Ohia et al., 2002; Rao and Sakaria, 1988; Sullivan and Triscari, 1977; Sullivan et al., 1974a; Vasselli et al., 1998). HCA is a potent competitive inhibitor of ATP-citrate lyase (EC 4.1.3.8) (Sullivan, 1977; Watson et al., 1969), which is an extramitochondrial enzyme catalyzing the cleavage of citrate to oxaloacetate and acetyl-CoA. This inhibitory action of HCA reduces the acetyl-CoA pool, thus limiting the availability of two-carbon units required for the initial steps of fatty acid and cholesterol biosynthesis (Berkhout et al., 1990; Chee et al., 1977; Sullivan et al., 1974b,c, 1977). This enzyme is particularly important during the hyperlipogenic nutritional state produced by high carbohydrate diet. The reduction in the acetyl-CoA pool is proposed to decrease the concentration of malonyl-CoA, thus resulting in the suppression of body fat accumulation through stimulation of carnitine palmitoyltransferase I activity and promotion of fatty acid oxidation (Ishihara et al., 2000; McCarty, 1994; Ruderman et al., 1999; Vasselli et al., 1998). Consequently, utilization of extra glucose from a high carbohydrate diet for lipogenesis is restricted, and utilization for glycogenesis is promoted through suppressed glycolysis (Hellerstein and Xie, 1993; McCarty, 1994; Sullivan et al., 1974c).

Pair-feeding studies also revealed a significant antilipogenic contribution of HCA treatment beyond its anorectic properties in CD strain rats (Sullivan et al., 1974b) and Zucker lean (Fa/-) rats (Greenwood et al., 1981). However, an usual level of HCA around 50 mmol/kg diet used in many previous studies (Chee et al., 1977; Greenwood et al., 1981; Rao and Sakaria, 1988; Sullivan and Triscari, 1977) was ineffective in suppressing body fat accumulation in developing Zucker obese (fa/fa) rats when the control group of rats was pair-fed with the HCA-treated rats (Greenwood et al., 1981). This ineffectiveness may be due to the several important metabolic characteristics that cause Zucker obese rats to become obese during early development, such as elevated adipose tissue lipoprotein lipase activity (Cleary et al., 1980; Gruen et al., 1978; Peinado-Onsurbe et al., 2001) and acyl-CoA synthetase activity (Shimomura et al., 1992), which contribute to increase lipogenesis. Thus, Zucker obese rats and other animal species with higher lipogenic properties appear to be insensitive to HCA treatment at the usual dietary levels. In addition, the duration of feeding experiments was generally short, and ad libitum feeding has been employed in most animal experiments conducted so far (Chee et al., 1977; Greenwood et al., 1981; Rao and Sakaria, 1988).

Therefore, this study was designed to validate the ability of HCA-containing *Garcinia cambogia* to suppress body fat accumulation in developing Zucker obese rats. We performed a dose–response study with a wide range of HCA levels in the diet, and used long-term pair-feeding with a constant energy intake. Hence, we also examined histopathologically the safety of high doses of *Garcinia cambogia* to avert adverse side effects that would be caused by its high doses.

2. Materials and methods

2.1. Animals and dietary treatment

The experimental procedures used in this study met the guidelines of the Animal Committee of Incorporated Administrative Agency, National Institute of Health and Nutrition (Tokyo, Japan).

Male Zucker obese (fa/fa) rats (Japan SLC, Hamamatsu, Japan), 6 week of age and weighing 140–150 g, were housed individually in stainless steel wire-bottomed cages at a constant temperature of 22 ± 1 °C and relative humidity of 50–60% with a 12-h light–dark cycle. The composition of the experimental diets, based on the AIN-93G purified diet for laboratory rodents (Reeves et al., 1993), is shown in Table 1. *Garcinia cambogia* powder S® was generously donated by Nippon Shinyaku Co. Ltd., Japan along with the various analytical data including its HCA content and the ratio of free to lactone form. The HCA content of the powder was 41.2 wt% and the ratio of its free to lactone form was 36.6 to 63.4. The experimental diets contained *Garcinia cambogia* powder S® at 0, 4.9, 24.4, 48.9 and 73.3 g/kg diet. These levels were equivalent to 0, 2.0, 10.1, 20.1 and 30.2 g HCA/kg diet, or 0, 10, 51, 102 and 154 mmol HCA/kg diet. The lipid content of the diets was 50 g/kg diet, representing 11.4–11.5% of the total energy.

Six rats in each group were fed the experimental diets with free access to water for 92 or 93 d. HCA has been reported to suppress food intake through appetite suppression (Greenwood et al., 1981; Rao and Sakaria, 1988; Sullivan and Triscari, 1977; Sullivan et al., 1974a), so the groups were pair-fed with the highest HCA group, which received ad libitum feeding. On the last experimental day, the rats were allowed to consume three-quarters of the food intake of the previous day and were then killed by cardiac puncture. Liver, kidney, spleen, testis, and epididymal fat pads were promptly excised, washed with isotonic saline and weighed. The liver, spleen and testis were fixed with 10% formalin neutral buffer solution, pH 7.4 and histopathological examinations were performed after hematoxylin-eosin (H.E.) staining. The histopathological scoring shown in Table 4 was blindly conducted by histopathologists in a separate institute. The rest of the tissue samples were stored at −80 °C until analysis. Serum and plasma were separated by centrifugation at 2700*g* for 15 min at 4 °C and also stored at −80 °C until analysis.

2.2. Assay procedures

Serum leptin concentration was determined with a rat leptin, ELIZA kit (Amersham Pharmacia Biotech Inc.,

Table 1
Composition of experimental diets fed to Zucker obese rats[a]

Ingredients (g/kg diet)	Group/HCA (mmol/kg)				
	G1/154	G2/102	G3/51	G4/10	G5 (control)/0
Cornstarch	326.7	351.1	375.5	395.1	400.0
Casein	200.0	200.0	200.0	200.0	200.0
Glucose	152.0	152.0	152.0	152.0	152.0
Sucrose	100.0	100.0	100.0	100.0	100.0
Soybean oil	50.0	50.0	50.0	50.0	50.0
Cellulose	50.0	50.0	50.0	50.0	50.0
Mineral mix (AIN-93G-MX)	35.0	35.0	35.0	35.0	35.0
Vitamin mix (AIN-93-VX)	10.0	10.0	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0	3.0	3.0
tert-Butylhydroquinone	0.014	0.014	0.014	0.014	0.014
Garcinia cambogia powder[b]	73.3	48.9	24.4	4.9	0.0
HCA content	30.2	20.1	10.1	2.0	0.0)
Total energy (kJ/kg diet)	16,338	16,388	16,443	16,480	16,493
Total energy (kcal/kg diet)	3903	3915	3928	3937	3940

HCA, (−)-hydroxycitric acid.
[a] The diet composition was based on the AIN-93G (Reeves et al., 1993) with a slight modification. The vitamin mixture (AIN-93-VX) contained choline bitartrate at 2.5 g/kg diet.
[b] *Garcinia cambogia* powder S® supplied by Nippon Shinyaku Co. Ltd. was used. The HCA content was 41.2% and the ratio of its free to lactone form was 36.6 to 63.4.

Piscataway, NJ, USA). Plasma non-esterified fatty acid (NEFA) concentration was measured enzymatically with the commercially available NEFA C-test WAKO. Other serum parameters such as total protein, albumin, L-aspartate:2-oxoglutarate aminotransferase (AST), L-alanine:2-oxoglutarate aminotransferase (ALT), γ-glutamyltranspeptidase (γ-GTP), alkaline phosphatase (ALP), creatinine, blood urea nitrogen (BUN), and total bilirubin were measured with commercially available kits.

Plasma testosterone was measured with radioimmunoassay method (Diagnostic Products Corporation, Los Angeles, USA). Plasma inhibin-B concentration was determined by a sandwich EIA kit (OXFORD BIO-INNOVATION LTD., Oxfordshire, UK). Plasma follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentrations were determined with a rat FSH IRMA kit (BIOCODE, Liége, Belgium) and a rat LH EIA kit (Amersham Biosciences, Buckinghamshire, UK), respectively.

ATP-citrate lyase (EC 4.1.3.8) activity in the liver and epididymal adipose tissue 10,000 g supernatant fraction was analyzed as described elsewhere (Takeda et al., 1969), and the protein content was measured by the method of Lowry et al. (1951). Liver glycogen concentration was determined according to the method of Good et al. (1933).

2.3. Statistical analysis

After confirming the normality of data and the homogeneity of variance of data for the treatment groups (the latter being evaluated by the Bartlett test), the significance of differences between mean values was assessed by 1-way ANOVA coupled with Duncan's multiple-range test at the 5% level of significance (Duncan, 1957).

3. Results

3.1. Food intake and growth

The rats consumed 14.7–15.1 g food/d and gained 2.4–2.6 g/d over the 92 or 93 d experiment (Table 2). There was no significant difference in body-weight gain among any of the treatment groups, although the food intake and body-weight gain were gradually suppressed with extended experimental duration in the rats fed the highest HCA diet (G1) ad libitum (data not shown). Additionally, a dietary HCA level over 3.0 wt% (154 mmol HCA/kg diet) caused severe diarrhea in the 6-week-old rats, and thus the upper tolerable intake level of *Garcinia cambogia* powder S® was 3.0 wt% in developing male Zucker obese rats.

3.2. Organ and epididymal fat pad weights

The testis weights in the highest and second highest HCA groups (G1 and G2) were half of those in the other groups (G3 to G5), and marked atrophy of the testis was observed in the former two groups (Table 3). There were no significant differences in weights of the liver, spleen and kidney in any of the treatment groups (data not

Table 2
Food consumption and body weight gain in Zucker obese rats fed graded levels of *Garcinia cambogia* for 92 days in G2 to G5 and 93 days in G1

		Group/HCA (mmol/kg)/wt%				
		G1/154/3.0	G2/102/2.0	G3/51/1.0	G4/10/0.2	G5 (control)/0/0.0
Food consumption	g/d	15.1 ± 1.1	14.8 ± 0.9	14.8 ± 1.0	14.9 ± 1.1	14.7 ± 1.2
HCA consumption[a]	mg/d	452.7	296.1	147.6	29.8	0.0
	mg/kg BW/d	1243.8	778.2	388.9	77.5	0.0
	mmol/d	2.30	1.51	0.75	0.15	0.00
	mmol/kg BW/d	6.33	3.96	1.98	0.39	0.00
Body weight gain	g/d	2.4 ± 0.3	2.5 ± 0.1	2.5 ± 0.2	2.5 ± 0.2	2.6 ± 0.2

HCA, (−)-hydroxycitric acid.
Each value is the mean ± S.D. $n = 6$.
[a] Average.

Table 3
Testis and epididymal fat pad weights, activities of ATP-citrate lyase in liver and epididymal fat pad, and concentrations of liver glycogen, plasma NEFA and serum leptin in Zucker obese rats fed graded levels of *Garcinia cambogia*

		Group/HCA (mmol/kg)				
		G1/154	G2/102	G3/51	G4/10	G5 (control)/0
Testis	g	0.85 ± 0.18a	0.88 ± 0.10a	1.98 ± 0.15b	1.83 ± 0.55b	1.97 ± 0.16b
Epididymal fat pad	g	9.67 ± 1.43a	12.73 ± 1.61b	12.17 ± 2.04b	13.65 ± 1.34b	13.22 ± 2.48b
	g/100 gBW	2.66 ± 0.31a	3.37 ± 0.57b	3.22 ± 0.59ab	3.55 ± 0.24b	3.50 ± 0.66b
ATP-citrate lyase						
Liver	nmol/mg protein/min	67.9 ± 14.0a	61.3 ± 11.8ab	46.9 ± 19.5bc	41.3 ± 9.1c	41.4 ± 17.5c
Epididymal fat pad	nmol/mg protein/min	5.48 ± 2.67a	8.62 ± 6.30a	8.33 ± 2.81a	8.48 ± 2.61a	9.27 ± 5.21a
Liver glycogen	mg/g liver	11.3 ± 4.2a	10.5 ± 4.0a	5.7 ± 2.4b	4.2 ± 2.6b	4.2 ± 3.0b
Plasma NEFA	mmol/l	0.47 ± 0.08a	0.69 ± 0.07bc	0.65 ± 0.07b	0.84 ± 0.24c	0.77 ± 0.12bc
Serum leptin	ng/ml	68.9 ± 30.0a	73.3 ± 23.0a	67.1 ± 22.3a	74.7 ± 25.5a	63.2 ± 29.0a

HCA, (−)-hydroxycitric acid; NEFA, non-esterified fatty acid.
Each value is the mean ± S.D. $n = 6$. Means in a row that are not followed by a common letter are different. Significance of differences between mean values was assessed by 1-way ANOVA coupled with Duncan's multiple-range test at the 5% level of significance.

shown). Epididymal fat pad weights (absolute and relative) were significantly lower in the highest HCA group (G1) than in the other groups.

3.3. ATP-citrate lyase activity and concentrations of liver glycogen, plasma NEFA and serum leptin

The activities of liver ATP-citrate lyase became lower as the dietary level of HCA decreased (Table 3), but there was no significant difference in the activities of the epididymal fat pad among any of the treatment groups, although that of the highest HCA group (G1) tended to be lower than those of the other groups. Liver glycogen concentrations became lower as the dietary level of HCA decreased, and those of the highest and second highest HCA groups (G1 and G2) were significantly higher than those of the other groups (G3 to G5). The plasma NEFA concentration was significantly lower in the highest HCA group (G1) and tended to become higher as the dietary level of HCA decreased, but the differences among groups G2 to G5 were not statistically significant. There was no significant difference in the serum leptin concentration among any of the treatment groups.

3.4. Histopathological examination

As marked atrophy of the testis was noticed at autopsy, histopathological examination of the testis, together with the liver and spleen, was performed by H.E. staining. Marked atrophy and degeneration of germ cells (Fig. 1C and D) were observed in the highest and second highest HCA groups (G1 and G2); the degeneration was dose-dependent and most marked in G1 (Table 4). However, abnormal findings were not seen morphologically in Sertoli cells and Leydig cells even in the highest and second highest HCA groups. No histopathological changes in the testis were observed in the other three groups, G3 to G5 (Table 4 and Fig. 1A and B). In the liver, fat accumulation as judged by fat vacuolation was observed in all treatment groups (Table 4), and the accumulation was conspicuous in both the highest HCA group (G1) and the control group (G5). No histopathological changes caused by *Garcinia cambogia* were observed in the spleen (data not shown).

3.5. Serum biochemical parameters

No obvious changes associated with tissue cell injury were observed in any of the treatment groups (Table 5). ALP activity was significantly lower and blood urea nitrogen (BUN) concentration was significantly higher in the highest HCA group (G1) compared with those in the control group (G5), respectively.

As shown in Table 6, there were no significant differences in the plasma concentrations of testosterone and

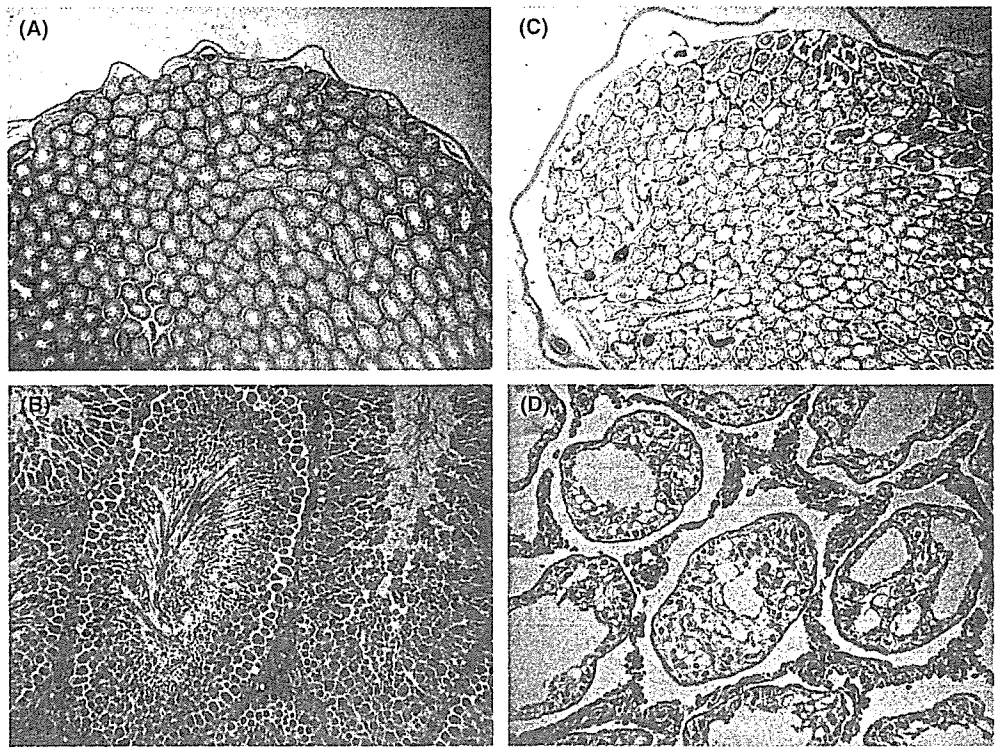

Fig. 1. Histopathological examination of the testis in Zucker obese rats fed graded levels of *Garcinia cambogia*. A: G5 (control) (H.E. stain, × 20), no significant changes; B: higher magnification of A (H.E. stain, × 200); C: G1 (H.E. stain, × 20), note marked atrophy (+++); D: higher magnification of C (H.E. stain, × 200), note marked degeneration of germ cells (+++). Abnormal findings were not seen morphologically in Sertoli cells and Leydig cells.

Table 4
Histopathological findings in testis and liver in Zucker obese rats fed graded levels of *Garcinia cambogia*

		Group/HCA (mmol/kg)/No. of animals examined				
		G1/154/6	G2/102/6	G3/51/6	G4/10/6	G5 (control)/0/6
Testis						
Germ cell degeneration	–	0	0	6	6	6
	+	0	2	0	0	0
	++	0	2	0	0	0
	+++	6	2	0	0	0
Atrophy	–	0	0	6	6	6
	+	0	0	0	0	0
	++	0	4	0	0	0
	+++	6	2	0	0	0
Liver						
Fat accumulation	–	0	0	0	0	0
	+	0	0	0	2	0
	++	0	2	2	1	0
	+++	6	4	4	3	6

HCA, (−)-hydroxycitric acid.
−: negative; +: mild; ++: moderate; +++: marked.

Table 5
Serum biochemical parameters in Zucker obese rats fed graded levels of *Garcinia cambogia*

		Group/HCA (mmol/kg)				
		G1/154	G2/102	G3/51	G4/10	G5 (control)/0
Total protein	g/l	64.2 ± 2.9a	61.8 ± 1.5ab	59.7 ± 1.4b	63.6 ± 2.9a	60.8 ± 4.1ab
Albumin	g/l	30.2 ± 1.9ac	30.3 ± 1.4ac	29.2 ± 1.7bc	31.8 ± 2.3a	29.7 ± 2.2ac
A/G		0.87 ± 0.08a	0.93 ± 0.05ac	0.97 ± 0.10ac	1.00 ± 0.10bc	0.97 ± 0.05ac
AST	μkat/l	3.6 ± 2.0a	3.5 ± 1.0a	4.9 ± 1.9a	3.4 ± 1.5a	3.6 ± 0.6a
ALT	μkat/l	4.1 ± 2.1a	4.5 ± 1.5a	7.1 ± 3.4a	3.9 ± 2.7a	4.6 ± 1.1a
γ-GTP	μkat/l	0.02 ± 0.00a	0.02 ± 0.00a	0.02 ± 0.00a	0.02 ± 0.00a	0.02 ± 0.00a
ALP	μkat/l	9.3 ± 1.3a	10.8 ± 1.3ac	12.4 ± 1.8bc	10.1 ± 3.2ac	12.0 ± 1.9bc
Creatinine	μmol/l	30.9 ± 4.8ab	29.5 ± 4.6ac	26.5 ± 0.0bc	33.6 ± 4.0a	29.5 ± 4.6ac
BUN	mmol/l	8.0 ± 2.0a	4.9 ± 1.1b	4.7 ± 0.5bc	3.4 ± 0.5c	4.0 ± 0.6bc
Total bilirubin	μmol/l	1.71 ± 0.00a	1.71 ± 0.00a	1.71 ± 0.00a	2.05 ± 0.76a	1.71 ± 0.00a

HCA, (−)-hydroxycitric acid; A/G, albumin/globulin; AST, L-aspartate:2-oxoglutarate aminotransferase; ALT, L-alanine:2-oxoglutarate aminotransferase; γ-GTP, γ-glutamyltranspeptidase; ALP, alkaline phosphatase; BUN, blood urea nitrogen. Each value is the mean ± S.D. $n = 6$. Means in a row that are not followed by a common letter are different.
Significance of differences between mean values was assessed by 1-way ANOVA coupled with Duncan's multiple-range test at the 5% level of significance.

Table 6
Concentrations of testosterone, LH, inhibin-B and FSH in plasma in Zucker obese rats fed graded levels of *Garcinia cambogia*

		Group/HCA (mmol/kg)				
		G1/154	G2/102	G3/51	G4/10	G5 (control)/0
Testosterone	ng/ml	0.19 ± 0.16a	0.20 ± 0.11a*	0.19 ± 0.09a*	0.15 ± 0.08a**	0.13 ± 0.03a
LH	ng/ml	3.85 ± 1.42a	2.32 ± 0.25a	5.22 ± 3.80a	4.92 ± 5.99a*	3.18 ± 0.98a
Inhibin-B	pg/ml	n.d. a	n.d. a	25.0 ± 15.8b	21.6 ± 3.9b*	26.5 ± 10.2b
FSH	ng/ml	37.62 ± 13.39a	31.35 ± 11.87a	9.95 ± 1.80b	9.66 ± 3.99b*	10.40 ± 2.13b

HCA, (−)-hydroxycitric acid; LH, luteinizing hormone; FSH, follicle-stimulating hormone. n.d., not detected. Each value is the mean ± S.D. $n = 6$. Means in a row that are not followed by a common letter are different.
Significance of differences between mean values was assessed by 1-way ANOVA coupled with Duncan's multiple-range test at the 5% level of significance.
* $n = 5$.
** $n = 4$.

LH among any of the treatment groups. The plasma concentrations of inhibin-B in the highest and second highest HCA groups (G1 and G2) were significantly lower and those of FSH in the same groups significantly higher than those of the other three groups (G3 to G5), but the concentrations between the latter three groups (G3 to G5) were not significantly different, respectively.

4. Discussion

The Zucker obese rat has been used extensively as a model of early-onset obesity. In addition to hyperphagia caused by leptin receptor missense mutation (Iida et al., 1996; Phillips et al., 1996) and hyperplastic–hypertrophic adipose depots (Greenwood et al., 1981), Zucker obese rats are characterized by hypercholesterolemia, hyperlipidemia, hyperleptinemia, hyperinsulinemia and insulin resistance as a recessive trait (Cleary et al., 1987; Shimomura et al., 1992), featuring similar to human obesity.

HCA-containing *Garcinia cambogia* has been shown to be active in suppressing appetite and body fat accumulation in experimental animals (Greenwood et al., 1981; Ishihara et al., 2000; Ohia et al., 2002; Rao and Sakaria, 1988; Sullivan and Triscari, 1977; Sullivan et al., 1974a; Vasselli et al., 1998). However, an usual level of HCA around 50 mmol/kg diet used in many previous studies (Chee et al., 1977; Greenwood et al., 1981; Rao and Sakaria, 1988; Sullivan and Triscari, 1977) was ineffective in suppressing body fat accumulation in developing Zucker obese (fa/fa) rats. This ineffectiveness may be due to the several important metabolic characteristics of Zucker obese rats, such as elevated adipose tissue lipoprotein lipase activity (Cleary et al., 1980; Gruen et al., 1978; Peinado-Onsurbe et al., 2001) and acyl-CoA synthetase activity (Shimomura et al., 1992), which contribute to increase lipogenesis. Thus, Zucker obese rats and other animal species with higher lipogenic properties appear to be insensitive to HCA treatment at the usual dietary levels. Therefore, in this study we administered higher levels of HCA in order to determine dose–response relationships in a long-term pair-feeding with a constant energy intake in developing Zucker obese rats.

As a result, a significant suppression of body fat accumulation was observed in epididymal adipose tissue in

the highest dietary level of HCA (Table 3). As ATP-citrate lyase activity in epididymal adipose tissue tended to be lower in the highest HCA group than in the other four groups (Table 3), high-dose HCA might suppress fatty acid synthesis, and thus suppress lipogenesis and epididymal fat accumulation, through the decreased activity of ATP-citrate lyase. This is not mediated by suppression of appetite and food intake because similar food intake with a constant energy and similar body-weight gain were observed in all of the treatment groups (Table 2). This presumption may be supported by the observations in this study that the ATP-citrate lyase activity and glycogen concentration in the liver became higher and the plasma NEFA concentration became lower as the dietary level of HCA increased (Table 3). Zucker obese rats are hyperphagic, and thus the rats receiving lower dietary levels of HCA but pair-fed with the highest HCA group may experience hunger. Similar results have been obtained in Sprague–Dawley rats, where animals consuming a diet devoid of HCA but pair-fed with HCA-treated free-feeding rats had significantly lower activities of ATP-citrate lyase and fatty acid synthetase in the liver (Chee et al., 1977). The anorectic action of HCA in the present study is not mediated by leptin, because Zucker obese rats with a leptin receptor missense mutation were used, and thus, the serum leptin concentrations were not different among any of the treatment groups (Table 3).

The ineffectiveness of the lower levels of dietary HCA against fat accumulation may be explained by the metabolic characteristics unique to Zucker obese rats, such as elevated adipose tissue lipoprotein lipase activity (Cleary et al., 1980; Gruen et al., 1978; Peinado-Onsurbe et al., 2001), with high incorporation of circulating lipids, and also elevated adipose tissue acyl-CoA synthetase activity (Shimomura et al., 1992), with enhanced lipogenesis.

Therefore, it appears from the results obtained herein that obese people who are genetically predisposed to the alterations in lipid metabolism, characteristic of Zucker obese rats, would be unlikely to benefit practically from HCA derived from *Garcinia cambogia* in terms of suppression of body fat accumulation. Moreover, acetyl-CoA production from glucose in humans has been reported to be approximately one-fortieth of that in rats because of low activity of ATP-citrate lyase in humans (Hoffmann et al., 1980), and thus it is further unlikely that obese people would experience a suppression of body fat accumulation by HCA except in the setting of an unphysiological high-carbohydrate and low-fat diet containing very high level of HCA. Actually, the results of weight loss by HCA intake in humans are very controversial (Heymsfield et al., 1998; Kovacs et al., 2001; Kriketos et al., 1999; Mattes and Bormann, 2000; Rothacker and Waitman, 1997; Sergio, 1988; Thom and Andrews, 1997; Westerterp-Plantenga and Kovacs, 2002).

HCA-containing *Garcinia* products have been on the market for more than 8 years with no adverse side effects reported so far (Ohia et al., 2002). The LD_{50} of HCA (Super CitriMax™, a calcium/potassium salt of 60% HCA) was greater than 5000 mg/kg BW when administered once orally via gastric intubation to fasted male and female albino rats (Ohia et al., 2002). There was also no evidence of acute systemic toxicity among rabbits that were dermally administered HCA at 2000 mg/kg BW (Ohia et al., 2002). The LD_{50} obtained after intraperitoneal and oral administration of HCA ((−)-hydroxycitrate trisodium salt) to mice was more than 2000 mg/kg BW and 4000 mg/kg BW, respectively (Sullivan and Triscari, 1977). Therefore, HCA-containing *Garcinia* products have been deemed to be safe.

However, as clearly demonstrated in this study, marked testicular atrophy was observed at autopsy in the groups given HCA at 154 and 102 mmol/kg diet, but not in the group given HCA at 51 mmol/kg diet. Similar testicular toxicity was also observed in Fischer 344 rats fed a diet containing high levels of HCA-containing *Garcinia cambogia* (personal communication; Sekita, S. 2004, and our unpublished data), and so the toxicity is not unique to the Zucker obese rats used in the present study. On histopathological examination, severe testicular injury was found in the 154 mmol HCA/kg diet group, characterized by marked degeneration (disappearance) and atrophy of germ cells. Similar findings were also observed in the 102 mmol HCA/kg diet group, but with less severity.

This is the first published report of the testicular toxicity of HCA-containing *Garcinia cambogia*, and therefore, we do not currently know which of the constituents of this preparation is responsible for the toxicity. The *Garcinia cambogia* powder S® used in this study has been used as an ingredient of commercially available dietary supplements for so-called dieting, and accordingly, contamination with heavy metals and environmental pollutants can be ruled out. If we tentatively suppose that the toxicity is caused by HCA, its toxic or non-toxic cutoff point exists between 102 and 51 mmol HCA/kg diet, and the lower level can be seemed to be the NOAEL. The average intake of HCA in the 102 mmol/kg diet group through the entire experiment was 778 mg HCA/kg BW/d (Table 2), and 778 mg are nearly the same amount as the lower limit of the recommended intake range of 750–1500 mg HCA/d per person in commercially available dietary supplements containing *Garcinia cambogia* in Japan (Hayamizu et al., 2001; Sawada et al., 1997). The NOAEL of 51 mmol HCA/kg diet corresponds to 389 mg HCA/kg BW/d (Table 2), and the recommended intake range for a subject with body weight 50 kg is 15–30 mg HCA/kg BW/d, which is one-twentysixth to one-thirteenth of the NOAEL. Therefore, safety for human consumption is not essentially ruled out. However, a variety of *Garcinia*

cambogia-containing so-called health foods are sold on the market, and the tablet- and capsule-type dietary supplements are easily taken in excess. Therefore, we cannot recommend their use based at least on ineffectiveness particularly in people with higher lipogenic properties.

Weight reduction of the testis in experimental animals, including testicular atrophy and degeneration in some cases, has been observed in high/excess intake of methylxanthines occurring in cocoa powder (Tarka et al., 1991), alcohol (Klassen and Persaud, 1978; Shirai and Ikemoto, 1992) and tea catechins (Satoh et al., 2002). This phenomenon has also been noticed in dietary imbalance and nutritional restriction, such as essential fatty acid deficiency (Leat et al., 1983), a protein-free, carbohydrate-free or low-fat diet (Brinkworth et al., 1992), and severe feed restriction (Levin et al., 1993). Hence, testis weight reduction may not be rare phenomenon in high/excess and/or unbalanced intake of food components. Currently, we are energetically conducting studies to elucidate the mechanisms underlying this toxicity. At least as shown in Table 6, the concentrations of Leydig cell-releasing testosterone and pituitary LH did not change, Sertoli cell-releasing inhibin-B concentrations decreased, and pituitary FSH concentrations increased in the highest and second highest HCA groups. Although abnormal findings were not seen morphologically even in Sertoli cells as well as Leydig cells (Table 4 and Fig. 1), subnormal Sertoli cell function and/or spermatogenesis is reported to result in elevated FSH level and lowered inhibin-B level (Pierik et al., 2003). Therefore, the plasma hormonal changes (Table 6) and histopathological findings (Table 4 and Fig. 1) support subnormal Sertoli cell function and/or derangement of spermatogenesis. Further detailed mechanisms of HCA-containing *Garcinia cambogia*-induced testicular toxicity remain to be solved.

In conclusion, the high dose of HCA-containing *Garcinia cambogia* (154 mmol HCA/kg diet) was effective in suppressing epididymal fat accumulation even in developing male Zucker obese rats. However, marked testicular atrophy and toxicity were observed at 778 mg HCA/kg BW/d (102 mmol HCA/kg diet) and higher, but not at 389 mg HCA/kg BW/d (51 mmol HCA/kg diet), and thus this level was deemed to be the NOAEL.

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Suppressive Effect of *Citrus aurantium* against Body Fat Accumulation and Its Safety

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Summary **A large number of so-called diet foods containing *Citrus aurantium* (CA) and its active constituent, synephrine, for suppressing body fat accumulation are currently on the market. However, only one human study has demonstrated the efficacy of CA, and its potential cardiotoxicity has been reported in a rat study. Therefore, we investigated the safety of CA and its suppressive effect against body fat accumulation in rats. High-fat (20% (w/w)) diets containing CA (synephrine content, 6.4%) at 0, 40, 200, 1,000, and 5,000 mg/kg diet were fed to rats *ad libitum* for 79 days. For dieting, the recommended daily intake of CA in many dietary supplements ranges from 100 to 1,000 mg/day, and the amount used in this experiment was approximately equivalent to 40 and/or 200 mg/kg diet. In the 5,000 mg/kg CA group, the adrenaline and dopamine concentrations in plasma were significantly higher, perirenal fat pad weight was significantly lower, and body weight tended to be lower than in the control group. Although no abnormalities of serum clinical and biochemical parameters were observed except for adrenaline and dopamine, and also no histopathological abnormalities were evident in the heart, heart weight in the 5,000 mg/kg CA group was significantly lower than in the control group. Therefore, it is necessary to examine more precisely the potential cardiotoxicity caused by excess intake of CA. Particularly, the elucidation of influences of the simultaneous intake of CA and some stimulants, such as caffeine, awaits further characterization.**

Key Words: ***Citrus aurantium*****, synephrine, dietary supplement, body fat accumulation, safety**

Introduction

Citrus aurantium (CA) is popularly used as an ingredient of many ephedra-free supplements for dieting. The active constituent of CA is considered to be synephrine (Fig. 1) [*1*], and its suppressive effect against body fat accumulation is thought to occur through β-adrenergic receptors [*2*, *3*]. Synephrine is an ephedrine-like alkaloid known to occur in citrus [*4*, *5*], and is used as a sympathomimetic drug in Europe.

It has been reported that in humans a suppressive effect against body fat accumulation occurs when synephrine-containing CA is taken with other stimulants such as caffeine [*6*]. However, there has been no other human study on the effect of synephrine on body fat accumulation. Marcus and Grollman [*7*] have warned that a combination of synephrine and caffeine has the potential to cause arrhythmia, hypertension and heart attack, as is the case for a combination of ephedra and caffeine.

Health Canada has warned consumers not to use

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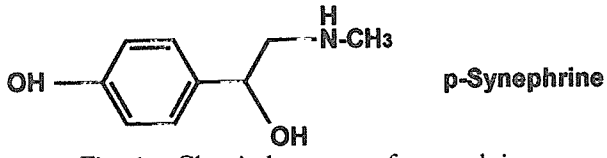

Fig. 1. Chemical structure of *p*-synephrine.

particular synephrine-containing products, especially those containing caffeine and other stimulants, and banned the import of one specific product in May, 2004[http://www.hc-sc.gc.ca/english/protection/warnings/2004/2004_30.htm] because it contained high levels of stimulants, caffeine and other ingredients that potentiate the effects of synephrine, leading to possibly serious adverse effects, including death.

On the other hand, a rat study using only CA, but not with stimulant such as caffeine, has revealed potential cardiotoxicity [*8*]. The amounts of CA used in this experiment were approximately equivalent to the recommended daily intake of CA in many dietary supplements for dieting.

In the present study, therefore, we focused on the suppressive effect of synephrine-containing CA against body fat accumulation and its safety in rats, particularly to see the sole influence of synephrine-containing CA.

Materials and Methods

Animals, diets and feeding trial

The experimental procedures used in this study met the guidelines of animal committee of Incorporated Administrative Agency, National Institute of Health and Nutrition (Tokyo, Japan). After being fed the basal diet containing 7% soybean oil for 7 days, 6–7 male Sprague-Dawley rats (CLEA Japan, Tokyo, Japan), 8 weeks of age and weighing 277–304 g, were housed individually in stainless steel wire-bottomed cages at a constant temperature of 22 ± 1°C and humidity of 50–60% with a 12-h light/dark cycle. The rats were maintained on experimental diets for 79 days. The experimental diets were formulated according to the AIN-93G diets for rodents [*9*]. The energy density of all the experimental diets was 20.1 MJ/kg diet (4,800 kcal/kg), using the Atwater energy factors for the energy calculation [*10*]. The basic components of the experimental diet given to all groups were as follows: casein, 200.0 g; L-cystine, 3.0 g; α-cornstarch, 399.5 g; sucrose, 102.5 g; cellulose powder, 50.0 g; AIN-93 vitamin mixture [*9*], 10.0 g; AIN-93G mineral mixture [*9*], 35.0 g; *tert*-butylhydroquinone, 0.014 g; soybean oil, 50 g; lard, 150 g. Lipid content of the diet was 20 wt% and 37.5% of total energy. Powdered extract of CA was added to each diet of the Groups 1–5 at the level of 0, 40, 200, 1,000, and 5,000 mg/kg, respectively, at the expense of α-cornstarch. The powdered extract of CA was purchased from Exquim, S.A. (Barcelona, Spain). As a result of the determination of synephrine by HPLC on a Sumichiral OA-5000 ligand-exchange column in accordance with the method of Kusu *et al.* [*11*], its concentration was 6.4%. Food and water were available *ad libitum*. The rats were killed by cardiac puncture. Their heart, kidney, liver, lung, spleen, testis and perirenal and epidydimal fat pads were promptly excised, washed with isotonic saline and weighed. The heart, liver and lung were fixed with 10% formalin neutral buffer solution, pH 7.4 and histopathological examinations were performed after hematoxylin-eosin staining. Serum and plasma were separated by centrifugation at 2,700 ×*g* at 4°C for 15 min and stored at −80°C until needed for analysis.

Assay procedures

Serum insulin and brain natriuretic peptide (BNP) were determined with Insulin ELISA Kit for rat (Morinaga Milk Industry Co. Ltd., Kanagawa, Japan) and Brain Natriuretic Peptide-32 (Rat) EIA Kit (Phoenix Pharmaceuticals, Inc., CA, USA), respectively. Other serum parameters determined were as follows: total protein, albumin, ratio of albumin/globulin (A/G), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), Lactate dehydrogenase (LDH), creatinine, blood urea nitrogen (BUN), glucose, glycosylated albumin, triacylglycerol, phospholipids, non-esterified fatty acid (NEFA), total cholesterol, HDL-cholesterol and total bilirubin; plasma parameters were as follows: adrenaline, noradrenalin, dopamine, thyroxine (T4) and triiodothyronine (T3); urinary parameters were as follows: adrenaline, noradrenalin, dopamine and homovanillic acid. These biochemical parameters were measured with commercially available kits.

Statistical analysis

After confirming the normality of data and the homogeneity of variance of data for the treatment groups (the latter being evaluated by the Bartlett test), the significance of differences between mean

Table 1. Influence of graded levels of dietary *Citrus aurantium* on food intake, body weight gain and tissue weights in rats.

Group (*Citrus aurantium* (mg/kg diet))	Group 1 (0)	Group 2 (40)	Group 3 (200)	Group 4 (1,000)	Group 5 (5,000)
Food intake (g/day)	18.8 ± 1.0^{NS}	18.3 ± 0.9^{NS}	18.8 ± 1.1^{NS}	19.2 ± 1.1^{NS}	18.8 ± 1.1^{NS}
Citrus aurantium intake (mg/day)	0.0 ± 0.0^{a}	0.7 ± 0.03^{ab}	3.8 ± 0.2^{b}	19.2 ± 1.1^{c}	93.9 ± 5.4^{d}
Synephrine intake (μg/day)	0 ± 0^{a}	47 ± 2^{ab}	244 ± 15^{b}	$1{,}228\pm71^{c}$	$6{,}008\pm344^{d}$
Body weight gain (g)	271.8 ± 18.2^{NS}	253.7 ± 21.9^{NS}	266.6 ± 27.6^{NS}	260.5 ± 48.0^{NS}	241.3 ± 31.0^{NS}
Initial body weight (g)	292.0 ± 7.9^{NS}	292.7 ± 9.2^{NS}	288.4 ± 7.5^{NS}	293.3 ± 10.9^{NS}	293.3 ± 6.2^{NS}
Final body weight (g)	563.8 ± 23.5^{NS}	546.3 ± 22.3^{NS}	555.0 ± 33.5^{NS}	553.8 ± 51.9^{NS}	534.6 ± 36.5^{NS}
Liver (g)	14.5 ± 2.0^{NS}	15.0 ± 1.1^{NS}	15.7 ± 1.9^{NS}	14.8 ± 2.8^{NS}	14.5 ± 1.9^{NS}
Kidney (g)	2.73 ± 0.19^{NS}	2.85 ± 0.15^{NS}	2.89 ± 0.11^{NS}	2.71 ± 0.12^{NS}	2.65 ± 0.10^{NS}
Heart (g)	1.38 ± 0.09^{a}	1.32 ± 0.03^{ab}	1.39 ± 0.08^{a}	1.37 ± 0.09^{a}	1.26 ± 0.06^{b}
Testis (g)	3.78 ± 0.14^{NS}	4.00 ± 0.22^{NS}	3.72 ± 0.53^{NS}	3.72 ± 0.26^{NS}	3.92 ± 0.14^{NS}
Spleen (g)	0.79 ± 0.13^{ab}	0.85 ± 0.10^{ab}	0.96 ± 0.22^{a}	0.81 ± 0.13^{ab}	0.76 ± 0.08^{b}
Lung (g)	1.54 ± 0.11^{NS}	1.66 ± 0.17^{NS}	1.63 ± 0.18^{NS}	1.69 ± 0.13^{NS}	1.70 ± 0.13^{NS}
Perirenal fat pad (g)	22.4 ± 2.5^{a}	18.9 ± 3.4^{ab}	22.8 ± 4.2^{a}	18.2 ± 4.4^{ab}	18.0 ± 2.9^{b}
Epididymal fat pad (g)	15.3 ± 1.7^{NS}	14.6 ± 2.9^{NS}	15.7 ± 2.9^{NS}	14.1 ± 4.1^{NS}	12.5 ± 3.5^{NS}

Each value is the mean±SD of 6–7 animals in each group. Means in a row that are not followed by a common letter are different, $p<0.05$. NS, not significant.

Table 2. Influence of graded levels of dietary *Citrus aurantium* on serum or plasma biochemical indicators in rats.

Group (*Citrus aurantium* (mg/kg diet))	Group 1 (0)	Group 2 (40)	Group 3 (200)	Group 4 (1,000)	Group 5 (5,000)
Total protein (g/liter)	59.0 ± 2.1^{NS}	58.0 ± 1.8^{NS}	57.4 ± 2.7^{NS}	58.8 ± 1.6^{NS}	59.3 ± 3.0^{NS}
Albumin (g/liter)	28.0 ± 0.9^{NS}	27.8 ± 0.8^{NS}	28.0 ± 1.2^{NS}	28.7 ± 0.5^{NS}	28.6 ± 1.5^{NS}
Ratio of albumin/globulin (A/G)	0.90 ± 0.00^{NS}	0.93 ± 0.02^{NS}	0.96 ± 0.02^{NS}	0.95 ± 0.02^{NS}	0.91 ± 0.01^{NS}
Asparate aminotransferase (U/liter)	88.2 ± 12.2^{NS}	111.0 ± 19.7^{NS}	89.4 ± 14.0^{NS}	85.8 ± 16.8^{NS}	107.7 ± 31.9^{NS}
Alanine aminotransferase (U/liter)	29.8 ± 11.5^{NS}	32.3 ± 12.2^{NS}	29.2 ± 9.3^{NS}	25.5 ± 10.5^{NS}	34.1 ± 19.1^{NS}
Alkaline phosphatase (U/liter)	252.0 ± 121.6^{NS}	253.8 ± 112.8^{NS}	204.6 ± 47.6^{NS}	233.3 ± 46.1^{NS}	259.1 ± 78.5^{NS}
Creatinine (μmol/liter)	36.7 ± 2.7^{NS}	34.6 ± 2.1^{NS}	31.1 ± 16.0^{NS}	38.0 ± 4.7^{NS}	36.1 ± 5.0^{NS}
Urea nitrogen (mmol/liter)	3.0 ± 0.2^{NS}	3.0 ± 0.2^{NS}	2.6 ± 0.3^{NS}	2.9 ± 0.2^{NS}	3.0 ± 0.5^{NS}
Glucose (mmol/liter)	12.3 ± 1.5^{NS}	11.8 ± 0.4^{NS}	12.2 ± 1.3^{NS}	12.4 ± 1.9^{NS}	12.4 ± 0.9^{NS}
Glycosylated albumin (%)	3.1 ± 0.1^{NS}	2.9 ± 0.3^{NS}	3.2 ± 0.3^{NS}	3.2 ± 0.3^{NS}	3.1 ± 0.3^{NS}
Triacylglycerol (mmol/liter)	0.85 ± 0.21^{NS}	0.87 ± 0.11^{NS}	0.89 ± 0.51^{NS}	1.04 ± 0.39^{NS}	1.05 ± 0.31^{NS}
Phospholipid (mmol/liter)	1.26 ± 0.04^{NS}	1.34 ± 0.14^{NS}	1.12 ± 0.57^{NS}	1.21 ± 0.11^{NS}	1.46 ± 0.29^{NS}
Non-esterified fatty acid (mmol/liter)	0.43 ± 0.04^{NS}	0.36 ± 0.06^{NS}	0.35 ± 0.14^{NS}	0.42 ± 0.08^{NS}	0.44 ± 0.11^{NS}
Total cholesterol (mmol/liter)	1.45 ± 0.13^{NS}	1.58 ± 0.35^{NS}	1.31 ± 0.39^{NS}	1.16 ± 0.20^{NS}	1.40 ± 0.23^{NS}
HDL-cholesterol (mmol/liter)	0.53 ± 0.07^{NS}	0.53 ± 0.06^{NS}	0.48 ± 0.09^{NS}	0.44 ± 0.08^{NS}	0.54 ± 0.11^{NS}
Total bilirubin (μmol/liter)	2.00 ± 0.29^{NS}	2.28 ± 0.36^{NS}	1.71 ± 0.00^{NS}	1.71 ± 0.00^{NS}	1.72 ± 0.36^{NS}
Insulin (ng/ml)	4.04 ± 2.50^{bc}	4.63 ± 1.65^{bc}	7.84 ± 2.99^{a}	5.58 ± 2.70^{ab}	2.75 ± 0.71^{c}
Brain natriuretic peptide (μg/liter)	4.13 ± 0.16^{b}	4.36 ± 0.18^{a}	4.17 ± 0.15^{b}	4.30 ± 0.09^{ab}	4.16 ± 0.07^{b}
Triiodothyronine (nmol/liter)	1.23 ± 0.10^{NS}	1.32 ± 0.13^{NS}	1.28 ± 0.14^{NS}	1.29 ± 0.26^{NS}	1.25 ± 0.16^{NS}
Thyroxine (nmol/liter)	46.3 ± 4.4^{NS}	49.5 ± 11.2^{NS}	49.4 ± 7.5^{NS}	44.4 ± 3.8^{NS}	43.2 ± 9.3^{NS}

Each value is the mean±SD of 6–7 animals in each group. Means in a row that are not followed by a common letter are different, $p<0.05$. NS, not significant.

values was assessed by 1-way ANOVA coupled with Duncan's multiple-range test at the 5% level of significance [*12*].

Results

The rats consumed 18.3-19.2 g food/day and gained 3.1–3.4 g body weight/day over the 79-day experiment. There was no significant effect of dietary CA on the food intake (Table 1). There was no significant difference in the body-weight gain between any of the treatment groups (Table 1). The weights of liver, kidney, testis, spleen, lung and epididymal fat pad in the rats fed CA were not signifi-

Table 3. Influence of graded levels of dietary *Citrus aurantium* on plasma concentrations of catecholamine in rats.

Group (*Citrus aurantium* (mg/kg diet))	Group 1 (0)	Group 2 (40)	Group 3 (200)	Group 4 (1,000)	Group 5 (5,000)
Adrenaline (nmol/liter)	53.85 ± 25.40^{a}	59.95 ± 16.63^{a}	69.97 ± 23.80^{ab}	60.04 ± 46.46^{a}	105.27 ± 34.77^{b}
Noradrenaline (nmol/liter)	53.99 ± 29.11^{NS}	38.62 ± 17.31^{NS}	53.08 ± 26.29^{NS}	53.39 ± 42.36^{NS}	68.06 ± 31.74^{NS}
Dopamine (nmol/liter)	1.29 ± 1.19^{a}	0.90 ± 0.57^{a}	1.20 ± 0.33^{ab}	0.81 ± 0.88^{a}	2.41 ± 1.30^{b}

Each value is the mean±SD of 6–7 animals in each group. Means in a row that are not followed by a common letter are different, $p<0.05$.NS, not significant.

Table 4. Influence of graded levels of dietary *Citrus aurantium* on urinary concentrations of catecholamine and homovanillic acid in rats.

Group (*Citrus aurantium* (mg/kg diet))	Group 1 (0)	Group 2 (40)	Group 3 (200)	Group 4 (1,000)	Group 5 (5,000)
Adrenaline (nmol/day)	1.26 ± 0.58^{a}	1.32 ± 0.74^{a}	1.08 ± 0.19^{a}	2.13 ± 1.09^{a}	4.22 ± 2.77^{b}
Noradrenaline (nmol/day)	7.67 ± 1.75^{NS}	9.11 ± 1.70^{NS}	7.13 ± 2.10^{NS}	9.45 ± 1.78^{NS}	7.75 ± 1.32^{NS}
Dopamine (nmol/day)	22.51 ± 3.07^{NS}	20.82 ± 2.07^{NS}	20.93 ± 2.81^{NS}	23.81 ± 3.73^{NS}	22.59 ± 4.97^{NS}
Homovanillic acid (nmol/day)	146.0 ± 7.5^{NS}	139.2 ± 30.1^{NS}	133.7 ± 22.1^{NS}	137.1 ± 20.5^{NS}	137.8 ± 36.9^{NS}

Each value is the mean±SD of 6–7 animals in each group. Means in a row that are not followed by a common letter are different, $p<0.05$. NS, not significant.

cantly different from the control group (Table 1). The weights of heart and perirenal fat pad were significantly lower in the 5,000 mg/kg CA group than in the control group. No abnormal histopathological changes were recognized in the liver, lung and heart (data not shown).

The variations of serum BNP concentration in the treatment groups were biologically negligible (Table 2). The concentration of serum insulin in the 200 mg/kg CA group was significantly higher and that of the 5,000 mg/kg CA group tended to be lower than in the control group (Table 2). Other serum or plasma parameters were not significantly different among any of the treatment groups (Table 2).

The plasma concentrations of adrenaline and dopamine were significantly higher in the 5,000 mg/kg CA group than in the control group (Table 3). The concentration of noradrenaline in the plasma was not significantly different among all the groups (Table 3). The urinary concentration of adrenaline was significantly higher in the 5,000 mg/kg CA group than in the other groups (Table 4). In contrast, those of the noradrenaline and dopamine were not significantly different among all the groups. The urinary concentration of homovanillic acid was not significantly different among any of the treatment groups.

Discussion

Calapai *et al.* [8] reported that administration of only CA (synephrine content, 6%), but not with any stimulants, to Sprague-Dawley rats (240–280 g) *via* a stomach tube at 2.5–20 mg/kg body weight caused a dose-dependent decrease of food intake and suppression of body weight gain after 7 days. Furthermore, mortality increased from 10 to 50% with increasing oral administration of only CA over a period of 15 days. In the present study, however, perirenal fat pad and heart weights were significantly lower and body weight gain tended to be lower only in the group receiving the maximal amount of CA (5,000 mg/kg diet), although these changes were small. The CA (and synephrine) intake values calculated from the final body weight of groups 2–5 were 1.3 (0.1), 6.8 (0.4), 34.7 (2.2), and 176.1 (11.3) mg/kg, respectively. In our study and that of Calapai *et al.*, the strain and sex of the rats were the same, and the age, body weight and synephrine content of the CA given to the rats were almost the same. The major differences between the two experiments were the way in which the CA was administered, in addition to the intake level and the amount of dietary lipid. When a CA-containing diet is fed to rats by *ad libitum*, it is thought that the absorption rate of CA and synephrine from the digestive tract is higher than that in the case for forced feeding by stomach intubation. However, stomach intubation may result

in a transient rapid rise in the blood concentration of ingested material compared with *ad libitum* feeding. Therefore, ingestion of CA in the form of a dietary supplement may enhance the efficacy of synephrine. At the same time, it is thought that if the influence of synephrine is rapidly elicited beyond the response threshold, harmful and/or toxic effects might easily occur. The precise mechanism of this action is remained to be solved. Such study using stomach intubation is now under way as an extension of our current study.

Since the rats in this study received a 20% (w/w) high-fat diet for 79 days, their accumulation of body fat was remarkable. β_3-Adrenergic receptors are localized in the white and brown adipose tissues of rats [*13*, *14*], and these receptors are known to control lipolysis in white adipose tissue and thermogenesis in brown adipose tissue. However, it is reported that the expression of β_3-adrenergic receptor mRNA in white and brown adipose tissue is decreased in obese rats, such as those of the Zucker strain [*13*]. Furthermore, the effect of synephrine on lipolysis in rats occurs mostly through its action on β_1- and β_2-adrenergic receptors, and thus its dependence on β_3-adrenergic receptors is relatively small [*3*]. Under the present experimental conditions, therefore, the suppressive effect of CA and/or synephrine against body fat accumulation might have been small. Hence, in a high-carbohydrate diet compared with a high-fat diet, this effect of CA and/or synephrine against mesenteric fat accumulation might be smaller than that in a high-fat diet, since the high-carbohydrate diet feeding resulted in a preferential accumulation of mesenteric adipose mass compared with the high-fat diet feeding [*15*].

β_3-Adrenergic receptors are considered to be the major receptors for catecholamines in rodent fat cells [*13*, *14*, *16–18*], but it is reported that the sensitivity of β_3-adrenergic receptors to adrenaline is lower than that of β_1- and β_2-adrenergic receptors [*19*, *20*]. In the maximal CA intake group (5,000 mg/kg diet), where a significant decrease of perirenal fat pad weight and a tendency for reduced body weight gain were recognized, the concentrations of adrenaline and dopamine in plasma and the excretion of adrenaline into urine were significantly higher than those in the other groups. Increased levels of synephrine and catecholamines in the blood might affect the few β_1- and β_2-adrenergic receptors present in the perirenal adipose tissue, and thus promote a decrease of stored fat. However, mechanisms of increase of catecholamines in plasma by CA intake are unclear. In rats, it has been reported that *m*- and *p*-synephrine were detected only in the adrenal medulla, and not in other organs, even after administration of a monoamine oxidase inhibitor [*21*]. As the adrenal medulla would be expected to receive stimulation with synephrine, its secretion of adrenaline and dopamine might be promoted by *p*-synephrine in CA. This speculation invites further empirical investigation. A full understanding of the mechanisms of increase of catecholamines in plasma by CA intake awaits future studies. Moreover, the finding that the serum insulin level in the maximal CA intake group (5,000 mg/kg diet) tended to be lower than that in the control group is consistent with the phenomenon that insulin competes with adrenaline in lypolysis. Additionally, the decrease of stored fat in rats might raise their sensitivity to insulin because the concentration of serum glucose in the maximal CA intake group did not differ from those of the other groups. However, since there was no change in the serum level of non-esterified fatty acid, the reason for this remains to be explained.

β_1-Adrenergic receptors are the major subtype controlling the rate and contraction of the heart [*22*]. In the rat heart, stimulation of β_1-adrenergic receptors by catecholamines accentuates cardiac function and cardiomyopathy, and thus causes leakage of lactate dehydrogenase (LDH) [*23*]. This phenomenon is suggested to be caused by adrenochrome, which is a non-physiological metabolite of adrenaline [*23*]. In the present experiment, these observations would be associated with the increased concentration of plasma adrenaline and the concomitant decrease of heart weight in the maximal CA intake group.

Actual maximal CA intake (Group 5) in this experiment was 176.1 mg/kg of final body weight per day, which was about 1/3 of the LD_{50} (476.94 mg/kg body weight/day) for mice [*24*]. The recommended daily intake of CA in humans ranges from 100 to 1,000 mg/day, and this is equivalent to 2 to 20 mg/kg body weight/day when calculated for a body weight of 50 kg. Therefore, this is within the range 1/90 to 1/9 of 176.1 mg/kg/day. Under the present experimental conditions, no histopathological abnormalities of the heart were observed by light microscopy even in the maximal CA intake group, and also there were few changes in the concentration of BNP, which is an index of chronic and acute heart failure, or serum clinical and biochemical parame-

ters. Therefore, if the suggested instructions on the label of dietary supplements of CA are properly followed, and its excessive intake with some stimulants including caffeine in teas and coffee is avoided, then no safety problems may occur.

On the other hand, however, Calapai *et al.* [*8*] reported that administration of only CA (synephrine content, 6%) at 20 mg/kg body weight to Sprague-Dawley rats (240–280 g) by stomach tube for 15 days caused electrocardiographic abnormalities in 80% of the rats, and that 50% of them died. Furthermore, Marcus and Grollman [7] have warned that a combination of synephrine and caffeine has the potential to cause cardiac arrhythmia, hypertension, heart attack, and stroke, as is the case for a combination of ephedra and caffeine. In fact, a case of acute lateral-wall myocardial infarction in a caucasian woman possibly caused by ingestion of a CA-containing dietary supplement has been reported [*25*]. Therefore, the possible association between CA intake and cardiotoxicity remains to be examined in more detail. Additionally, a determination of the influences of simultaneous intake of CA and some stimulants, such as caffeine, awaits further characterization.

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Note

Evaluation of the Correlation Between Amount of Curcumin Intake and its Physiological Effects in Rats

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We examined the correlation between amount of curcumin intake and its physiological effects on indices of liver function, serum and liver lipid profiles in rats. Animals were fed diets containing 0.5, 5 and 50 mg curcumin per 100 g body weight for 28 days.

HDL-cholesterol concentrations of rats fed curcumin diets were significantly higher ($P<0.05$) than those of the control group, and serum TG concentration of rats fed the x100 curcumin diets was significantly lower ($P<0.05$) than that of the x1 curcumin group. Serum TG concentration of rats fed curcumin diets tended to decrease in a curcumin dose-dependent manner. These results indicate that curcumin intake can improve serum lipid profiles effectively.

Keywords: Curcumin, Rat serum, Triglyceride, HDL-cholesterol, Liver function.

Introduction

Turmeric has long been used as a traditional remedy in Asia. Its commercial derivatives currently demand high prices in the so-called health food market of Japan because it is well known that they have various beneficial effects on human health. These physiological effects are emphasized by manufacturers and promote sales in the so-called health food market.

The main physiological ingredient of turmeric is considered to be the 3–5% of curcumin contained in *Curcuma longa*. Curcumin is a well-known natural anti-oxidant (Sharma, 1976). In addition to such a function, it has been reported that curcumin has various physiological functions such as lowering cholesterol (Ramirez-Tortosa *et al.*, 1999), improving liver function (Park *et al.*, 2000), suppressing tumor activity (Surh, 2002), and it can be used as an anti-inflammatory (Rao *et al.*, 1982).

Although curcumin is thought to be the main active ingredient of turmeric, whether there is a clear relation between the physiological function of turmeric and curcumin content has not yet been established. This is because turmeric consists of various natural materials such as minerals, dietary fiber, tannin, curcumin, flavonoids, camphor, azulene and similar compounds. In addition, the curcumin content in turmeric is about 5% at most (Hiserodt *et al.*, 1996), and absorptivity is considered to be very low (Asai and Miyazawa, 2000). Therefore, it seems unlikely that curcumin acts independently when turmeric is ingested because similar effects are observed in *Curcuma aromatica* (Salisb) and *Curcuma zedoaria* (Roscoe), which hardly contain curcumin. Thus, the relation between the amount of curcumin contained in turmeric and substantial physiological effects are still poorly understood. Therefore, it is important to examine the actual effects associated with ingestion of a certain amount or excessive intake of curcumin.

In the current study, to investigate the relation between amount of curcumin intake and its physiological effects on indices of liver function, serum and liver lipid profiles and other biochemical parameters, male Wistar rats were fed 0.5 mg (x1), 5 mg (x10), 50 mg (x100) curcumin-containing diets or a curcumin-free diet for 28 days. In addition, we also conducted histological observations of metabolic organs such as liver and kidney to gain more insight into the metabolic state resulting from excessive curcumin.

Materials and Methods

Male Wistar rats (8 weeks old) were purchased from Japan SLC Inc. (Shidzuoka, Japan). After acclimation for 1 week, rats were randomly divided into four groups ($n=6$/group). Rats were fed experimental diets ad libitum for 4 weeks. Experimental diets were based on AIN-93G formula of the following ingredients (g/kg diet): casein, 200; corn starch, 150; test oil, 100; AIN-93G mineral mixture, 35; AIN-93G vitamin mixture, 10; cellulose, 50; D, L-methionine, 3; choline bitartrate, 2 and ~1000 sucrose. The experimental diets contained 0.5 (x1), 5 (x10) or 50 (x 100) mg curcumin per 100 g body weight, while the control group received the AIN-93G type diet without curcumin. The reagent-grade curcumin was purchased from Wako Pure Chemical Industries, Ltd. The basal daily amount of curcumin in rats was estimated by weight conversion based on the recommended daily intake for humans. At the end of the experimental period, after overnight fast-

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ing, rats were anesthetized and sacrificed for analysis. Care and use of laboratory animals was in accordance with the guidelines of the National Institute of Health and Nutrition.

Blood samples were obtained from the rat abdominal aorta after overnight fasting, and centrifuged at 3,000 rpm for 15 min. Sera were stored at −80℃ before analysis. Serum lipids (total cholesterol, high density lipoprotein (HDL)-cholesterol and triglyceride (TG)), liver function indices (GOT, GPT, γ-GPT, ALP and LDH) and other biochemical parameters (total protein and blood glucose) were analyzed enzymatically using commercially available assay kits (Wako Pure Chemical, Osaka). Serum insulin concentrations were measured using a commercially available EIA kit (Biotrak, Amersham Pharmacia Biotech, MO).

Liver was excised, weighed and stored at −80℃ until analysis. The liver lipids were extracted with chloroform-methanol (2: 1 v/v) (Folch *et al.*, 1957). Liver cholesterol and TG concentrations were determined using a Cholesterol E-test and Triglyceride E-test (Wako, Osaka) as described elsewhere with minor modifications (Carr *et al.*, 1993).

The liver and kidneys obtained from rats fed control and x100 curcumin diets were fixed with 10% formaldehyde solution (pH=7.4) and embedded in paraffin. Paraffin-embedded specimens were prepared, and stained with hematoxylin and eosin. Sample preparation and microscopic examination was performed at the Sapporo Pathology Research Institute.

Data are presented as means±SEM (standard error of the mean). The statistical significance of the difference was evaluated by ANOVA followed by Fisher's PLSD test. Differences at $P<0.05$ were considered to be significant.

Results and Discussion

There were no significant differences in body weight gain, food intake, and relative weights of liver, kidney, spleen, testis, epididymal and perirenal adipose tissue between groups by curcumin intake. These findings indicate that excessive curcumin intake did not affect the growth of rats. Therefore, it is thought that curcumin as a component of food is not harmful on rat growth in the range of curcumin consumption measured in the present study. Pathological examination revealed lipid deposition or extramedullary hematopoiesis in one sample and small granuloma in the livers of two rats fed the x100 curcumin diet. However, these manifestations were not considered a pathological problem.

Serum HDL-cholesterol concentrations of rats fed curcumin diets were significantly higher ($P<0.05$) than that of control group. Serum TG concentration of rats fed the x100 curcumin diets was significantly lower ($P<0.05$) than that of the x1 curcumin group. Serum TG concentration tended to decrease in a curcumin dose-dependent manner (Fig. 1). No significant differences in serum and liver cholesterol, and liver TG concentrations were observed between groups. The hypocholesterolemic effect of curcumin can probably be explained by its effect

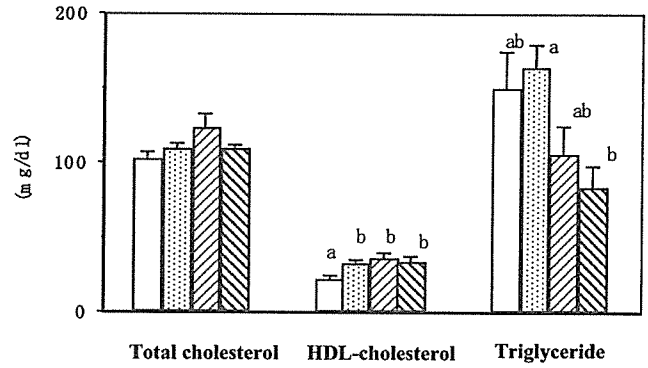

Fig. 1. Serum lipid profiles of rats maintained on different curcumin diets.
Values are means±SEM for 6 rats. Values not sharing a common letter differ, $P<0.05$.
□: Control diet, ⬚: x1 Curcumin diet, ▨: x10 Curcumin diet, ▧: x100 Curcumin diet.

on the stimulation of bile fluid and biliary cholesterol secretion and enhanced excretion of bile acids and cholesterol in feces (Ramprasad and Sirsi, 1957; Patil and Srinivasan, 1971; Srinivasan and Sambaiah, 1991). Although, in this study, we did not observe a marked reduction in serum cholesterol concentrations in rats fed curcumin diets, the results agree with a previous report, which indicated that the plasma cholesterol levels of animals fed cholesterol-free diet were not affected by curcumin intake (Rao *et al.*, 1970). Furthermore, curcumin ingestion contributed to an increase in HDL-cholesterol concentration regardless of the amount of curcumin ingestion. These results indicate that curcumin intake modulated HDL- and LDL-cholesterol concentrations, maintaining proportions at desirable levels. It is known that a decrease in the ratio of LDL- to HDL-cholesterol concentrations leads to improvement in the arteriosclerosis index (Hostmark *et al.*, 1990). Moreover, although Asai *et al.* also observed a reduction in serum TG concentration (Asai and Miyazawa, 2001), the critical regulatory mechanism of curcumin on the reduction in serum TG concentration has not yet been clarified. Thus, the alterations in serum lipid profiles observed in the present study could not be explained by the increase in fecal bile acid excretion alone, and further detailed study will be required to examine the regulatory mechanism of curcumin on serum lipid profiles.

Liver function indices were not significantly influenced by curcumin ingestion, although GOT and GPT slightly increased, and γ-GTP and ALP tended to decrease in accordance with an increase in curcumin intake (Table 1). It is well known that effects of liver function improvement are the typical physiological effect associated with curcumin (Nirmala and Puvanakrishnan, 1996; Rukkumani *et al.*, 2004). Since the present study was carried out using a normal animal model, a marked improvement in liver function might not be observed under these experimental conditions. However, use of an impaired liver function animal model, a heavy cholesterol diet, or over-

Table 1. Serum biochemical parameters in rats maintained on different curcumin diets.

	Control	Curcumin		
		x1	x10	x100
		(IU/L)		
GOT	41.2±4.54	54.9±2.25	49.7±1.84	52.8±5.26
GPT	1.57±0.45	3.53±0.53	0.47±0.30	2.59±0.59
γ-GTP	6.11±0.76	5.67±0.51	4.11±0.25	3.93±0.47
		(K-A unit)		
ALP	88.5±13.6	63.9±8.28	56.1±10.1	57.2±4.68
		(mg/dl)		
LDH	736.2±3.23	727.9±5.99	731.1±6.66	672.2±67.3
		(g/dl)		
Total protein	6.33±0.20	6.16±0.11	5.84±0.18	5.97±0.06
		(mg/dl)		
Blood glucose	96.9±12.9a	120.6±4.89ab	140.6±8.68bc	159.0±9.00c
		(ng/ml)		
Insulin	1.13±0.11	1.11±0.18	1.75±0.34	2.36±0.63

Values are means±SEM, n=6. Values not sharing a common letter differ significantly ($P<0.05$).

dose of curcumin under the cruel experimental conditions in animal models might be better suited for illustrating the effect of the amount of curcumin intake on improved liver function (Park *et al.*, 2000; Patil and Srinivasan, 1971; Rao *et al.*, 1970). In the present study, however, we found that moderate amounts of curcumin consumption improved the ratio of HDL- and LDL-cholesterol concentrations, even in a healthy animal model. While curcumin intake increased both blood glucose and serum insulin concentrations in this study (Table 1), previous studies on blood glucose, serum insulin or diabetes found that the antioxidant properties of curcumin had antidiabetic effects (Srivivasan *et al.*, 2003; Arun and Nalini, 2002; Nishizono *et al.*, 2000). It may therefore be necessary to consider changes in these biochemical characteristics in future research.

In conclusion, it is noteworthy in this study that curcumin intake improved the proportion of HDL- and LDL-cholesterol concentrations, even at curcumin concentrations found in turmeric, and excessive curcumin intake reduced the serum TG concentration in healthy rats. These results imply that curcumin may contribute to the regulation of lipid metabolism. The mechanisms of action are not yet clear and further study will be necessary to understand how curcumin affects liver function. In addition, the physiological effect of other turmeric ingredients, such as natural polyphenolic compounds, needs to be considered.

To clarify the substantial physiological benefits of turmeric or curcumin, we intend to study the effects of long-term or excessive feeding in an impaired animal model, such as animals with liver dysfunction and hyperlipidemia. A series of such experiments will increase our understanding of the regulatory mechanisms of lipid metabolism and the correlation between physiological function and amount of turmeric intake.

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Selective protection of curcumin against carbon tetrachloride-induced inactivation of hepatic cytochrome P450 isozymes in rats

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Abstract

We investigated the effects of curcumin, a major antioxidant constituent of turmeric, on hepatic cytochrome P450 (CYP) activity in rats. Wistar rats received curcumin-containing diets (0.05, 0.5 and 5 g/kg diet) with or without injection of carbon tetrachloride (CCl_4). The hepatic CYP content and activities of six CYP isozymes remained unchanged by curcumin treatment, except for the group treated with the extremely high dose (5 g/kg). This suggested that daily dose of curcumin does not cause CYP-mediated interaction with co-administered drugs. Chronic CCl_4 injection drastically decreased CYP activity, especially CYP2E1 activity, which is involved in the bioactivation of CCl_4, thereby producing reactive free radicals. Treatment with curcumin at 0.5 g/kg alleviated the CCl_4-induced inactivation of CYPs 1A, 2B, 2C and 3A isozymes, except for CYP2E1. The lack of effect of curcumin on CYP2E1 damage might be related to suicidal radical production by CYP2E1 on the same enzyme. It is speculated that curcumin inhibited CCl_4-induced secondary hepatic CYPs damage through its antioxidant properties. Our results demonstrated that CYP isozyme inactivation in rat liver caused by CCl_4 was inhibited by curcumin. Dietary intake of curcumin may protect against CCl_4-induced hepatic CYP inactivation via its antioxidant properties, without inducing hepatic CYPs.

Keywords: Curcumin; Carbon tetrachloride; Free radicals; CYP2E1; Hepatotoxicity

Introduction

Recently, interest in complementary and alternative medicine has grown rapidly in industrialized countries, and the demand for herbal remedies has currently increased (De Smet, 2002; Ammon and Wahl, 1991). Turmeric, the rhizome of *Curcuma longa* L., has traditionally been used for treatment of gastrointestinal colic, flatulence, hemorrhage, hematuria, menstrual difficulties and jaundice. The anti-inflammatory and hepatoprotective characteristics of turmeric and its constituents have been widely investigated (Govindarajan, 1980; Luper, 1999; Miquel et al., 2002). The most well-researched component of turmeric is curcumin (diferuloylmethane, Fig. 1). Curcumin is the major yellow pigment comprising 3–6% of turmeric, and has been widely used in curry, mustard, cosmetics and drugs (Govindarajan, 1980; Miquel et al., 2002). Curcumin is well known for its pharmacological properties including antioxidant, anti-inflammatory, antimutagenic and anticancer activity (Miquel et al., 2002; Okada et al., 2001; Asai and Miyazawa, 2001; Ramirez-Tortosa et al., 1999; Sharma et al., 2004). The preventive and improved effects of curcumin on symptoms of liver diseases are shown to stem from its antioxidant effects (Rukkumani et al., 2004; Park et al., 2000; Nanji et al., 2003).

Many alternative remedies including turmeric may be taken with medicine; hence, pharmacological interactions are a concern in clinical therapy (Ernst, 2002; Williamson, 2001). Changes in the pharmacokinetics and pharmacodynamics of co-administered drugs affects clinical efficacy, and occasion-

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