

Figure 2. Scanning electron micrographs of the longitudinal sections of the proximal tibia sampled at 7 weeks after ovariectomy from (A) sham-operated, (B) ovariectomized (OVX), (C) OVX given chlorella growth factor (CGF) solution rats.

effect independent of the increased food intake. However, ovariectomy resulted in a decrease in fluid consumption under our *ad libitum* condition (Table 1; Hidaka *et al.*, 1997), our result is also in opposite to previous findings (Tartelin and Gorski, 1971; Geiselman and Almi, 1978). Further study is needed to resolve this discrepancy.

Ovariectomy also resulted in a dramatic decrease in the uterine weight (Table 2). Since the CGF solution did not increase the uterine weight of OVX rats (Table 2), it did not function as an oestrogen agonist. Our result suggests that the reduction of the weight gain is caused by an action other than the oestrogenic action.

The ovariectomy increased the level of both total and high-density lipoprotein (HDL) cholesterol, but decreased the triglyceride level (Table 3). The injection of 17 β -oestradiol to ovariectomized (OVX) rat normalized these parameters to the level of Sham rats (Hidaka *et al.*, 2001). Similarly, Lundeen *et al.* (1997) reported that increased levels of total and HDL cholesterol were reduced to the level of Sham rats by the administration of oestrogens to the OVX rats. By the administration of the CGF solution, total and HDL cholesterol decreased, and triglyceride increased to the levels of Sham rats (Table 3). However, our result showed that the CGF solution did not have an oestrogenic action. Further study is needed to elucidate the mechanism of action of the CGF solution.

Watkins *et al.* (1972) reported that ovariectomy reduced the secretion of triglyceride by the liver, which may explain our results. Contrary to our result and that of Watkins *et al.* (1972), Van Lenten *et al.* (1983) reported that the ovariectomy resulted in the elevation of plasma triglyceride. Depending on the metabolic status, oestrogen may have variable and even an opposite effect on the blood triglyceride level. Since the level of triglyceride in ovariectomized rats depends on the hepatic secretion (Valette *et al.*, 1986), the CGF solution may increase the level of hepatic triglyceride secretion. In clinical trials done by Merchant and Andre (2001), the subjects, who were diagnosed as suffering from hypertension and consumed for 2 months both the solid tablets and the liquid (CGF), decreased the levels of total and HDL cholesterol, but had no effects on the level of triglycerides. Therefore, their results, as ours, strongly suggest that single administration of the CGF solution would be beneficial for cardiovascular disease and high cholesterol for women after menopause. Since a prominent inhibitory effect on the bone loss is the primary proof as an antiosteoporotic agent, the CGF solution is not an antiosteoporotic agent (See Table 2 and Fig. 2).

The administration of the CGF solution to Sham rats had no effects on the growth, although this solution claims to have growth promoting effect on plant and microorganism (Merchant and Andre, 2001). Furthermore, the CGF solution had no effects on Sham rats in this study (Tables 1–3).

The consumption of natural 'whole foods' rich in macro-nutrients has been suggested to have beneficial effects for those who regularly ingest normal, nonvegetarian diets. The extract from *Chlorella pyrenoidosa*, a unicellular fresh water green alga rich in proteins, vitamins, and minerals, has been recommended as a dietary supplement. Our study revealed that a hot water extract of *C. pyrenoidosa* may have a therapeutic potential to relieve symptoms, improve quality of life, and normalize body functions in patients with fibromyalgia, hypertension, or ulcerative colitis, as reviewed by Merchant and Andre (2001). A possible usefulness of chlorella products against these chronic diseases should be further investigated using animal experiments to define its mechanism of action.

Cardiovascular disease and osteoporosis are serious concerns for women after menopause. Therefore, we evaluated the efficacy by the CGF solution against these problems using an ovariectomized rat model.

We found that the ovariectomy induced an increase of body weight and decreases in serum lipid metabolism and the tibial BMD. Although the CGF solution did not recover the bone loss, this agent normalized lipid metabolism and body weight. In conclusion, this

commercial chlorella liquid may have beneficial effects for the prevention of cardiovascular diseases.

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(45–50%). Food and drinking water were supplied *ad libitum*.

Daily food and fluid consumptions as well as their body weight were measured at a specified time of the day during the experiment. After euthanizing the animal under chloroform anaesthesia, the right legs were dissected from each animal and stored at -83°C . The uterus of each rat was dissected and weighed (Hidaka *et al.*, 1999).

Serum lipids. After completion of the experiment, food was removed 12 h prior to euthanasia. Rats were anaesthetized by ether and blood samples were collected into 50 ml beakers by cutting the carotid artery. After coagulation, serum was separated by low speed centrifugation. Total serum cholesterol and triglyceride were determined using diagnostic kits from Sigma Chemicals Co. (St. Louis, MO, USA) according to the manufacturer's instructions (Hidaka *et al.*, 2001). After low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) were precipitated from the serum using the dextran sulphate/magnesium chloride precipitation method, high-density lipoprotein (HDL) cholesterol was determined, using the HDL cholesterol diagnostic kits, as described by the same manufacturer (Hidaka *et al.*, 2001).

Measurement of bone mineral density (BMD). After removing adherent soft tissues, longitudinal sections of tibiae were made by the manual grinding with whetstones (# 600). The right tibiae were dehydrated by a step-wise application of 70% and 99.9% (v/v) ethanol solutions. These samples were used for both the measurement of BMD and scanning electron microscopic observation.

The BMD was quantitatively determined by the computed x-ray densitometry (CXD) method (Bonalyzer; Teijin Company, Tokyo, Japan) (Hidaka *et al.*, 1997; 1999). The radiographs of the longitudinal sections of tibiae were taken along with an aluminium step-wedge using an x-ray apparatus (Model CMB: Softex Company, Tokyo, Japan) set at 4 mA, 40 cm, 120 s, and 35 kV. The densitometric pattern of the proximal tibia on an x-ray picture was read by a personal computer (PC-9801; NEC, Tokyo, Japan) using software program for rat bone density (Version 2.10A-M; Teijin Company, Tokyo, Japan). As an index of BMD, $\Sigma\text{GS/D}$ (mm Al) was used where ΣGS is a value obtained by integrating the pattern area which was obtained optically and was converted into a number of steps in an aluminium step-wedge, and D represents the bone width. The area that covers the epiphysis and a part of metaphysis in the proximal tibia was calculated for $\Sigma\text{GS/D}$. The determination of the area and the precision of the measurements were described by Hidaka *et al.* (1997).

Electron microscopic observations. Longitudinal sections of tibiae were then shadowed with carbon before examination in a scanning electron microscope (SEM) (JSM-6330F, Nihon Denshi, Tokyo, Japan) at 10 kV.

Statistics. Data were obtained from 3–5 measurements and were expressed as the means \pm standard deviations. Statistical comparisons were made by ANOVA and Scheffé's tests using a statistical software program.

The difference was considered significant when $p < 0.05$.

RESULTS

Food and fluid consumptions and body weight

As shown in Table 1, the mean daily food consumption of the OVX rats on 1–7 weeks was higher than that of Sham rats by 28%. Food consumption of OVX + CGF rats in 1–7 weeks was almost the same as those in Sham and Sham + CGF rats.

Daily fluid consumption of OVX rats in 1–7 weeks was lower than that of Sham rats by 19% (Table 1). Fluid consumption of OVX + CGF rats in 1–7 weeks was comparable to that of OVX rats.

The increase of body weight in ovariectomized (OVX) rats was significantly higher than that in sham control (Sham) rats (Fig. 1). After 7 weeks, the mean body weight \pm SD in Sham and OVX rats were 286 ± 13 ($n = 6$) and 355 ± 14 ($n = 6$), respectively (Table 2). The increase of body weight of OVX + CGF rats was smaller than that in OVX rats. With the administration of the CGF solution to Sham rats (Sham + CGF), the increase was reduced to almost the same level as that in Sham rats (Fig. 1 and Table 2).

Uterine weight

As shown in Table 2, the uterine weight of Sham and OVX rats differed significantly ($p < 0.01$). The weight in the OVX + CGF group was almost the same as that in the OVX group. The weight in the Sham + CGF group was also almost the same as that in the Sham group.

Serum lipids

As shown in Table 3, the total cholesterol levels of Sham and OVX rats were 88.7 ± 9.5 and 101 ± 9.8 (mg/dl), respectively. This value increased by 14% in OVX rats. High-density lipoprotein (HDL) cholesterol also significantly increased by 18% in OVX rats. The triglyceride level of OVX rats decreased by 36% as compared to that of Sham rats. The administration of the CGF solution to the OVX rats reduced these serum parameters to the level of Sham rats, while the administration of CGF solution to the Sham rats had no effects.

Table 1. Mean daily food and fluid consumptions in sham-operated and of ovariectomized rats treated with and without chlorella growth factor (CGF) solution for 7 weeks

	Food per rat (g)	Fluid per rat (ml)
Sham	14.5 ± 1.1	35.1 ± 1.5
Sham + CGF	15.2 ± 1.1	36.0 ± 1.4
OVX	$18.5 \pm 1.3^*$	$28.5 \pm 1.5^*$
OVX + CGF	16.3 ± 1.0	$28.1 \pm 1.5^*$

Rats were divided into 4 groups, sham-operated (Sham), Sham given CGF solution (Sham + CGF), ovariectomized (OVX), OVX given CGF solution (OVX + CGF), each having 6 rats. * Significant difference ($p < 0.05$) when compared with Sham rats.

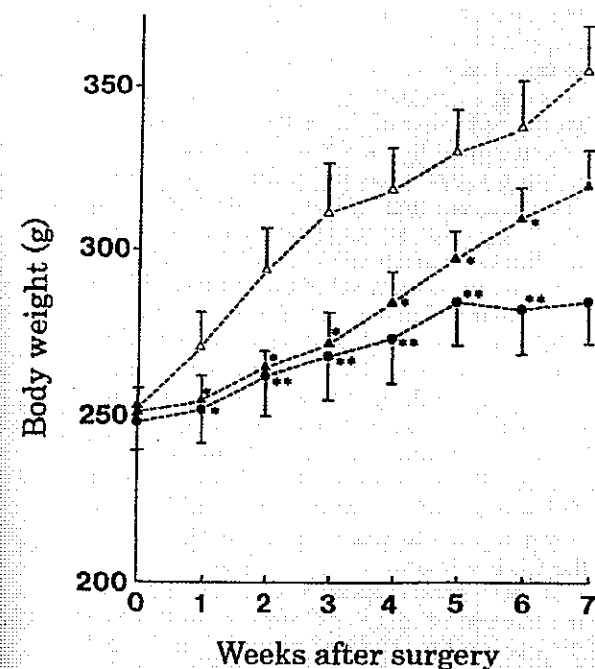


Figure 1. Changes in body weight in rats. Rats were sham administered chlorella growth factor (CGF) solution (●), ovariectomized (OVX) (Δ), and OVX administered chlorella growth factor (CGF) solution (▲). Since the changes of sham-operated were the same as sham-administered CGF solution, its curve was not shown. Each point represents the mean value \pm SD ($n = 6$). * $p < 0.05$ and ** $p < 0.01$, significant difference from the ovariectomized group at corresponding times.

Table 2. Body weight, uterine weight, and tibial bone mineral density (BMD) of sham-operated and of ovariectomized rats treated with and without chlorella growth factor (CGF) solution for 7 weeks

	Body weight (g)	Uterine weight (mg)	Bone mineral density ($\Sigma\text{GS/D}$)
Sham	286 ± 13^b	534 ± 33	0.99 ± 0.02
Sham + CGF	295 ± 13^b	522 ± 35	1.01 ± 0.02
OVX	$355 \pm 14^*$	$112 \pm 9^*$	$0.80 \pm 0.02^*$
OVX + CGF	322 ± 11^{ab}	$115 \pm 10^*$	$0.78 \pm 0.02^*$

Abbreviations are as given in Table 1.

* Significant difference ($p < 0.05$) when compared with Sham rats.

^a Significant difference ($p < 0.05$) when compared with OVX rats.

Bone mineral density (BMD)

As shown in the right side column of Table 2, the tibial bone mineral densities (BMD) of Sham and OVX rats were 0.99 ± 0.02 and 0.80 ± 0.02 , respectively. This indicated that the ovariectomy decreased the BMD by 19%. The administration of the CGF solution to OVX rats did not recover the BMD. The administration of the CGF solution to Sham rats had no effects.

SEM analyses

The scanning electron micrograph of the proximal tibiae taken at 7 weeks after the ovariectomy is shown in Fig. 2b. Compared to that of Sham rats (Fig. 2a), the connectivity of cancellous bone in the epiphysis and

Table 3. Total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglyceride contents in the sera of sham-operated and of ovariectomized rats treated with and without chlorella growth factor (CGF) solution for 7 weeks

	Total cholesterol (mg/dl)	HDL cholesterol (mg/dl)	Triglyceride (mg/dl)
Sham	88.7 ± 9.5	59.7 ± 6.1	56.7 ± 10
Sham + CGF	87.5 ± 9.9	60.3 ± 6.5	59.8 ± 10
OVX	$101 \pm 9.8^*$	$70.3 \pm 7.5^*$	$36.4 \pm 2.6^*$
OVX + CGF	88.3 ± 7.9	62.3 ± 5.1	60.0 ± 6.1^{ab}

Abbreviations are as given in Table 1.

* Significant difference ($p < 0.05$) when compared with Sham rats.

^a Significant difference ($p < 0.05$) when compared with OVX rats.

that of trabecular bone in the metaphysis exhibited greater loss as compared to those in the Sham rats. The administration of the CGF solution did not restore the connectivity in the metaphysis to those of the Sham rats (Fig. 2c). The administration of the CGF solution to Sham rats had no effects (data not shown).

DISCUSSION

To characterize the lipid metabolism in women after menopause, ovariectomized rat models have been used, for example, a 8–9-week-old Sprague-Dawley (Valette *et al.*, 1986; Lundeen *et al.*, 1997) or a 12-week-old Wistar rat (Van Lenten *et al.*, 1983). We used 90-day-old Sprague-Dawley rats which were ovariectomized to make them sex-hormone deficient. This mimics the situation occurring in women following menopause (Kalu, 1991).

Ovariectomy caused an increase in body weight (Fig. 1 and Table 2). This obesity following oestrogen depletion has been postulated to provide a partial protection against the development of osteoporosis (Roudebush *et al.*, 1993). The increase in the food consumption in ovariectomized rats (28% increase in OVX over Sham rats) may correlate with the increase of body weight (Clark and Tarttelin, 1982). By the administration of the CGF solution to OVX rats, the body weight decreased significantly during the experimental period. We noticed that the food consumption of OVX + CGF rats during the experiment was 88% of OVX rats (Table 1). It is possible that the suppression on appetite, which is controlled by ventromedial hypothalamus, may have caused the decrease of the body weight. Since food consumption of Sham + CGF rats was almost the same as that in Sham rats, it does not seem that the CGF is an unpleasant drink to the rats. There remains a possibility that the CGF solution prevented the lipid deposition in the adipose tissues.

The OVX rats consumed fluid 28.1 ± 1.5 ml per day per rat (Table 1). For a human adult people, the daily dosage of the CGF solution recommended by the manufacturer is 30 ml. Provided that an average body weight of the OVX rat is 300 g (Fig. 1) and a body weight of a human adult is 60 kg, the consumed CGF solution by the OVX rat (92.7 OD/kg/day) was approximately the same as the human adult (100 OD/kg/day).

Geiselman and Almlil (1978) stated that an increased water intake following ovariectomy has a hyperdipsic

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A Hot Water Extract of *Chlorella pyrenoidosa* Reduces Body Weight and Serum Lipids in Ovariectomized Rats

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The effects of a hot water extract of *Chlorella pyrenoidosa*, which contains chlorella growth factor (CGF), on the body weight, serum lipids, and the bone mass were evaluated using an ovariectomized rat as a model for postmenopausal bone loss. Rats were divided into four groups: sham-operated (Sham), Sham given the CGF solution, ovariectomized (OVX), and OVX given the CGF solution, respectively. Administration of the extract to OVX rats suppressed the body weight gain. After 7 weeks, the administration of the extract to the OVX group reduced increases in both serum total cholesterol and high-density lipoprotein (HDL) cholesterol. It also normalized the decrease of triglyceride level in the OVX group. The ovariectomy decreased the tibial bone mineral density (BMD) by 19%, and the administration of the extract to OVX rats did not inhibit this decrease. These results suggest that a dietary supplement of CGF may be useful to control the body weight and improve lipid metabolism of menopausal women. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: ovariectomized rats; chlorella extract (CGF solution); body weight; serum lipids; bone mineral density.

INTRODUCTION

Since the population of elderly people has increased, new and effective treatments for chronic and protracted diseases, for examples, chronic pain, diabetes, arthritis, osteoporosis, and cancer, are needed. Recently, more and more unconventional therapies are being used for the treatment of chronic diseases, and we have previously reported the effects of Japanese herbal medicine and soybean isoflavones in the prevention of osteoporosis using an ovariectomized rat model (Hidaka *et al.*, 1999, 2001).

The broken cell wall preparations and extracts of *Chlorella pyrenoidosa* as well as some other *Chlorella* species have been used as a dietary supplement by many Japanese people (Merchant and Andre, 2001). A number of studies and anecdotal reports have suggested that such dietary supplements promote growth and healing (Merchant and Andre, 2001), stimulate the immune system (Miyazawa *et al.*, 1988) and offer protection from infection (Tanaka *et al.*, 1986). We decided therefore, to evaluate the effects of chlorella extract in preventing hyperlipidemia and osteoporosis, in ovariectomized rats.

MATERIALS AND METHODS

Chlorella extract and chemicals. 'Miyabi Chlorella 900 CGF solution' was supplied by MIYABI Co. Ltd (Osaka, Japan). This extract from *Chlorella pyrenoidosa* [concentration; OD (Optical Density) = 200 at 50% (v/v) measured at a wavelength of 260 nm] contains a chlorella growth factor (CGF) which is a water-soluble extract and contains a variety of substances including amino acids, peptides, proteins, vitamins, sugars, and nucleic acids. The CGF content in raw *C. pyrenoidosa* is estimated to be approximately 5% (Merchant and Andre, 2001). All other reagents were purchased from ABIOZ Co. Ltd (Osaka, Japan).

Ovariectomy and administration of Chlorella extract. 24 female Sprague-Dawley rats, aged 9 weeks, were purchased from Seac Yoshitomi Ltd (Fukuoka Prefecture, Japan). 27 days later [90-days-old; a mature rat model (Kalu, 1991) was used], 12 rats were given a sham operation (they were equally divided into first and second groups) under nembutal (Pentobarbital sodium; 50 mg/kg body weight; Abott Lab., IL., U.S.A.) anaesthesia. The remaining rats were ovariectomized (they were also equally divided into the third and fourth groups). The second (Sham + CGF) and fourth (OVX + CGF) groups received a 0.5% (v/v; 200 times dilution) CGF solution, whereas the first (Sham) and third (OVX) groups received tap water. All groups received MF pellets (Oriental Yeast Co. Ltd, Tokyo, Japan) as their food. To develop bone loss in ovariectomized rats, all animals were maintained for 7 weeks under regulated 12 hr/12 hr light-dark illumination cycles at constant temperature (24 ± 0.5 °C) and humidity

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