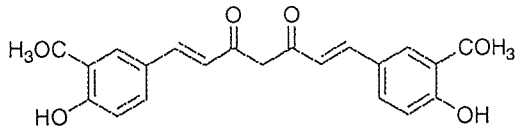

Fig. 1. Chemical structure of curcumin.

ally severe adverse reactions occur. We have examined cytochrome P450 (CYP)-mediated interactions with herbal remedies and medication (Umegaki et al., 2002; Kubota et al., 2004; Sugiyama et al., 2004). Regarding the metabolic functions of the liver, the effects of curcumin on metabolic enzymes, especially hepatic CYP activity, have not been completely elucidated. In addition, many liver diseases are related to lipid peroxidation in liver tissue, and some antioxidant components have a protective effect against liver damage. Curcumin has antioxidative properties and prevents some oxidative stress, and the action of curcumin has been shown to be beneficial for inhibition of tissue injury (Luper, 1999; Miquel et al., 2002; Okada et al., 2001; Khopde et al., 2000). Carbon tetrachloride (CCl_4), a well-known model compound for producing chemical hepatic injury, requires biotransformation by hepatic microsomal CYP to produce toxic metabolites, namely trichloromethyl free radicals (Recknagel et al., 1989; Brattin et al., 1985; Brautbar and Williams, 2002). CYP2E1 is the major isozyme involved in bioactivation of CCl_4 and subsequent production of free radicals (Recknagel et al., 1989). It has been proposed that the antioxidative action of curcumin plays an important role in its hepatoprotective effects against CCl_4-induced liver injury (Park et al., 2000). However, the mechanism by which curcumin protects the liver against CCl_4-induced toxicity is unclear, particularly in association with CYP activity.

This study was undertaken to evaluate the effect of repeated curcumin ingestion on hepatic CYP enzymes and to examine the protective effect of curcumin on CCl_4-induced hepatic CYP damage in rats.

Materials and methods

Materials

Curcumin was purchased from Wako Pure Chemical Ltd. (Osaka, Japan). Resorufin, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, testosterone, 6β-hydroxytestosterone, corticosterone, *p*-nitrophenol, 4-nitrocatechol and 7-ethoxycoumarin were purchased from Sigma (St. Louis, MO, USA). (*S*)-Warfarin and 7-hydroxywarfarin were obtained from Ultrafine (Manchester, England). NADPH was obtained from Oriental Yeast (Tokyo, Japan). Other reagents were obtained from Wako Pure Chemical Ltd. (Osaka, Japan).

Animal experiments

Male Wistar rats (5 weeks old) obtained from Japan SLC (Shizuoka, Japan) were housed individually in stainless steel, wire-bottomed cages at a constant temperature (23 ± 1 °C) under a 12 h light–dark cycle. Rats were given AIN-93G based diets (containing 53.2% (w/w) α-corn starch, 20% milk casein, 10% sucrose, 7% corn oil, 5% cellulose, 3.5% mineral mix (AIN-93G-MX), 1.0% vitamin mix (AIN-93G-VX), 0.3% L-cysteine and 0.0014% *tert*-butylhydroquinone) (Reeves et al., 1993) with or without curcumin (0.05, 0.5 and 5 g/kg diet) for 4 consecutive weeks. In order to examine the protective effects of curcumin on the liver damage, rats were subcutaneously injected with CCl_4 (50% (v/v in olive oil) for 0.2 ml/100 g body weight) twice a week during the 7 weeks of curcumin ingestion. After these treatments, rats were anesthetized with pentobarbital and sacrificed, the blood was collected, and the livers were immediately removed and weighed. The glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) activities in plasma were determined using an assay kit, transaminase CII-Test Wako (Wako Pure Chemical Ltd., Osaka, Japan).

All procedures were in accordance with the National Institute of Health and Nutrition guidelines for the Care and Use of Laboratory Animals.

Preparation of microsome and cytosolic fractions from the liver

The liver was rinsed with 0.9% (w/v) NaCl solution and homogenized in 50 mmol/L Tris–HCl buffer (pH 7.4) containing 0.25 mol/L sucrose. The homogenate was centrifuged at 10,000×*g* at 4 °C for 30 min. The supernatant was further centrifuged at 105,000×*g* at 4 °C for 60 min. The supernatant was used as the cytosolic fraction for the assay of glutathione *S*-transferase, the activity of which was determined using 1-chloro-2,4-dinitrobenzene as a substrate (Habig and Jakoby, 1981). The pellet was washed once with 50 mmol/L Tris–HCl buffer (pH 7.4) containing 0.25 mol/L sucrose by centrifugation at 105,000×*g* at 4 °C for 60 min, and the concentration and activities of CYP were analyzed.

Protein concentrations of microsomal and cytosolic fractions were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA).

Analysis of CYP enzyme activities

The CYP content was quantified by the method of Omura and Sato (1964). The activities of various CYP enzymes were determined by HPLC methods as reported previously (Umegaki et al., 2002). The subtypes of CYP enzymes examined and the corresponding CYPs were ethoxyresorufin *O*-deethylase, CYP1A1; methoxyresorufin *O*-demethylase, CYP1A2; pentoxyresorufin *O*-dealkylase, CYP2B; (*S*)-warfarin 7-hydroxylase, CYP2C9; *p*-nitrophenol hydroxylase, CYP2E1; and testosterone 6β-hydroxylase, CYP3A (Hanioka et al., 2000; Mishin et al., 1996; Lang and Bocker, 1995).

Statistical analysis

The data are presented as means with standard deviation (S.D.) for the individual groups. Statistical analysis of the data was carried out using ANOVA followed by a post hoc test of Fisher's

Table 1
Effects of curcumin on the weights of body and liver, and hepatic drug metabolizing enzymes in rats

	Untreated control	Curcumin		
		(0.05 g/kg)	(0.5 g/kg)	(5 g/kg)
Body weight (g)	211.8±9.9	216.1±6.7	215.1±5.5	219.9±9.6
Liver weight (%/body weight)	2.90±0.08	2.93±0.13	3.02±0.11	3.04±0.09
Hepatic metabolizing enzymes				
Cytochrome P450 content (nmol/mg protein)	0.615±0.077	0.584±0.088	0.686±0.044	0.675±0.055
Glutathione *S*-transferase (μmol/mg protein/min)	0.346±0.075	0.522±0.088*	0.416±0.030	0.476±0.068*

Wistar rats were given diets containing curcumin (0.05, 0.5 and 5 g/kg diet) for 4 weeks. Each value is the mean±S.D. for five rats. Significant difference from the untreated control group is indicated by $*P<0.01$.

PLSD. A P-value <0.05 was considered to be significant. These statistical analyses were performed using a computer program (Stat View 5.0, ASA Institute Inc., Cary, NC, USA).

Results

Dose-dependent effects of curcumin on the hepatic CYP activity

During the curcumin treatment, there was no difference in the dietary intake or the body weight gain between each group. The average intake dose of curcumin was calculated based on the intake amount of diet and the body weight of the group, at a dose of 0.05 g/kg diet for about 6.2 mg/kg body weight per day. The effects of curcumin on the weights of body and liver, and hepatic metabolizing enzymes of rats are shown in Table 1. Curcumin had no influence on the liver weight and hepatic CYP content in rats (Table 1). Glutathione *S*-transferase activity increase correlated with the ingestion of curcumin (Table 1). The effects of curcumin on the various CYP activities of rats are shown in Table 2. Treatment with curcumin (0.05 and 0.5 g/kg diet) did not change the activity of the six types of CYP, while the extremely high dose (5 g/kg diet) of curcumin tended to increase the activity of pentoxyresorufin *O*-dealkylase as corresponding to CYP2B and (*S*)-warfarin 7-hydroxylase as CYP2C9 (Table 2).

Effects of curcumin on the changes of hepatic CYP activities induced by chronic CCl_4 injection in rats

Liver weight was increased with CCl_4 treatment, by 1.2-fold (4.32±0.12%/body weight, $P<0.05$) compared with the untreated control group (3.69±0.13%), and slight inductions were observed by co-administration of curcumin. The liver weights were 4.60±0.22% in 0.05 g/kg diet group, 4.59±0.17% in 0.5 g/kg diet group and 4.64±0.21% in 5 g/kg diet group, respectively. The GOT and GPT activities in plasma were significantly increased by chronic CCl_4 treatment: 40.3±4.8 IU/L and 17.6±2.2 IU/L in the untreated control group, 120±7.4 IU/L and 78.9±19.3 IU/L in the CCl_4-treated group. Repeated administrations of curcumin did not influence the increases in GOT and GPT activities, even high dose 0.5 g/kg diet: 105±11.1 IU/L and 87.7±10.9 IU/L, respectively. Effects of curcumin on the changes in the content of CYP and the activities of the CYPs and glutathione *S*-transferase in CCl_4-treated rats are shown in Fig. 2 and Table 3. Chronic CCl_4 treatment markedly decreased hepatic total CYP content to 29%, compared to the level of the untreated control group (Fig. 2A). In contrast, the ingestion of higher doses of curcumin (0.5 and 5 g/kg diet) significantly moderated the reduction of CYP content to 55% of the level of the untreated control group. Similarly, the activities of the six types of CYPs were drastically decreased by CCl_4 treatment, while higher doses of curcumin (0.5 and 5 g/kg diet) inhibited the decreases of CYP activity, except for *p*-nitrophenol hydroxylase corresponding to CYP2E1 (Table 3). Glutathione *S*-transferase activity was reduced by CCl_4 treatment, while the effects of co-administered curcumin were not significant (Fig. 2B).

Discussion

The objectives of the present study were two-fold: firstly, to examine the effects of curcumin on hepatic CYP activity in order to analyze hepatic drug-metabolizing function and CYP-

Table 2
Effects of curcumin on the activity of various hepatic CYPs in rats

	Untreated control	Curcumin		
		(0.05 g/kg)	(0.5 g/kg)	(5 g/kg)
	Activity (pmol/mg protein/min)			
Ethoxyresorufin *O*-deethylase (CYP1A1)	10.1±2.80	10.1±1.22	9.94±1.17	12.7±2.02
Methoxyresorufin *O*-demethylase (CYP1A2)	6.19±1.34	6.41±0.93	5.95±0.92	6.68±1.19
Pentoxyresorufin *O*-dealkylase (CYP2B)	2.71±0.76	2.71±0.36	2.69±0.23	3.58±0.70*
(*S*)-Warfarin 7-hydroxylase (CYP2C9)	2.09±0.61	2.05±0.25	2.13±0.24	3.02±0.52*
p-Nitrophenol hydroxylase (CYP2E1)	7330±1235	7162±674	6531±527	7331±872
Testosterone 6β-hydroxylase (CYP3A)	1641±506	1425±157	1505±115	1693±157

Wistar rats were fed diets containing curcumin (0.05, 0.5 and 5 g/kg diet) for 4 weeks. Each value is the mean±S.D. for five rats. Significant difference from the untreated control group is indicated by $*P<0.05$.

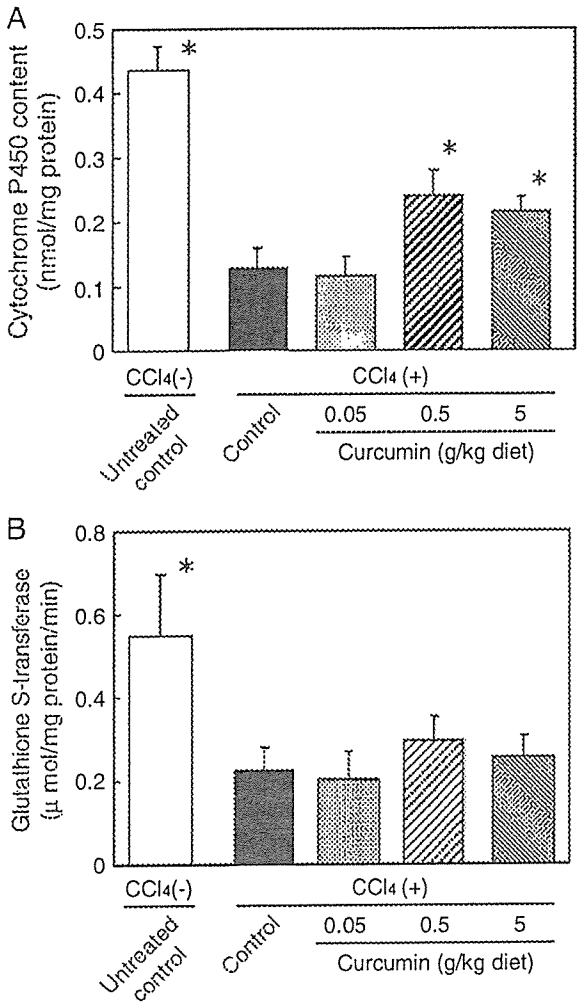

Fig. 2. Effects of curcumin on the reduction of hepatic cytochrome P450 content and glutathione *S*-transferase activity induced by CCl_4 injection. Wistar rats were fed diets containing curcumin (0.05, 0.5 and 5 g/kg diet) for 7 weeks and intraperitoneally injected with CCl_4 twice a week. (A) Cytochrome P450 content, (B) glutathione *S*-transferase activity. Each column is the mean±S.D. for five rats. Significant difference from the CCl_4-treated control group is indicated by $^*P<0.01$.

mediated drug interaction, and secondly, to determine the protective effect of curcumin on hepatic CYP damage induced by chronic CCl_4 injection.

As shown in the results, rats were given curcumin-containing diets for 4 consecutive weeks. The dose of curcumin was calculated to be about 5 mg/kg body weight per day based on the recommended dose of curcumin (500 mg/day or more) in human therapy (Sharma et al., 2004; Cheng et al., 2001), and the 10- and 100-fold doses (50 and 500 mg/kg body weight) were also tested. Even in the highest dose group (5 g/kg in diet; about 500 mg/kg body weight), the repeated ingestion of curcumin had no effect on body weight gain, liver weight or the total content of hepatic CYP enzyme of rats (Table 1). Likewise, the activities of six CYP isozymes remained unchanged after curcumin treatment at doses of 0.05 g/kg diet, i.e. 5 mg/kg body weight (Table 2). These results indicate that daily doses of curcumin have no influence on hepatic CYP activities, namely phase I drug-metabolizing enzymes. On the other hand, curcumin increased the activity of glutathione *S*-transferase, one of phase II drug-metabolizing enzymes (Table 1), as previous reports (Iqbal et al., 2003; Okada et al., 2001). Glutathione *S*-transferase is a soluble protein located in the cytosol, and plays an important role in the detoxification and excretion of xenobiotics (Mannervik, 1985; Mannervik et al., 1985). Compounds that increase the glutathione *S*-transferase activity and convert toxic substances to nontoxic substances are known to protect the liver. Some reports have indicated that curcumin increases intracellular glutathione levels and activities of glutathione *S*-transferase and some antioxidative enzymes (Okada et al., 2001; Rinaldi et al., 2002; Piper et al., 1998; Iqbal et al., 2003). These results indicate that curcumin might be beneficial for glutathione-mediated detoxification of electrophilic products of lipid peroxidation.

Recently, herb–drug interactions have become a concern in clinical therapy. Alternative remedies containing curcumin or turmeric are consumed by many patients receiving medical therapy for liver disease (Luper, 1999; Miquel et al., 2002). The fact that repeated intake of curcumin has no influence on hepatic CYP activity suggests that curcumin does not change the efficacy or pharmacokinetics of co-administered medicines. Moreover, because CYPs mediate the biosynthesis and metabolisms of various hormones, it is unlikely that a daily dose of curcumin cause adverse reactions involved in changes of CYP activity.

The free radical scavenging activity of curcumin is beneficial to liver injury caused by a variety of hepatotoxic substances, including CCl_4, ethanol, pentobarbital and acetaminophen (Luper, 1999; Miquel et al., 2002; Park et al., 2000). However, the changes of various CYP activities are not clear in simultaneous injection of CCl_4 and curcumin in rats. Thus, we focused on the changes in CYP activity in CCl_4-induced hepatopathy-modeled rats. Chronic CCl_4 injection increased the liver weight, while drastically reducing the content and activity of CYP enzymes, especially CYP2E1, as shown by the *p*-nitrophenol hydroxylase activity (Fig. 2A, Table 3). These results support recent reports of the reductions of mRNA expression and activity of some CYP enzymes in the liver of rats given various doses of CCl_4 (Lee et al., 2004). CYP2E1 is the major isozyme involved in CCl_4 bioactivation and generated cytotoxic trichloromethyl radicals are thought to cause hepatotoxicity (Recknagel et al., 1989; Wong et al., 1998; Williams and Burk, 1990). Furthermore, alterations in CYP2E1 activity can affect susceptibility to hepatic injury from CCl_4 (Wong et al., 1998; Takahashi et al., 2002). Moreover, the reactive free radicals inactivate CYP enzymes and subsequent depletion of CYP2E1 (Guengerich et al., 1991; Jeong, 1999; Zhou et al., 2004). In this way, CCl_4 injection decreased the CYPs 1A, 2B, 2C and 3A isozymes activities, similar to CYP2E1 (Table 3). In contrast, repeated curcumin ingestion in higher doses (0.5 and 5 g/kg diet) significantly relieved the CCl_4-caused reductions of total CYP content (Fig. 2A) and the activities, except for CYP2E1

Table 3
Effects of curcumin on the activity of various hepatic CYPs in rats treated with and without CCl_4

	Untreated control	CCl_4-treated			
		Control	Curcumin		
			(0.05 g/kg)	(0.5 g/kg)	(5 g/kg)
	Activity (pmol/mg protein/min)				
Ethoxyresorufin *O*-deethylase (CYP1A1)	16.92±1.78*	3.70±1.15	3.16±0.51	7.67±3.42*	6.67±2.67
Methoxyresorufin *O*-demethylase (CYP1A2)	10.31±0.49*	1.97±0.27	1.65±0.14	3.43±0.49*	3.22±0.47
Pentoxyresorufin *O*-dealkylase (CYP2B)	3.25±0.37*	1.28±0.18	1.24±0.13	1.82±0.36*	1.77±0.49*
(*S*)-Warfarin 7-hydroxylase (CYP2C9)	1.00±0.25*	0.146±0.044	0.192±0.042	0.416±0.142*	0.419±0.276
p-Nitrophenol hydroxylase (CYP2E1)	6444±1043*	462±147	419±127	458±148	462±67
Testosterone 6β-hydroxylase (CYP3A)	917±171*	340±63	224±63	579±263*	502±237

Wistar rats were fed diets containing curcumin (0.05, 0.5 and 5 g/kg diet) for 7 weeks and intraperitoneally injected with CCl_4 twice a week. Each value is the mean±S.D. for five rats. Significant difference from the CCl_4-treated control group is indicated by $*P<0.05$.

(Table 3). Glutathione *S*-transferase activity was also decreased by CCl_4 treatment and co-administered curcumin (0.5 g/kg diet) tended to recover the decreased activity, but there was no significance. The activities of GOT and GPT, well-known biomarkers, were markedly elevated by CCl_4 injection, indicating severe tissue damage. Co-administered curcumin, even in high dose, did not inhibit the increase in these activities. These results suggested that curcumin did not significantly relieve tissue damage by CCl_4 as indicated by the transaminase activities, but relieved the decreased hepatic CYPs activity in the present experimental condition. Interestingly, among the six CYP enzymes examined, CYP2E1 was degraded the most by CCl_4 injection and no amelioration was observed with curcumin ingestion (Table 3). CYP2E1-mediated metabolism of CCl_4 generated reactive free radicals, and CYP2E1 protein might be more susceptible to CCl_4 toxicity than other CYP isozymes. Curcumin could not moderate the decrease of CYP2E1 activity. In other words, curcumin was unavailable to additionally precipitate the bioactivation of CCl_4 and exacerbated liver damage. Curcumin did not change the hepatic CYP activity in normal rats (Tables 1 and 2), indicating that curcumin indirectly improved the inactivation of CYPs induced by severe CCl_4 toxicity.

Many previous investigations regarding CCl_4-induced liver injury have focused only on CYP2E1 activity, but not on other CYP isoforms (Yokogawa et al., 2004; Jeong et al., 2002; Jeon et al., 2003). In this study, different susceptibilities to CCl_4 were observed between in CYP2E1 and other isozymes, i.e. CYPs 1A, 2B, 2C and 3A, and the effects of curcumin were also different. The mechanism underlying the CCl_4-induced degradation of CYP activity may be different between CYP2E1 and other isoforms. CYP2E1 mediated CCl_4 bioactivation and produced reactive free radicals, and accordingly, the most suicidal damaged among the CYP isozymes. It is speculated that the antioxidant properties of curcumin inhibit the secondary inactivation of CYPs caused by reactive free radicals.

In conclusion, curcumin ingestion has no influence on hepatic CYP activity in rats, indicating no pharmacokinetic interaction with co-administered drugs. Curcumin does not prevent the decrease of CYP2E1 activity related to the first step of metabolic activation of CCl_4. However, curcumin is beneficial for ameliorating the subsequent inactivation of other CYP isozymes caused by CCl_4. The antioxidant properties of curcumin may contribute to the inhibition of the reactive free radicals produced from CCl_4 bioactivation. Further detail study will be needed to clarify the mechanism of curcumin against CCl_4-induced liver injury.

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Relationship between *Garcinia cambogia*-Induced Impairment of Spermatogenesis and Meiosis-Activating Sterol Production in Rat Testis

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Summary **Dietary supplements for body fat reduction have become popular, particularly in developed countries. *Garcinia cambogia* (GA) is one such supplement, and its active component is (–)-hydroxycitric acid ((–)-HCA), a competitive inhibitor of ATP citrate lyase, which is responsible for producing acetyl CoA from citric acid. Recently we have found that administration of (–)-HCA-containing GA markedly reduces testis weight in male Zucker obese rats. In particular, histopathological examinations revealed testicular atrophy and impairment of spermatogenesis. In the present study, we investigated the cause of the impaired spermatogenesis after ingestion of GA containing (–)-HCA at 102 mmol/kg diet in young Fischer 344 male rats. Among hormones related to spermatogenesis, the serum level of inhibin-B was significantly lower and that of follicle-stimulating hormone (FSH) was higher in the GA group. The level of testis meiosis-activating sterol (T-MAS), which is an intermediate in cholesterol biosynthesis from acetyl CoA and is presumed to transmit a signal for spermatogenesis, was statistically lower in the testes of rats administered GA. We hypothesize from these results that (–)-HCA-mediated inhibition of ATP citrate lyase in rats fed GA leads to diminished accumulation of MAS substances, thus resulting in impairment of spermatogenesis.**

***Key Words*: *Garcinia cambogia*, (–)-hydroxycitric acid, testis-meiosis activating sterol, inhibin-B, spermatogenesis**

Introduction

Dietary supplements for body fat reduction have become popular, particularly in developed countries. One ingredient of such dietary supplements is an extract of *Garcinia cambogia* (GA), a fruit grown in Southeast Asia and India. The rind of GA contains hydroxycitric acid (HCA), and four isomers of HCA with their free and lactone forms are found in the extract [*1*]. Among them, only (–)-HCA is a potent competitive inhibitor of ATP citrate lyase (EC 4.1.3.8) [*2*].

Citric acid, produced by glycolysis and then transported into the cytosol from mitochondria, is an important substrate for ATP citrate lyase, which converts citric acid to acetyl CoA and oxaloacetic acid. Hence, ATP citrate lyase is a key enzyme in the supply of acetyl CoA for both *de novo* fatty acid and cholesterol biosyntheses. Lowenstein [*3*] determined the effect of (–)-HCA on fatty acid biosynthesis in rat liver by measuring the incorporation of ^{3}H from $^{3}H_2O$ and showed that fatty acid biosynthesis was inhibited strongly by (–)-HCA. Sullivan *et al.* [*4*] observed the effect of isomers of HCA on lipogenesis in rat liver by using [^{14}C]citrate and [^{14}C]alanine, and obtained similar results.

Recently, our group [*5*] examined the effect of GA

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administration on body fat accumulation in male Zucker obese rats. The rats were fed diets containing GA powder S® ((−)-HCA levels ; 0, 10, 51, 102 and 154 mmol/kg diet) for 92 or 93 days. Surprisingly, the high doses of (−)-HCA-containing GA (102 mmol/kg diet or higher) caused testicular atrophy and impairment of spermatogenesis. From the results, a diet containing 51 mmol/kg was considered to be the no observed adverse effect level (NOAEL) in these rats.

It has been shown recently that 4,4-dimethyl-5α-cholesta-8,24-diene-3β-ol (testis meiosis-activating sterol; T-MAS) is a specific intermediate product of cholesterol biosynthesis in testicular germ cells [*6*]. T-MAS was isolated and characterized from bull testes [*7*]. Similarly, 4,4-dimethyl-5α-cholesta 8,14,24-triene-3β-ol (follicular fluid meiosis-activating sterol; FF-MAS) was isolated from human follicular fluid [*7*]. These MAS substances are produced from lanosterol by the action of lanosterol 14α-demethylase (CYP51) and sterol Δ14-reductase in the cholesterol biosynthetic pathway (Fig. 1). Interestingly, FF-MAS and T-MAS are presumed to be signaling substances that trigger the start of meiotic division of the oocyte and spermatocyte, respectively [*8*]. Indeed, Grondahl *et al.* have demonstrated that FF-MAS has the ability to reinitiate meiosis in a mouse oocyte assay *in vitro* [*9*].

Therefore, in the present study, we examined the relationship between impaired spermatogenesis and MAS substances production in rat testis after administration of (−)-HCA-containing GA.

Materials and Methods

Materials

Garcinia cambogia powder S® was generously donated by Nippon Shinyaku Co.Ltd., Japan. The (−)-HCA content of this powder was 41.2wt% and the ratio of its free to lactone form was 36.6 to 63.4.

Animals

This experiment was carried out under the guidelines of the Animal Committee of Incorporated Administrative Agency, National Institute of Health and Nutrition (Tokyo, Japan).

Three-week-old male Fischer 344/DuCrj rats were purchased from Charles River Japan, Inc. (Yokohama, Japan). They were kept individually in stainless steel cages at 22 ± 1°C and 50–60% humidity with a 12 h light/dark cycle. The feed and water were supplied *ad libitum*. The composition of diets based on the AIN-93G purified diet for

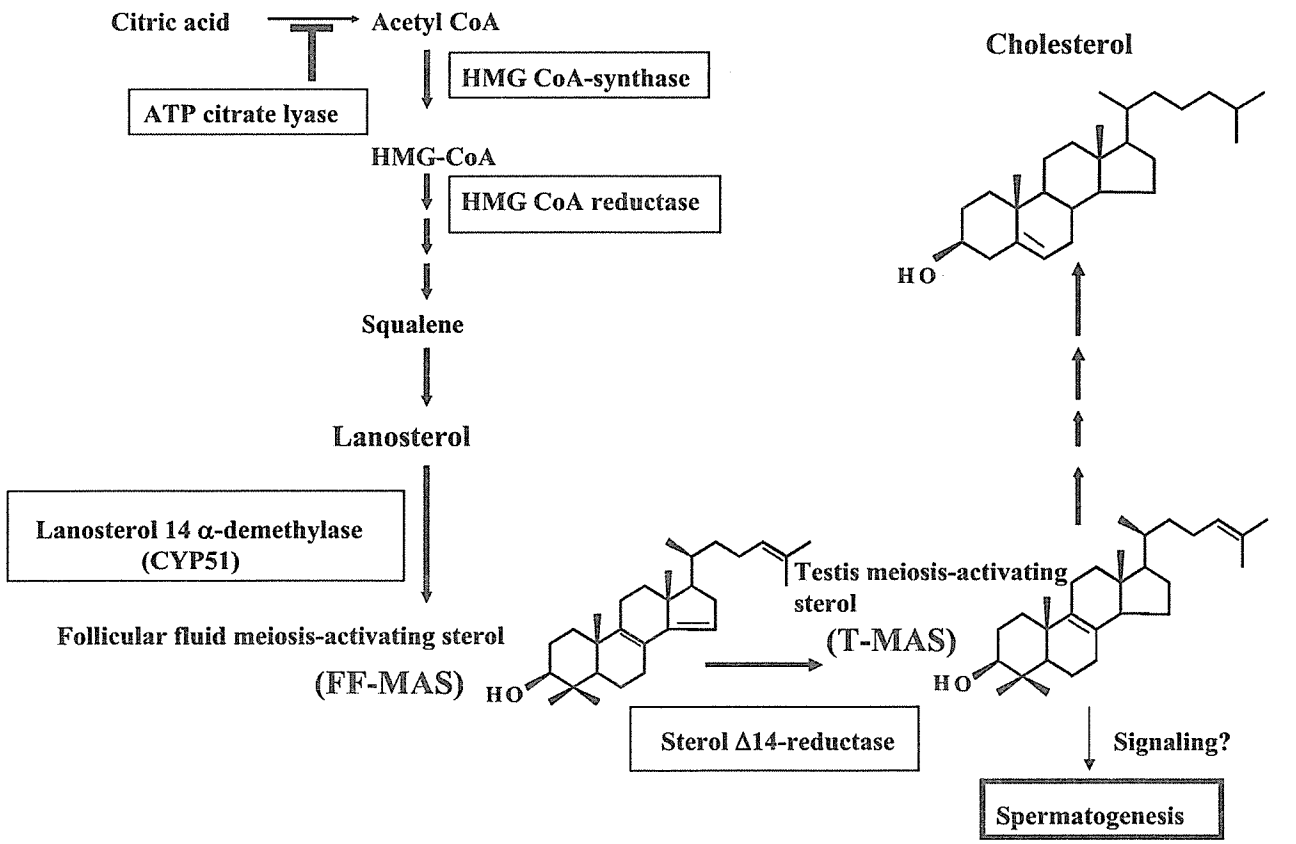

Fig. 1. Proposed biosynthetic pathway of follicular fluid meiosis-activating sterol (FF-MAS), testis meiosis-activating sterol (T-MAS) and cholesterol in rat testis.

Table 1. Composition of the experimental diets

Dietary Component	Group	
	Control	Garcinia
	g/kg	
alpha-Cornstarch	400.0	351.1
Casein	200.0	200.0
Glucose	152.0	152.0
Sucrose	100.0	100.0
Soybean oil	50.0	50.0
Cellulose	50.0	50.0
Mineral Mix (AIN-93)	35.0	35.0
Vitamin Mix (AIN-93G)[1]	10.0	10.0
L-Cystine	3.0	3.0
Tert-butylhydroquinone	0.014	0.014
Garcinia cambogia[2]	0	48.9

1. Vitamin Mix contained choline bitartrate at 2.5 g/kg diet.
2. Garcinia powder S® supplied by Nippon Shinyaku Co., Ltd. was used. The (−)-hydroxycitric acid ((−)-HCA) content was 41.2%(102 mmol/kg diet) and the ratio of its free to lactone form was 36.6:63.4.

laboratory rodents [*10*] is shown in Table 1. All of the animals were initially fed the control diet prepared in our laboratory for five days to allow them to adapt to the new environment, and then divided into two groups. One group was fed the control diet and the other group the GA diet. The (−)-HCA content was 102 mmol/kg in GA diet. Each group was further divided into two subgroups: a feeding period for two weeks and that for four weeks, respectively (totally four groups).

Experimental procedure

On the day before the autopsy, rats were given diets to consume three-quarters of the food intake of the previous day and were then killed by cardiac puncture. Liver and left and right testes were quickly excised and weighed. Right testis was cut in half, and then one part was fixed with 10% formalin neutral buffer solution, pH 7.4, and the other part was stored at −80°C together with other tissues. The histopathological examination was performed after hematoxylin-eosin staining. Serum was separated by centrifugation at 2,700×g for 15 min at 4°C and stored at −80°C until analysis for hormones.

Measurement of sexual hormones

Serum inhibin-B concentration was determined by a sandwich EIA kit (OXFORD BIO-INNOVATION LTD., Oxfordshire, UK). Serum 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. Serum testosterone was measured with radioimmunoassay method (Diagnostic Products Corporation, Los Angeles, CA, USA).

Determinations of FF-MAS, T-MAS and Cholesterol in rat testis

FF-MAS and T-MAS in rat testis were measured by modification of Tacer *et al.*'s method [*6*]. Briefly, testis tissue (0.2 g) was placed in the glass tube with 0.9% NaCl solution, and then homogenized. The homogenate was extracted with chloroform/methanol (1:1, vol/vol) solution, and this mixture was centrifuged at 2,700×g for 15 min. Bottom layer was collected and evaporated. The residue was dissolved in n-hexane to fractionate 4,4-dimethyl sterols with straight-phase high-performance liquid chromatography (HPLC). The HPLC system consisted of pump (LC-10ADvp), degasser (DGU-14A), column oven (CTO-10Avp), ultraviolet (UV) detector (LC-10Avp) and integrator (C-R7A plus) (Shimadzu Co., Kyoto, Japan). The fractionation of 4,4-dimethyl sterols was performed at 28°C by using ChromoSpher Si column (5 μm, 250 × 4.6 mm i.d.; Varian, CA, U.S.A.). The mobile phase was n-hexane/isopropanol (99.5:0.5, vol/vol) at a flow rate of 1.0 ml/min. The effluent was monitored with an UV detector set at 249 nm. The fraction containing 4,4-dimethyl sterols (8 min–11 min) was collected, and then dried using evaporator. The residue was dissolved in acetonitrile for the analysis of FF-MAS and T-MAS by reverse-phase HPLC. The analysis was performed at 40°C by using LiChrospher 100 RP-8 column (5 μm, 250 × 4.6 mm i.d.; MERCK, Germany). The mobile phase was acetonitrile/water (92.5:7.5, vol/vol) at a flow rate of 1.0 ml/min. Each collected peak of FF-MAS and T-MAS was identified by gas chromatography-mass spectrometry (GC-MS). Total cholesterol level in the testis was measured enzymatically with a commercially available cholesterol assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) after extraction of lipids by chroloform/methanol (1:1,vol/vol).

Statistical analysis

All results were expressed as means ± SD. The significance of difference between the Control and the GA groups was evaluated using independent-samples *t*-test (two-samples *t*-test) (Dr. SPSS II for Windows, SPSS Japan Inc. Tokyo Japan). The difference was considered significant at $p<0.05$.

Results

Final body weight, food intake and tissue weights

Final body weight, food intake and tissue weights are shown in Table 2. After two weeks of the experimental period (six weeks old), the final body weight of the GA

Table 2. Final body weight, food intake and each tissue weight

	Two weeks		Four weeks	
	Control	Garcinia	Control	Garcinia
Final body weight (B.W.) (g)	108.08 ± 1.96	101.33 ± 3.98**	162.20 ± 7.57	150.70 ± 9.60*
Food intake (g/day)	10.02 ± 0.29	9.65 ± 0.20	11.05 ± 0.39	10.71 ± 0.24
Liver (g/100 g B.W.)	3.73 ± 0.12	4.17 ± 0.21**	3.77 ± 0.23	3.98 ± 0.32
Liver (whole g)	4.03 ± 0.19	4.22 ± 0.25	6.12 ± 0.51	6.01 ± 0.68
Left testis (g/100 g B.W.)	0.48 ± 0.10	0.49 ± 0.07	0.65 ± 0.06	0.47 ± 0.13**
Left testis (whole g)	0.52 ± 0.11	0.50 ± 0.09	1.06 ± 0.13	0.71 ± 0.21**
Right testis (g/100 g B.W.)	0.49 ± 0.10	0.50 ± 0.07	0.66 ± 0.06	0.44 ± 0.12**
Right testis (whole g)	0.53 ± 0.11	0.51 ± 0.08	1.06 ± 0.12	0.67 ± 0.20**

Values are means ± SD of 6–7 rats in each group.
The significant differences between the Control group and GA group for each period were analyzed by two samples *t*-test (Dr. SpSS II for windows).
Asterisks show significant difference; *: $p<0.05$, **: $p<0.01$.

Table 3. Histopathological examination of testis in rats administered Garcinia

Two weeks	Control						Garcinia						
Rat No.	1	2	3	4	5	6	1	2	3	4	5	6	
Degeneration of germ cell	–	–	–	–	–	–	+	++	++	+	+	+	
Spermatogenesis	a	a	a	a	a	a	a	a	a	a	a	a	
Four weeks	**Control**						**Garcinia**						
Rat No.	1	2	3	4	5	6	1	2	3	4	5	6	7
Degeneration of germ cell	–	–	–	–	–	–	+++	++	+++	+++	+	+	+++
Hypospermatogenesis	–	–	–	a	–	–	+++	++	+++	+++	+	+	+++

Marks show the degree of influence for rat testis; –: negative, +: mild, ++: moderate, +++: marked
a: immature spermatogenesis.

Judged point:

	Degeneration of germ cell	Hypospermatogenesis
–: negative	Rate of degenerated cell < 3%	No remarkable change
+: mild	3% ≤ rate of degenerated cell < 20%	Decrease in number of sperm
++: moderate	20% ≤ rate of degenerated cell < 50%	Decrease in number of sperm and spermatid
+++: marked	50% ≤ rate of degenerated cell	Disappearance of sperm and spermatid

group was already statistically lower than that of the control group. However, there were no significant differences in the food intake and the weights of individual tissues between the control group and the GA group, except the relative liver weight of the GA group which was higher. After four weeks of feeding (eight weeks old), there was also no significant difference in the food intake between the two groups. But, the final body weight of the GA group was lower than that of the control group. The testis weight of the GA group was also remarkably less than that of the control group.

Histopathological examination of rat testis

As the testis weight of the GA group was markedly lower than that of the control group, we performed histopathological examinations of the testis. The germ cells of all rats of the control group at two weeks (six weeks old) were normal, but there was mild to moderate degeneration of the cells in all rats of the GA group (Table 3). Spermatogenesis was immature in both the control and GA groups at two weeks (six weeks old). Spermatogenesis of the control rats was normal at eight weeks old, as shown by the abundance of elongating and elongated spermatids in all the seminiferous tubules (Table 3, Fig. 2A). In contrast, there is complete absence of spermatid elongation in the eight-week-old GA-fed rats, with some of the round spermatids being released in clusters (Table 3, Fig. 2B).

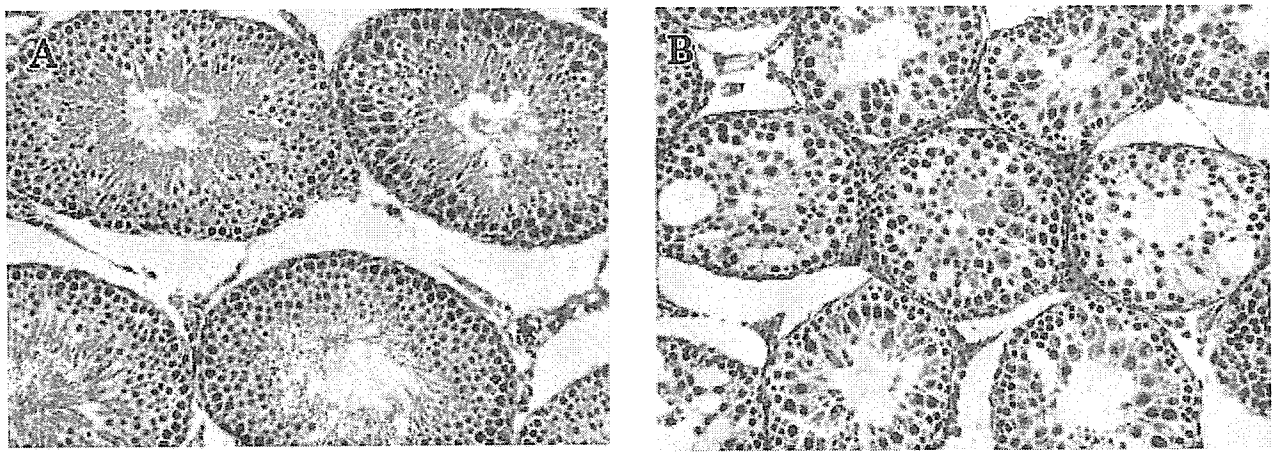

Fig. 2. Histopathological examination of testes. A; control rat kept for four weeks (magnification ×200), B; *Garcinia*-fed rat kept for four weeks (magnification ×200).

Table 4. Hormone analysis in serum of rat administered Garcinia

	Two weeks		Four weeks	
ng/mL	Control	Garcinia	Control	Garcinia
Inhibin-B	0.33 ± 0.04	0.20 ± 0.02***	0.19 ± 0.05	0.10 ± 0.03**
Follicle-stimulating hormone	6.50 ± 1.80	11.30 ± 2.30**	7.00 ± 1.30	13.50 ± 4.00**
Luteinizing hormone	4.20 ± 1.50	4.00 ± 1.60	4.30 ± 1.70	3.30 ± 1.20
Testosterone	0.07 ± 0.03	0.16 ± 0.18	0.73 ± 0.76	0.28 ± 0.19

Values are means ± SD of 6–7rats in each group.
The significant differences between the Control group and GA group for each period were analyzed by two samples *t*-test (Dr. SPSS II for windows).
Asterisks show significant difference; **: $p<0.01$, ***: $p<0.001$.

Testosterone, LH, FSH and inhibin-B concentrations in rat serum

The hormone levels related to spermatogenesis were measured (Table 4). There were no significant differences in LH concentration between the control group and the GA group after two or four weeks of feeding. Although the concentration of testosterone in serum was different into control group and GA group after two or four weeks of feeding, there was no difference statistically. However, the inhibin-B concentrations of the GA group were statistically lower than those of the control, and the FSH concentrations of the GA group were remarkably higher than those of the control, after both two and four weeks of feeding.

Changes in FF-MAS, T-MAS and cholesterol concentrations in rat testis

Several investigators have suggested that FF-MAS and T-MAS may have roles not only in cholesterol biosynthesis but also in signaling to trigger spermatogenesis. We thought that the accumulation of MAS in rat testis might be affected by the competitive inhibition of ATP citrate lyase by (−)-HCA, and we measured the concentrations of FF-MAS, T-MAS and cholesterol in rat testis (Fig. 3). Interestingly, the FF-MAS and T-MAS concentrations in the testis of the GA group were significantly lower (or tended to be lower; T-MAS at two weeks) than those of the control group after both two and four weeks of GA feeding. Nevertheless, there was no significant difference in the cholesterol concentration between the control and the GA groups after two weeks of feeding. The cholesterol concentration in the GA group after four weeks of feeding was higher than in the control group, but the difference was very slight and negligible.

Discussion

The present study was designed to investigate the relationship between impaired spermatogenesis and production of MAS substances in rat testis after administration of (−)-HCA-containing GA. This is our first attempt to reveal the testicular toxicity caused by (−)-HCA-containing GA at high doses [5].

In the previous study, we examined the effect of GA

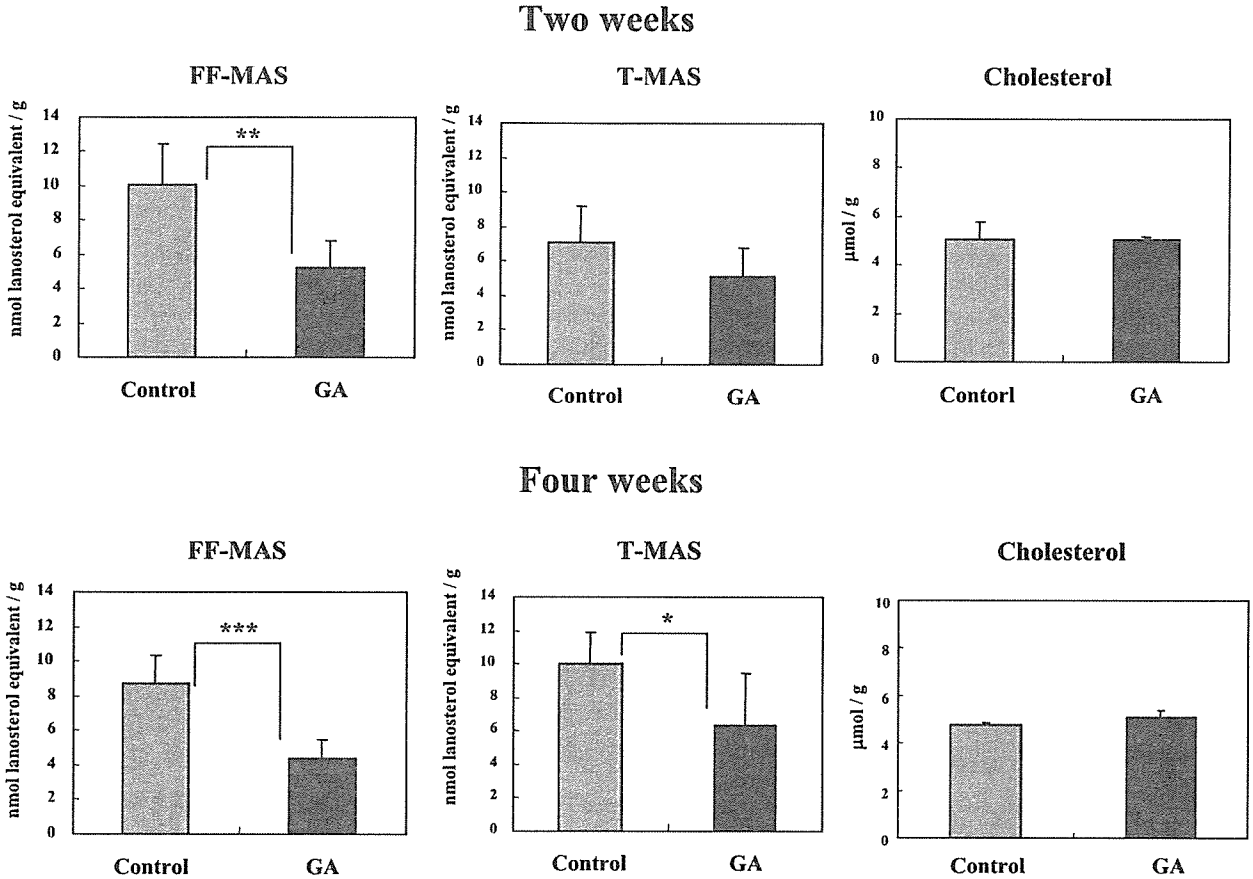

Fig. 3. Concentrations of follicular fluid meiosis-activating sterol (FF-MAS), testis meiosis-activating sterol (T-MAS) and cholesterol in testes of rats administered each diet for two weeks (up) and four weeks (down). Values are means ± SD of 6–7 rats. Differences between the control group and the GA group were analyzed by two-sample *t*-tests (Dr. SPSS II for Windows). Asterisks show significant differences: *p<0.05, **p<0.01, ***p<0.001.

administration on body fat accumulation using Zucker obese rats [*5*]. However, we found unexpectedly that the high dose of GA ((−)-HCA levels; 102 mmol/kg diet or higher) caused testicular atrophy and impairment of spermatogenesis. Since the aim of the current study was to elucidate the relationship between impaired spermatogenesis and MAS substance production in rat testis, we used Fischer 344 strain rats, being generally recognized as standard strain in toxicology. Then, this study was examined by using a diet containing (−)-HCA at 102 mmol/kg diet to be low observed adverse effect (LOAEL) in these rats from the former study.

In addition, we used weanling, immature rats for the present study because we considered that they would be a good model for observing the temporal (for two weeks and for four weeks) influences of GA administration on germ cell development, spermatogenesis, sexual maturity and hormonal levels related to spermatogenesis, where rats at six weeks old are thought to be sexually immature and at eight weeks old sexually mature. Actually, after two weeks of feeding, the rats were six weeks old, and spermatogenesis had not yet started (Table 3). But, spermatogenesis had already begun in rats after four weeks of feeding (eight weeks old). Consequently, we observed the influence of GA administration before and after the start of spermatogenesis.

We measured the serum concentrations of four hormones related to spermatogenesis. There were no significant differences in testosterone and LH concentrations between the control and GA groups. However, in the GA group, the inhibin-B concentration was statistically lower, and the FSH concentration higher, than those in the control group after two weeks of GA feeding, as was also the case after four weeks of feeding. Pierik *et al.* [*11*] examined the relationship between serum inhibin-B levels and the classical markers of spermatogenesis in subfertile men, and demonstrated a significant positive correlation between the serum inhibin-B level and sperm concentration, sperm count, and testicular volume. Moreover, the serum inhibin-B level was negatively correlated with the serum FSH concentration (Table 4). Other researchers have also demonstrated a negative correlation between inhibin-B and FSH levels in normal and

infertile men [*12–14*]. Therefore, inhibin-B is an important marker of the function of Sertoli cells and spermatogenesis, and a combination of a low inhibin-B level and a high FSH level indicates disturbed spermatogenesis [*15*]. This relationship implies that the low inhibin-B level and high FSH level in the serum of rats administered GA might be caused by arrest of spermatogenesis, and this supposition was supported by the results of histopathological examination, as shown in Fig. 2 and Table 3.

What causes the impairment of spermatogenesis after ingestion of GA? The GA powder used in this study contained 41.2wt% (−)-HCA. Sekita *et al.* [*16*] preliminarily examined whether (−)-HCA causes testicular toxicity, and suggested that GA-induced testicular toxicity may be due to its principal ingredient, (−)-HCA itself. Accordingly, in this study, we tentatively hypothesized that the GA-mediated toxic effect might be related to the ingestion of (−)-HCA, which is known to inhibit fatty acid biosynthesis, as mentioned in the Introduction section. However, its influence on cholesterol and the intermediates of cholesterol biosynthesis, i.e. FF-MAS and T-MAS, is unknown in rat testis. These intermediates are presumed to be signaling substances that initiate meiotic division of the oocyte and spermatocyte, respectively [*6–9*]. The concentrations of FF-MAS and T-MAS in the testis in the GA group were significantly lower than those in the control group (Fig. 3), although GA administration did not exert a significant influence on the cholesterol concentration. These data demonstrate that ingestion of (−)-HCA-containing GA prevents the accumulation of MAS substances in rat testis, and that the concentrations of FF-MAS and T-MAS in the testis do not simply reflect the concentration of cholesterol. Cholesterol is quite abundant in the testis in comparison with MAS substances, and its concentration may not change even if ATP citrate lyase is competitively inhibited by (−)-HCA. FF-MAS concentration in GA-administered rat testis decreased in addition to T-MAS concentration, and we thought that the formation of FF-MAS was reduced through the suppression of acetyl CoA production by the inhibition of ATP citrate lyase, followed by the reduction of T-MAS. However, it is remained to be solved how FF-MAS is associated with spermatogenesis.

This result is interesting and novel, supporting the idea that MAS substances may be connected with signal transmission, and also suggests that the cholesterol biosynthetic pathway in rat testis might exist primarily to produce MAS substances rather than cholesterol [*6*]. The level of cholesterol may be retained independently through different cholesterol biosynthetic pathways and/or incorporation from the blood circulation in rat testis, since cholesterol is essential for cell membrane structure and signaling, and also essential as a precursor for steroid hormones. In particular, Cholesterol is an important precursor of testosterone in the testis. Furthermore, it has been shown that cholesterol plays an important role in sperm capacitation [*17*]. Therefore, like MAS substances, cholesterol is also indispensable for the process of spermatogenesis. Nevertheless, in this study, there was no significant difference in the cholesterol concentration between the control and GA groups. Consequently, we suggest that MAS substances play more essential roles than cholesterol in spermatogenesis.

Our results indicate severe impairment of spermatogenesis in the testes of rats administered (−)-HCA-containing GA. The impaired spermatogenesis in these animals might be associated with blockade of MAS substances accumulation.

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Effect of *Garcinia cambogia* Administration on Female Reproductive Organs in Rats

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Summary **We investigated the effect of administering *Garcinia cambogia* (GA), which contains (–)-hydroxycitric acid ((–)-HCA), to female rats, focusing especially on changes in sexual hormones and its safety in terms of histopathological changes. A diet containing a high level of (–)-HCA (154 mmol/kg diet) was given to seven-week-old female rats with matched estrous cycles for two or four weeks. There were no significant differences in any of the serum hormones tested (follicle-stimulating hormone, luteinizing hormone, estradiol and progesterone) between the control group and the GA group during the observation period. Also, there were no abnormal morphological findings in the follicle and corpus luteum, nor were there any significant differences in the ovarian 4,4-dimethyl-5α-cholesta 8,14,24-triene-3β-ol (follicular fluid meiosis-activating sterol; FF-MAS) and 4,4-dimethyl-5α-cholesta-8,24-diene-3β-ol (testis meiosis-activating sterol; T-MAS) concentrations between the control and the GA groups. On the other hand, the final body weight of the GA group was statistically lower than that of the control group. The weight of abdominal fat in the GA group was markedly lower than that in the control group even after two weeks of feeding. Therefore, although we had confirmed in our previous study that GA had a marked effect on sexual maturation in male rats, no such deleterious influence was observed in the female rats.**

***Key Words*: *Garcinia cambogia*, (–)-hydroxycitric acid, female rat, ovary, abdominal fat accumulation**

Introduction

Obesity has become a serious social problem in developed countries. In the United States, for instance, 34% of the adult population is now classified as overweight (29.9≥ BMI≥25.0) and 31% as obese (30.0≥BMI) [*1*]. In Japan, approximately 30% of the adult male population and 20% of the adult female population are classified as obese (BMI≥25). However, only about 7% of young women in their twenties are classified as obese [*2*], and this low rate of obesity may reflect the concern of young women about their appearance.

Dietary supplements for body fat reduction have become popular, particularly in developed countries. One ingredient of such dietary supplements is an extract of *Garcinia cambogia* (GA), a fruit grown in Southeast Asia and India. The rind of GA contains hydroxycitric acid (HCA), and four isomers of HCA in their free and lactone forms are present in the extract [*3*]. Among them, only (–)-HCA is a competitive inhibitor of ATP citrate lyase (EC 4.1.3.8) with citric acid [*4*].

Citric acid produced by glycolysis and then transported into the cytosol from mitochondria is an important substrate for ATP citrate lyase, which converts citric acid to acetyl CoA and oxaloacetic acid. Accordingly, ATP citrate lyase is a key enzyme in the supply of acetyl CoA for both *de novo* fatty acid biosynthesis and cholesterol biosynthesis.

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Lowenstein [*5*] determined the effect of (−)-HCA on fatty acid biosynthesis in rat liver by measuring the incorporation of ^{3}H from $^{3}H_2O$ and showed that fatty acid biosynthesis was inhibited strongly by (−)-HCA. Sullivan *et al.* [*6*] observed the effect of isomers of (−)-HCA on lipogenesis in rat liver by using [^{14}C]citrate and [^{14}C]alanine, and obtained similar results.

Recently, our group [*7*] examined the effect of GA administration on body fat accumulation in male Zucker obese rats. The rats were fed diets containing GA powder S® ((−)-HCA levels; 0, 10, 51, 102 and 154 mmol/kg diet) for 92 or 93 days. Surprisingly, the high doses of (−)-HCA (102 mmol/kg diet or higher) caused testicular atrophy and impairment of spermatogenesis. Also, the plasma concentration of inhibin-B, a marker of spermatogenesis, in the groups given 154 mmol/kg diet or 102 mmol/kg diet of (−)-HCA groups was significantly lower than those in the other three groups. So, 51 mmol/kg diet was deemed to be the no observed adverse effect level (NOAEL) in these rats. Moreover, we suggested that (−)-HCA-mediated inhibition of ATP citrate lyase in rats fed GA leads to diminished accumulation of T-MAS in rat testis, resulting in impairment of spermatogenesis [*8*]. Hence, care should be taken with GA intake in males. However, the effect of GA administration in female animals has received little attention. In the present study, we investigated how GA administration affected the female rats matched the estrous cycle.

Materials and Methods

Materials

Garcinia cambogia powder S® was generously donated by Nippon Shinyaku Co. Ltd., Japan. The (−)-HCA content of this powder was 41.2wt% and the ratio of its free to lactone form was 36.6 to 63.4.

Animals

This experiment was carried out under the guidelines of the Animal Committee of Incorporated Administrative Agency, National Institute of Health and Nutrition (Tokyo, Japan).

Seven-week-old female SD-IGS (International Genetic Standard) rats matched the estrous cycles were purchased from Charles River Japan, Inc. (Yokohama, Japan). They were kept individually in stainless steel cages at 22 ± 1°C and 50–60% humidity with a 12 h light/dark cycle. The feed and water were supplied *ad libitum*. The composition of diets based on the AIN-93G purified diet for laboratory rodents [*9*] is shown in Table 1. All of the animals were initially fed the control diet prepared in our laboratory for five days to allow them to adapt to the new environment, and then divided into two groups. One group was fed the control diet and the other group the GA diet. The (−)-HCA content was 154 mmol/kg in the GA diet. Each group was further divided into two subgroups: a feeding period for two weeks and that for four weeks (totally four groups).

Table 1. Composition of experimental diets

Dietary Component	Group	
	Control	Garcinia (GA)
	g/kg	
alpha-Cornstarch	400.0	326.7
Casein	200.0	200.0
Glucose	152.0	152.0
Sucrose	100.0	100.0
Soybean oil	50.0	50.0
Cellulose	50.0	50.0
Mineral mix (AIN-93)	35.0	35.0
Vitamin mix (AIN-93G)[1]	10.0	10.0
L-Cystine	3.0	3.0
Tert-butylhydroquinone	0.014	0.014
Garcinia cambogia[2]	0	73.3

1. Vitamin mix contained choline bitartrate at 2.5 g/kg diet.
2. *Garcinia cambogia* powder S® supplied by Nippon Shinyaku Co., Ltd. was used. The (−)-hydroxycitric acid ((−)-HCA) content was 41.2% (154 mmol/kg diet) and the ratio of its free to lactone form was 36.6 to 63.4.

The female rat has an estrous cycle of 4 to 5 days, consisting of four sequential stages: estrus, metestrus, diestrus and proestrus. During the estrous cycle, the level of each sexual hormone changes in a complex manner [*10–12*]. Accordingly, all rats must be matched for estrous cycle stage to allow proper comparison of group data. During the experimental period, the estrous cycles of all rats were checked by measuring the electrical impedance in the vagina (EIV) every day. All female rats used in the present study were killed at the proestrus stage, determined by measuring the EIV, during the last week of the feeding period.

Experimental procedure

Each rat was killed by collecting blood from the abdominal aorta at day of the proestrus stage in the last week of the experimental period. Accordingly, the experimental period of each rat was not two weeks or four weeks strictly. Liver, left and right ovaries, uterus and abdominal fat were quickly excised and weighed. Right ovary was divided into two pieces, and one of them and all the uterus were fixed with 10% formalin neutral buffer solution at pH 7.4. The other parts of the ovary were stored at −80°C together with the other tissues. The histopathological examinations of the ovary and uterus were performed after hematoxylin-eosin staining. Serum was separated by centrifugation at 2,700 ×g for 15min at 4°C and stored at −80°C until analysis for hormones and biological parameters.

Measurement of sexual hormones and biological parameters in serum

Serum estradiol concentration was determined by a CAC estratdiol kit (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). Serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were determined with a rat FSH IRMA kit (BIOCODE, Liége, Belgium) and with a rat LH EIA kit (Amersham Biosciences, Buckinghamshire, UK), respectively. Serum progesterone was measured with COAT-A-COUNT progesterone kit (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan).

Other parameters such as total protein (TP), albumin (Alb), L-asparatate: 2-oxoglutarate aminotransferase (AST), L-alanine: 2-oxoglutarate aminotransferase (ALT), alkaline phosphatase (ALP), creatinine (CRN), blood urea nitrogen (BUN), glucose (Glc), non-esterified fatty acid (NEFA), phospholipids (PL), triglyceride (TG), total cholesterol (TCho), total ketone body, cholinesterase (ChE), leucine amino peptidase (LAP), and lactate dehydrogenase (LDH) were measured with commercially available kits.

Determinations of FF-MAS, T-MAS and Cholesterol in rat ovary

FF-MAS and T-MAS in rat ovary were measured by modification of Tacer *et al.*'s method [*13*]. Briefly, ovary tissue was homogenized with 0.9% NaCl solution. The homogenate was extracted with chloroform/methanol (1:1, vol/vol) solution, and the mixture was centrifuged at 2,700×g for 15 min. Bottom layer was collected and then evaporated. The residue was dissolved in n-hexane to fractionate 4,4-dimethyl sterols with straight-phase high-performance liquid chromatography (HPLC). The HPLC system consisted of pump (LC-10ADvp), degasser (DGU-14A), column oven (CTO-10Avp), ultraviolet (UV) detector (LC-10Avp) and integrator (C-R7A plus) (Shimadzu Co., Kyoto, Japan). The fractionation of 4,4-dimethyl sterols was performed at 28°C by using ChromSpher Si column (5 μm, 250 × 4.6 mm i.d.; Varian, CA, U.S.A.). The mobile phase was n-hexane/isopropanol (99.5:0.5, vol/vol) at a flow rate of 1.0 ml/min. The fraction containing 4,4-dimethyl sterols (8 min–11 min) was collected, and then dried using evaporator. The residue was dissolved in acetonitrile for the analysis of FF-MAS and T-MAS by reverse-phase HPLC. The analysis was performed at 40°C using LiChrospher 100 RP-8 column (5 μm, 250 × 4.6 mm i.d.; MERCK, Germany). The mobile phase was acetonitrile/water (92.5:7.5, vol/vol) at a flow rate of 1.0 ml/min. Total cholesterol in rat ovary was measured enzymatically with a commercially available cholesterol assay kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) after extraction of lipids by chroloform/methanol (1:1, vol/vol).

Statistical analysis

All results were expressed as means ± SD. The significance of difference between the Control and the GA groups was evaluated using independent-samples *t*-test (two-samples *t*-test) (Dr. SPSS II for Windows, SPSS Japan Inc. Tokyo Japan). The difference was considered significant at $p<0.05$.

Results

Final body weight, food intake and tissue weights

Final body weight, food intake and tissue weights are shown in Table 2. After two weeks of the experimental period, although the food intake was different between control group and GA group, there was no difference statistically. There were also no significant differences in the final body weight between the control group and the GA group. After four weeks of feeding, although the food intake was different between the two groups, there was no difference statistically. However, the final body weight of the GA group was statistically lower than that of the control group. In terms of abdominal fat weight, that in the GA group was already lower than in the control group after two weeks of feeding. Hence, we assumed that the reduction of body weight resulting from GA administration was caused by diminution of abdominal fat accumulation. No other differences were recognized in other tissues observed.

Histopathological examination of rat ovary

To consider the safety of GA administration in female rats, compared with that in male rats in our previous studies [*7*, *8*], we performed histopathological examination of the ovaries (Table 3). After two weeks of GA feeding, there were no evident morphological abnormalities in the follicle and corpus luteum. Moreover, no histopathological changes induced by GA were observed in the ovary (Table 3) and also in the uterus (data not shown) after four weeks of feeding.

Estradiol, FSH, LH and progesterone concentrations in rat serum

The levels of four hormones (estradiol, FSH, LH and progesterone) related to the estrous cycle were measured (Table 4), but there were no significant differences in any of them between the control group and the GA group after two or four weeks of feeding.

Serum biochemical parameters

Serum ALP and LAP activities were higher in the GA group than in the control group after the four weeks of treatment (Table 5). Other parameters showed no differences between the two groups.

Table 2. Final body weight, food intake and each tissue weight

	Two weeks		Four weeks	
	Control	Garcinia	Control	Garcinia
n	5	4	6	6
Final body weight (B.W.) (g)	233.50 ± 16.92	224.38 ± 14.97	261.00 ± 17.97	235.25 ± 15.37**
Food intake (g/day)	16.94 ± 1.70	15.24 ± 1.14	17.12 ± 1.37	15.61 ± 0.89
Liver (whole g)	8.03 ± 0.93	8.10 ± 0.58	8.44 ± 2.06	7.89 ± 0.91
(/100 g B.W.)	3.44 ± 0.37	3.61 ± 0.10	3.23 ± 0.22	3.36 ± 0.33
Left ovary (whole g)	0.07 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.06 ± 0.01
(/100 g B.W.)	0.03 ± 0.01	0.03 ± 0.001	0.03 ± 0.003	0.03 ± 0.004
Right ovary (whole g)	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.04	0.06 ± 0.01
(/100 g B.W.)	0.03 ± 0.004	0.03 ± 0.003	0.03 ± 0.003	0.03 ± 0.003
Uterus (whole g)	0.73 ± 0.20	0.81 ± 0.15	0.79 ± 0.26	0.84 ± 0.24
(/100 g B.W.)	0.32 ± 0.10	0.36 ± 0.05	0.31 ± 0.12	0.36 ± 0.12
Abdominal fat (whole g)	7.58 ± 1.67	4.57 ± 1.46*	11.42 ± 2.11	6.47 ± 1.63**
(/100 g B.W.)	3.22 ± 0.52	2.02 ± 0.56*	4.39 ± 0.77	2.74 ± 0.62**

Values are means ± SD of 4–6 rats in each group.
Significant differences between the Control group and GA group for each test period were analyzed by two samples *t*-test (Dr. SPSS II for windows).
Asterisks show significant difference; *: $p<0.05$, **: $p<0.01$.

Table 3. Histopathological examination of ovary in rats administered Garcinia

Two weeks	Control					Garcinia					
Rat No.	1	2	3	4	5	1	2	3	4		
Decrease in number of follicle	–	–	–	–	–	–	–	–	–		
Decrease in number of corpus luteum	–	–	–	–	–	–	+	–	–		
Atrophy of ovary	–	–	–	–	–	–	+	–	–		
Four weeks	**Control**						**Garcinia**					
Rat No.	1	2	3	4	5	6	1	2	3	4	5	6
Decrease in number of follicle	–	–	–	–	–	–	–	–	–	–	–	–
Decrease in number of corpus luteum	–	–	–	–	–	–	–	–	–	+	–	–
Atrophy of ovary	–	–	–	–	–	–	–	–	–	+	–	–

Marks show the degree of influence in rat overy; –: negative, +: mild, ++: moderate, +++: marked
Judged point:

	Decrease in number of follicle	Decrease in number of corpus luteum	Atrophy of ovary
–: negative	5 ≤ number of follicle	Number of corpus leteum ≥ 3	No remarkable change
+: mild	3 ≤ number of follicle ≤ 4	Number of corpus leteum ≥ 2	Width of ovary ≥ 60%
++: moderate	2 ≤ number of follicle ≤ 1	Number of corpus leteum ≥ 1	60%> width of overy ≥ 50%
+++: marked	Number of follicle: 0	Number of corpus leteum: 0	50%> width of ovary

Changes in FF-MAS, T-MAS and cholesterol concentrations in rat ovary

From the results obtained in male rat testis [*7*, *8*], we hypothesized that the accumulation of MAS substances in rat ovary might be affected by the competitive inhibition of ATP citrate lyase by (–)-HCA. So, we measured the concentrations of FF-MAS, T-MAS and cholesterol in rat ovary (Fig. 1), but no prominent differences were noted in any of them between the control and GA groups after two weeks or four weeks of feeding.

Table 4. Hormone analysis of serum in rats administered Garcinia

	Two weeks		Four weeks	
	Control	Garcinia	Control	Garcinia
	ng/ml		ng/ml	
Estradiol	49.80 ± 11.45	40.75 ± 17.52	39.60 ± 20.84	46.17 ± 12.84
Follicle-stimulating hormone	3.62 ± 2.23	4.28 ± 1.72	4.46 ± 1.00	4.52 ± 1.79
Luteinizing hormone	2.43 ± 0.61	3.03 ± 1.41	3.26 ± 2.55	3.38 ± 1.48
Progesterone	20.72 ± 9.16	15.93 ± 7.44	18.48 ± 10.82	13.98 ± 7.94

Values are means ± SD of 4–6 rats in each group.

Table 5. Serum biochemical parameters of rats administered Garcinia

Group		Two weeks		Four weeks	
		Control	Garcinia	Control	Garcinia
TP	g/dl	5.98 ± 0.08	5.50 ± 0.32	6.08 ± 0.24	6.03 ± 0.49
Alb	g/dl	3.18 ± 0.11	2.88 ± 0.10	3.20 ± 0.22	3.05 ± 0.29
A/G		2.80 ± 0.10	2.63 ± 0.26	2.88 ± 0.04	2.98 ± 0.21
BUN	mg/dl	13.40 ± 1.14	12.50 ± 1.73	14.40 ± 0.89	14.83 ± 1.94
CRN	mg/dl	0.27 ± 0.03	0.25 ± 0.03	0.28 ± 0.02	0.29 ± 0.02
Glc	mg/dl	237.20 ± 23.29	227.20 ± 11.52	211.80 ± 17.91	209.00 ± 18.31
NEFA	mEq/l	0.48 ± 0.12	0.32 ± 0.04	0.39 ± 0.07	0.36 ± 0.07
PL	mg/dl	155.80 ± 18.21	140.00 ± 20.38	160.20 ± 31.12	139.33 ± 21.91
TG	mg/dl	37.80 ± 16.02	32.75 ± 10.97	53.80 ± 34.90	28.67 ± 10.23
TCho	mg/dl	76.80 ± 6.72	71.25 ± 8.46	76.40 ± 16.95	67.17 ± 11.86
ALP	IU/l	514.20 ± 115.09	447.25 ± 94.61	296.40 ± 39.45	476.67 ± 81.52**
ALT	IU/l	24.60 ± 3.91	21.75 ± 2.36	23.80 ± 2.17	23.33 ± 4.03
AST	IU/l	58.60 ± 6.31	59.25 ± 6.65	55.20 ± 7.26	61.17 ± 10.01
ChE	IU/l	389.00 ± 98.35	297.75 ± 42.67	492.00 ± 276.32	358.33 ± 71.25
LAP	U	134.60 ± 16.73	144.00 ± 11.34	126.40 ± 6.23	142.17 ± 4.79***
LDH	IU/l	107.00 ± 41.76	94.50 ± 51.20	117.00 ± 47.58	139.33 ± 66.10
Ketone body	μmol/l	170.80 ± 57.01	182.75 ± 48.88	139.52 ± 25.36	174.87 ± 75.65

Values are means ± SD of 4–6 rats in each group.
Significant differences between the Control group and GA group for each test period were analyzed by two samples *t*-test (Dr. SPSS II for windows).
Asterisks show significant difference; **: $p<0.01$, ***: $p<0.001$.
Abbreviations: TP; total protein, Alb; albumin, A/G; albumin/globulin, BUN; blood urea nitrogen, CRN; creatinine, Glc; glucose, NEFA; non-estrified fatty acid, PL; phospholipids, TG; triglyceride, TCho; total cholesterol, ALP; alkaline phospatase, ALT; L-alanine: 2-oxoglutarate aminotransferase, AST; L-asparatate: 2-oxoglutarate aminotransferase, ChE; cholinesterase, LAP; leucine amino peptidase, LDH; lactate dehydrogenase.

Discussion

We have reported recently that the high dose of (−)-HCA-containing GA ((−)-HCA levels; 102 mmol/kg diet or higher) causes potent testicular atrophy and toxicity in male rats [7], and suggested that (−)-HCA-mediated inhibition of ATP citrate lyase leads to diminished accumulation of T-MAS in rat testis [8], thus probably resulting in impairment of spermatogenesis. In the present study, we examined the effect of GA administration on safety with special reference to female reproductive organs in rats.

In the former study, we focused the effect of GA administration on body fat accumulation using Zucker obese rats [7], and in the previous study, we examined the effect of GA administration on testicular toxicity in Fischer 344 (F344) strain rats [8]. Since the aim of the current study was to elucidate safety in female rats, we used rats of SD-IGS strain, a strain often used lately in toxicological study together with F344 strain, and a diet containing (−)-HCA at 154mmol/kg diet, the highest dose in the former study [7].

In general, it is known that the physical properties of the mammalian vaginal mucosa change before and after the

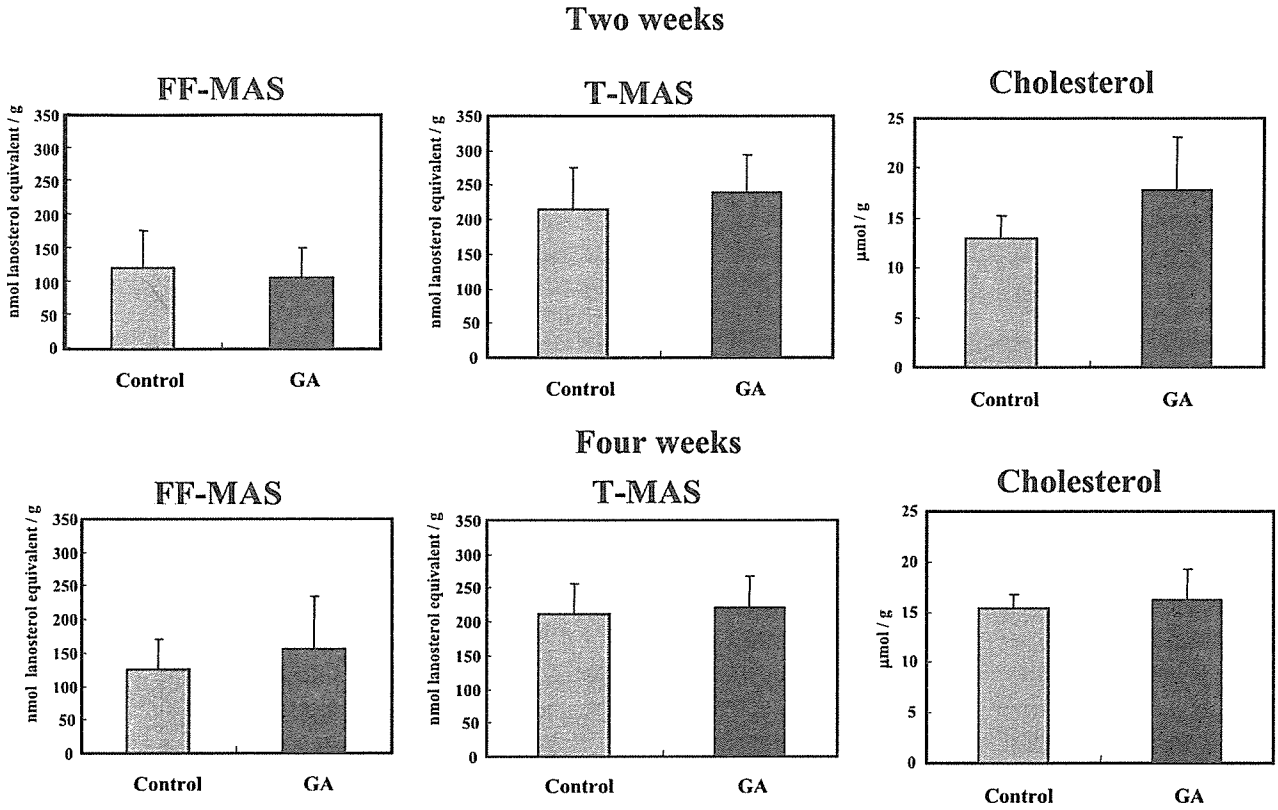

Fig. 1. Concentrations of follicular fluid meiosis-activating sterol (FF-MAS), testis meiosis-activating sterol (T-MAS) and cholesterol in ovaries of rats administered each diet for two weeks (up) and four weeks (down). Values are means ± SD of 4–6 rats in each group. Differences between the control group and the GA group were analyzed by two-sample *t*-tests (Dr. SPSS II for Windows). No significant differences were noticed in any of the analytical items.

estrous stage, and EIV is a method to decide the proestrous stage by making use of this property [*14*]. During the experimental period, EIV was measured in all rats daily, and the rats were sacrificed on the proestrus day in the last week of the feeding period. Accordingly, rats that did not have the estrous cycle were excluded from each experimental group.

The plasma estradiol concentration is known to increase to a peak in the proestrous stage [*12*]. Our data also showed that estradiol had the highest concentration among the four hormones examined. But, there were no significant differences in any of the hormones between the control group and the GA group. Also, the histopathological appearances of the ovary and uterus, and serum biochemical parameters in most of the rats administered GA were normal. Consequently, we suggest that GA administration for a short period (four weeks) may not affect the ovarian function of female rats. In male rats, GA administration even for a short period affected their testicular function (e.g., decline of testis weight, decrease in the serum inhibin-B concentration, hypospermatogenesis etc.) [*7*, *8*]. From the results obtained in male rats, we presumed that these influences of GA were due to prevention of MAS accumulation *via* the inhibition of ATP citrate lyase in the testis [*8*].

With regard to MAS substances, it has been shown recently that T-MAS is a specific intermediate product of cholesterol biosynthesis in testicular germ cells [*13*]. Bull testis T-MAS has been isolated and characterized [*15*]. Similarly, FF-MAS has also been isolated from human follicular fluid [*15*]. These MAS substances are produced from lanosterol by the action of lanosterol 14α-demethylase (CYP51) and sterol Δ14-reductase in the cholesterol biosynthetic pathway. FF-MAS and T-MAS are presumed to be signaling substances that trigger the start of meiotic division of the oocyte and spermatocyte, respectively [*16*]. Hence, we measured the concentrations of FF-MAS and T-MAS in rat ovary. There were no significant differences in the concentrations of either of the MAS substances or cholesterol between the control and GA groups after two or four weeks of feeding. Therefore, GA administration did not affect the accumulation of FF-MAS in rat ovary. Although it is unknown why the accumulation of MAS substances differs in male and female rats, some possible reasons can be considered. For example, HCA may not enter the ovary directly, or there may be an alternative biosynthetic pathway of cholesterol for the accumulation of FF-MAS in the ovary.

But, the precise reason remains to be clarified.

(−)-HCA or (−)-HCA-containing GA has been shown to suppress body fat accumulation in experimental male rodents [*7*, *17–20*]. Few data are available for female rodent [*21*, *22*]. In this study, therefore, we also examined the effect of GA administration on body fat accumulation in female rats. Although the food intake was different between the control group and the GA group, there was no difference statistically. However, both the final body weight and abdominal fat weight in the GA group were statistically lower than those in the control group. From these results, we assumed that the body weight loss caused by GA administration may be due mainly to the decrease in body fat weight. Abdominal fat weight in the GA group was about 56–60% of that in the control group. In male rats, epididymal fat weight in the group given the highest dose of GA was about 73% of that in the control group [*7*]. Consequently, we think that the effect of (−)-HCA on body fat accumulation may differ between males and females. But, the precise reasons also remain to be clarified.

A high dose of (−)-HCA-containing GA (154 mmol HCA/kg diet) was effective for decreasing abdominal fat weight and did not affect ovarian function (in term of histopathological features and sex hormone levels) in female rats. The latter result is based on a short study period (four weeks), and therefore, a safety study conducted over a long period will be required in order to judge whether or not GA is really safe for females.

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