p<0.05, ### p<0.005 compared to controls.

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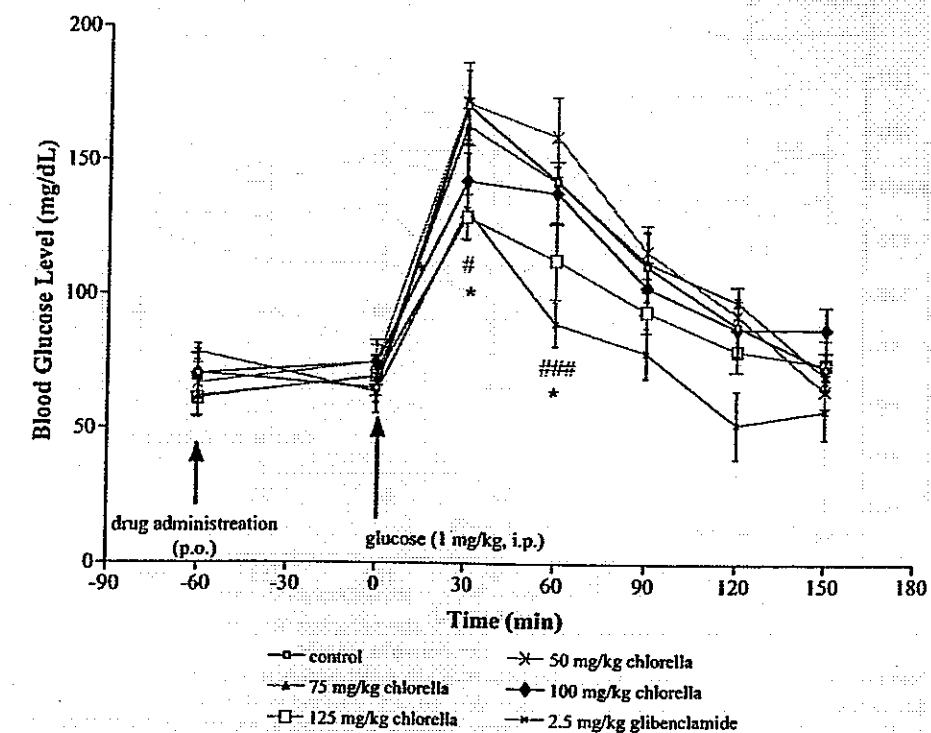


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Potential hypoglycemic effects of *Chlorella* in streptozotocin-induced diabetic mice

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Abstract

Chlorella, a type of unicellular fresh water algae, has been a popular foodstuff in Japan and Taiwan. *Chlorella* has been shown to produce hypoglycemic effects in alloxan-induced diabetic animals. However, there are no other reports of the effects of this substance in other diabetic animal models. Here we have used streptozotocin (STZ)-induced diabetic mice to study the hypoglycemic effects of *Chlorella*. Diabetes was induced in ICR strain mice by the i.p. injection of STZ. Vehicle-treated ICR mice were used as normal control animals and glibenclamide was used as a positive drug control. The effects of *Chlorella* on basal blood glucose, exogenous insulin sensitivity test and plasma insulin levels were measured. In normal mice *Chlorella* produced a transient hypoglycemic effect at 90 min after acute administration; whereas glibenclamide produced a more sustained hypoglycemic effect between 90 min and 180 min after acute administration. *Chlorella* did not affect the basal blood glucose level in STZ mice. However, *Chlorella* enhanced and prolonged the hypoglycemic effects of injected insulin in STZ mice for a further 60 min compared to the normal vehicle-treated group. Plasma insulin levels were increased in normal mice after treatment with glibenclamide, whereas *Chlorella* had no such effect. The current results indicate that *Chlorella* enhances the hypoglycemic effects of exogenous insulin at a dose which does not produce hypoglycemia in STZ mice, suggesting that insulin sensitivity is increased in these mice.

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Keywords: STZ diabetic mice; *Chlorella*; Insulin sensitivity; Insulin level; Glucose tolerance

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