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).

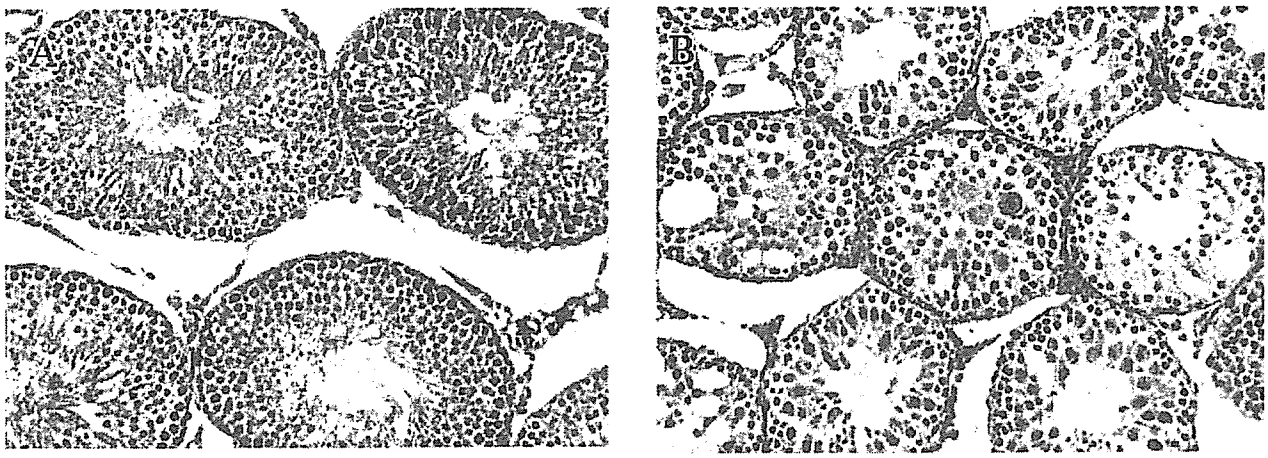

Fig. 2. Histopathological examination of testes. A; control rat kept for four weeks (magnification ×200), B; *Garcinia*-fed rat kept for four weeks (magnification ×200).

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

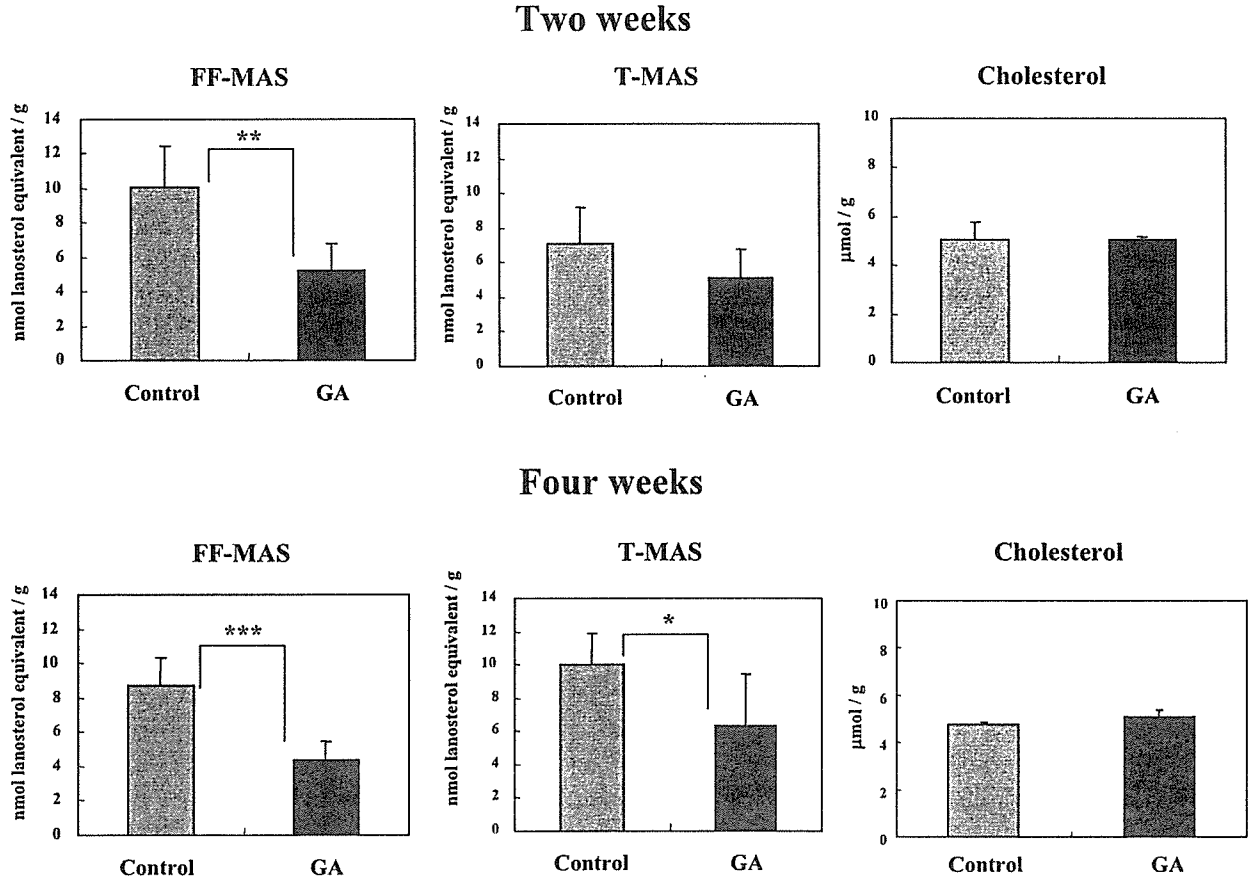

Fig. 3. Concentrations of follicular fluid meiosis-activating sterol (FF-MAS), testis meiosis-activating sterol (T-MAS) and cholesterol in testes of rats administered each diet for two weeks (up) and four weeks (down). Values are means ± SD of 6–7 rats. Differences between the control group and the GA 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

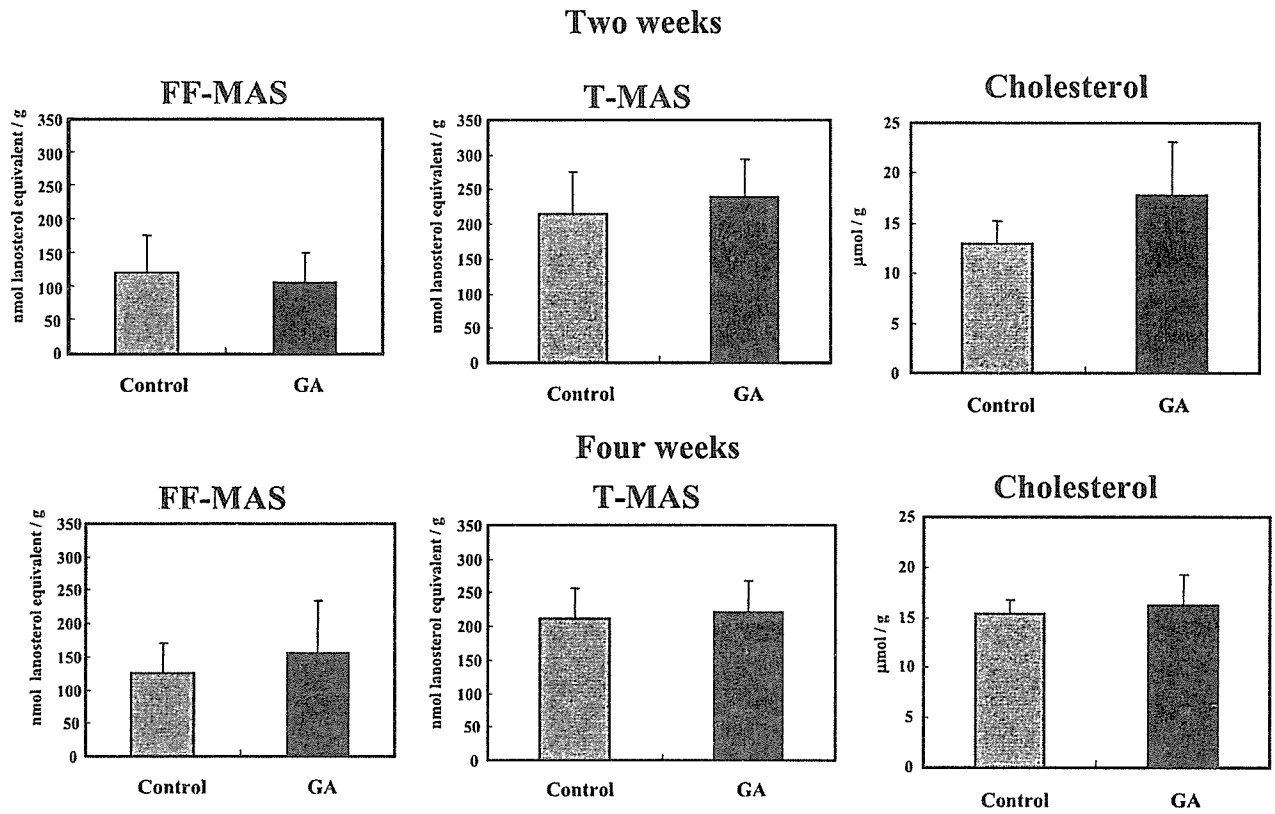

Fig. 1. Concentrations of follicular fluid meiosis-activating sterol (FF-MAS), testis meiosis-activating sterol (T-MAS) and cholesterol in ovaries of rats administered each diet for two weeks (up) and four weeks (down). Values are means ± SD of 4–6 rats in each group. Differences between the control 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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Effect of *Citrus Aurantium* Combined with Caffeine and/or Tea Catechins on Body Fat Accumulation and Its Safety in Rats

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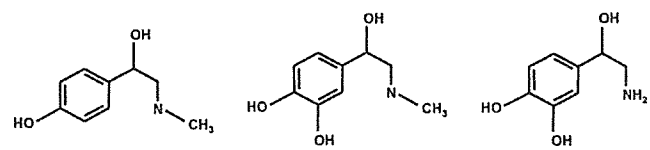
Summary **We investigated the weight loss efficacy and safety of *Citrus aurantium* (CA, 1,000 mg/kg diet) along with usual levels of adrenergic stimulants in foods, *i.e.* caffeine (100 mg/kg diet) and/or tea catechins (500 mg/kg diet), in rats for 44 or 45 days. Even in combination with caffeine and tea catechins, the suppressive effect of CA against body fat accumulation was negligible, whereas no deleterious influences concerning cardiotoxicity were observed. Thus, although the efficacy of CA for weight loss seems to be questionable, no safety problems may occur even in people who habitually consume coffee and tea. However, it is noteworthy that the intake of CA markedly elevated the urinary excretion of adrenaline, and this was not affected by intake of caffeine and/or tea catechins at the usual levels. Therefore, the safety of CA intake with high levels of caffeine and tea catechins, especially in people at risk of heart disease, remains to be elucidated further.**

***Key Words*: *Citrus aurantium*, caffeine, tea catechins, body fat accumulation, safety**

Introduction

Herbal supplements containing *Citrus aurantium* (CA) extracts have recently been marketed for claiming weight loss (reviewed in references [*1–3*]). Similarly to ephedra, CA contains several adrenergic amines, and the most abundant one is synephrine [*4*]. Synephrine is structurally similar to adrenaline and noradrenaline (Fig. 1), and has been suggested to stimulate thermogenesis and lipolysis in adipocytes through the activation of β_3-adrenergic receptor and thereby facilitate weight loss. Some clinical trials showed that CA-containing combination products lost body weight and/or body fat [*5–7*], but these results are likely to arise from ingredients other than CA, particularly ephedrine or caffeine. Therefore, little



Synephrine **Adrenaline** **Noradrenaline**

Fig. 1. Chemical structures of synephrine, adrenaline and noradrenaline.

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Abbreviations: A/G, ratio of albumin/globulin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; BW, body weight; CA, *Citrus aurantium*; Caf, caffeine; Cat, tea catechins; HDL, high-density lipoprotein; HPLC, high performance liquid chromatography; LDH, lactate dehydrogenase; NOAEL, no observed adverse effect level; PLs, phospholipids; T3, triiodothyronine; T4, thyroxine; TG, triacylglycerol.

evidence supports the efficacy of CA itself for weight loss in human at present.

On the other hand, synephrine acts as an α- and β-adrenergic antagonist [*8*, *9*] and thus is predicted to have hemodynamic effects, with potential implication risks for adverse effects. In fact, there were one case report of acute lateral-wall myocardial infarction in a 55-year-old woman [*10*], one case report of exercise-induced syncope with QT prolongation in a 22-year-old woman [*11*], and one case report of ischemic stroke in a 38-year-old man [*12*], which were associated with use of ephedra-free dietary supplements containing CA. In addition, Calapai *et al.* [*13*] reported that in rats the oral administration of CA alone significantly reduced body weight gain but high dose of CA caused ventricular arrhythmias and death.

Therefore, we have previously investigated the safety of CA alone and its suppressive effect against body fat accumulation in rats [*14*]. Dietary CA intakes less than or equal to 1,000 mg CA/kg diet (recommended daily intake of CA products is nearly equivalent to 200 mg CA/kg diet) did not suppress body weight gain or body fat accumulation, and not cause any biochemical and histopathological changes concerning toxicity. Thus, CA alone seemed to be ineffective against body fat accumulation but safe. However, an excessive intake of 5,000 mg/kg diet of CA suppressed significantly perirenal fat accumulation, but induced concomitantly heart weight loss and increased plasma levels of adrenaline and dopamine and urinary excretion of adrenaline [*14*].

There are some food components that could stimulate adrenergic antagonists, e.g. caffeine and catechin-polyphenols [*15*]. These stimulants may be synergistic with CA for weight loss, but may also increase the risk of cardiotoxicity as Marcus and Grollman [*16*] and Jordan *et al.* [*17*] have warned. It needs to be noted that some weight-loss products contain CA together with such stimulants at the same time. In 2004, Health Canada banned a certain product containing synephrine, caffeine, catechins and other stimulants [*18*]. Therefore, in the present study, we investigated the influences of simultaneous intake of CA with caffeine and/or tea catechins on body fat accumulation and safety in rats.

Materials and Methods

Animals, diets and feeding trial

The experiments were done in accordance with the guidelines of the Animal Committee of the Incorporated Administrative Agency, National Institute of Health and Nutrition (Tokyo, Japan).

Male Sprague-Dawley rats (CLEA Japan, Tokyo, Japan), 8 week of age and weighing 260–290 g, were housed individually at a controlled temperature of 22 ± 1°C and humidity of 50–60% with a 12-h light:dark cycle. They were first fed the AIN-93G purified diet for laboratory rodents [*19*] and had access to water *ad libitum* for 7 days. The rats were then randomly assigned by weight and weight gain to 7 diet groups, (i) control group, (ii) CA group, (iii) caffeine (Caf) group, (iv) CA + Caf group, (v) tea catechins (Cat) group, (vi) CA + Cat group and (vii) CA + Caf + Cat group, and raised for 44 or 45 days. Food and water were available *ad libitum*.

The composition of the high-fat experimental diets, based on the AIN-93G purified diet for laboratory rodents, is shown in Table 1. Lipid content of the diets was 20 w/w % and 37.5% of total energy. Powder extract of CA was purchased from Exquim, S.A. (Barcelona, Spain); this product contains 6.4% of synephrine according to manufacturer's data sheet and we confirmed this by HPLC [*14*]. Caffeine anhydride was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Polyphenon-60 (green tea catechins, Lot. 0409171) was purchased from Mitsui Norin Co., Ltd. (Tokyo, Japan); this product contains 60.3% of catechins (27.2% of (−)-epigallocatechin gallate, 15.2% of (−)-epigallocatechin, 7.7% of (−)-epicatechin gallate, 6.8% of (−)-epicatechin, 2.9% of (−)-gallocatechin gallate and 0.5% of (−)-catechin gallate) according to manufacturer's data sheet and 7.4% of caffeine measured by HPLC as reported by Goto *et al.* [*20*].

After 13, 28 and 39 days, 24-hr urine was collected using metabolic cages and was stored at 4°C until use. At the end of the experiment (44 or 45 days), the rats were killed by cardiac puncture. Their liver, kidney, heart, testis, spleen, lung, and perirenal and epididymal fad pads were promptly excised, washed with isotonic saline and weighed. The hearts of all rats 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 use.

Assay procedures

Serum inhibin-B concentration was determined by a sandwich EIA kit (Oxford Bio-Innovation Ltd., Oxfordshire, UK). 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 (TG), phospholipids (PLs), free fatty acids, total cholesterol, HDL-cholesterol, total bilirubin, triiodothyronine (T3) and thyroxine (T4); plasma parameters were as follows: adrenaline, noradrenalin and dopamine; urinary parameters were as follows: adrenaline, noradrenalin, dopamine and homovanillic acid. These biochemical parameters were measured with commercially available kits.

Table 1. Composition of experimental diets given to rats

Group	Control	CA	Caf	CA + Caf	Cat	CA + Cat	CA + Caf + Cat
				g/kg diet			
Casein	200.0	200.0	200.0	200.0	200.0	200.0	200.0
L-Cystine	3.0	3.0	3.0	3.0	3.0	3.0	3.0
α-Cornstarch	399.5	398.5	399.4	398.4	399.0	398.0	397.9
Sucrose	102.5	102.5	102.5	102.5	102.5	102.5	102.5
Cellulose powder	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Vitamin mix (AIN-93-VX)	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Mineral mix (AIN-93G-MX)	35.0	35.0	35.0	35.0	35.0	35.0	35.0
tert-Butylhydroquinone	0.014	0.014	0.014	0.014	0.014	0.014	0.014
Soybean oil	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Lard	150.0	150.0	150.0	150.0	150.0	150.0	150.0
				mg/kg diet			
Citrus aurantium[a]	0	1000	0	1000	0	1000	1000
Caffeine anhydride	0	0	100	100	0	0	100
Polyphenon-60 (tea catechins)[b]	0	0	0	0	500	500	500
Synephrine content	0	64	0	64	0	64	64
Caffeine content	0	0	100	100	37	37	137
Tea catechins content	0	0	0	0	302	302	302
Total energy (kcal/kg diet)[c]	4800	4796	4800	4796	4798	4794	4794

CA, *Citrus aurantium*; Caf, caffeine; Cat, tea catechins

[a]*Citrus aurantium* contained 6.4% of synephrine.

[b]Polyphenon-60 contained 60.3% of tea catechins and 7.4% of caffeine.

[c]The energy density of all diets was calculated using Atwater energy factors for energy calculation.

Statistical analysis

Statistical analysis was performed using one-way ANOVA followed by Tukey-Kramer post-hoc test for multiple comparisons. The *p*-values less than 0.05 were considered statistically significant. Data that failed the Levene's test for equality of variances were log-transformed to stabilize variances before analysis.

Results

The rats consumed 22.8–26.7 g food/day and gained 4.8–5.7 g BW/day over the 44- or 45-day-experiment (Table 2). There were no significant differences in the food intake and body weight gain among the groups. The relative weights of liver, kidney, testis, spleen, lung, and perirenal and epididymal fat pad did not change significantly in any of the treatment groups.

The hearts showed no histopathological abnormalities attributable to three dietary components of CA, caffeine and tea catechins, even in the CA + Caf + Cat group (data not shown). The plasma concentrations of adrenaline, noradrenaline and dopamine were not significantly different between the groups (Table 3). Furthermore, no obvious changes in the serum biochemical parameters were observed in any of the treatment groups (Table 4). The variations of serum albumin concentration were within the normal range. The 24-h urinary excretion of adrenaline was already increased in the CA intake groups on the 13th day of the experiment, and remained elevated thereafter (Table 5). Caffeine and tea catechins did not further increase the urinary excretion of adrenaline even in the combination with CA. On the other hand, there were not notable changes in the urinary excretion of noradrenaline, dopamine and homovanillic acid between the groups.

Discussion

The dose of CA used in this study was 1,000 mg/kg diet, which was no observed adverse effect level (NOAEL) of CA alone in our previous study [*14*]. CA intakes calculated using final body weights in the present study were *ca.* 40–50 mg CA/kg BW/day (*ca.* 3 mg synephrine/kg BW/day); this is *ca.* 2,000–2,500 mg CA/day (*ca.* 150 mg synephrine/day) for a 50 kg BW human, and 2–25 times excess of recommended daily intake on the labels of many CA products (100–1,000 mg CA/day). In a similar manner, caffeine intakes were *ca.* 2–6 mg caffeine/kg BW/day; this is *ca.* 100–300 mg caffeine/day for a 50 kg BW human, and

Table 2. Influence of dietary *Citrus aurantium* (CA) combined with caffeine (Caf) and/or tea catechins (Cat) on food intake, body weight gain and tissue weight in rats.

Group	Control	CA	Caf	CA + Caf	Cat	CA + Cat	CA + Caf + Cat
Food intake (g/day)	24.3 ± 2.2^{NS}	22.8 ± 2.4	25.2 ± 2.1	25.1 ± 2.7	24.0 ± 2.3	26.7 ± 1.4	25.7 ± 1.4
Citrus aurantium intake (mg/day)	0	22.8 ± 2.4	0	25.1 ± 2.7	0	26.7 ± 1.4	25.7 ± 1.4
Synephrine intake (mg/day)	0	1.5 ± 0.2	0	1.6 ± 0.2	0	1.7 ± 0.1	1.6 ± 0.1
Caffeine intake (mg/day)	0	0	2.5 ± 0.2	2.5 ± 0.3	0.9 ± 0.1	1.0 ± 0.1	3.5 ± 0.2
Tea catechins intake (mg/day)	0	0	0	0	7.2 ± 0.7	6.9 ± 0.4	7.7 ± 0.4
Body weight gain (g)	246 ± 41^{NS}	220 ± 41	241 ± 21	240 ± 37	211 ± 14	248 ± 25	243 ± 37
Initial body weight (g)	312 ± 14^{NS}	309 ± 9	310 ± 10	310 ± 9	310 ± 14	310 ± 12	311 ± 8
Final body weight (g)	558 ± 51^{NS}	530 ± 47	551 ± 25	550 ± 45	521 ± 24	558 ± 35	554 ± 43
Liver (g/100 g BW)	3.28 ± 0.21^{NS}	3.04 ± 0.12	3.25 ± 0.17	3.15 ± 0.47	2.99 ± 0.23	3.10 ± 0.10	3.06 ± 0.11
Kidney (g/100 g BW)	0.59 ± 0.04^{NS}	0.58 ± 0.03	0.60 ± 0.04	0.61 ± 0.06	0.62 ± 0.04	0.61 ± 0.05	0.61 ± 0.05
Heart (g/100 g BW)	0.26 ± 0.02^{NS}	0.25 ± 0.01	0.25 ± 0.02	0.25 ± 0.02	0.25 ± 0.01	0.25 ± 0.01	0.24 ± 0.02
Testis (g/100 g BW)	0.70 ± 0.05^{NS}	0.74 ± 0.10	0.73 ± 0.07	0.76 ± 0.10	0.75 ± 0.06	0.73 ± 0.07	0.69 ± 0.07
Spleen (g/100 g BW)	0.18 ± 0.02^{NS}	0.18 ± 0.05	0.16 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	0.15 ± 0.02	0.17 ± 0.01
Lung (g/100 g BW)	0.29 ± 0.05^{NS}	0.29 ± 0.03	0.29 ± 0.03	0.29 ± 0.04	0.31 ± 0.01	0.30 ± 0.02	0.30 ± 0.02
Perirenal fat pad (g/100 g BW)	3.08 ± 0.50^{NS}	3.03 ± 0.77	3.28 ± 0.72	3.17 ± 0.91	3.05 ± 0.90	2.91 ± 0.61	3.12 ± 0.58
Epididymal fat pad (g/100 g BW)	2.63 ± 0.32^{NS}	2.48 ± 0.46	2.62 ± 0.45	2.58 ± 0.60	2.47 ± 0.35	2.60 ± 0.43	2.44 ± 0.34

Each value is the mean ± SD of 6–7 rats in each group. NS, not significant.

Table 3. Influence of dietary *Citrus aurantium* (CA) combined with caffeine (Caf) and/or tea catechins (Cat) on plasma concentrations of catecholamines in rats.

Group	Control	CA	Caf	CA + Caf	Cat	CA + Cat	CA + Caf + Cat
Adrenaline (nmol/liter)	36.0 ± 12.4^{NS}	47.8 ± 17.2	45.9 ± 21.1	48.3 ± 25.1	47.7 ± 20.6	44.4 ± 22.1	32.6 ± 7.7
Noradrenaline (nmol/liter)	25.4 ± 12.5^{NS}	30.2 ± 21.8	32.5 ± 12.4	33.3 ± 21.6	36.7 ± 26.7	31.3 ± 14.3	21.0 ± 12.4
Dopamine (nmmol/liter)	1.15 ± 0.54^{NS}	1.21 ± 0.46	1.01 ± 0.43	0.77 ± 0.31	1.06 ± 0.28	1.06 ± 0.56	0.57 ± 0.23

Each value is the mean ± SD of 6–7 rats in each group. NS, not significant.

equivalent to caffeine level in a few cups of coffee. Tea catechins intakes were *ca.* 14 mg tea catechins/kg BW/day; this is *ca.* 700 mg tea catechins/day for a 50 kg BW human, and equivalent to tea catechins level in *ca.* 1 liter of green tea. In our present study, caffeine or tea catechins alone did not suppress body fat accumulation although they have been reported to reduce body fat mass. This may be because the doses used in this study were below an effective dose, e.g., 250 mg caffeine/kg diet, in rats, reported by Kobayashi-Hattori *et al.* [*21*], and 2,000 mg tea catechins/kg diet, in mice, reported by Murase *et al.* [*22*], respectively.

Our results suggest that excess level of CA did not suppress body weight gain and body fat accumulation even in combination with usual levels of caffeine and tea catechins in rats. However, Colker *et al.* [*5*] reported that human subjects (BMI>25 kg/m^2) received CA (975 mg/day), caffeine (528 mg/day) and St. John's wort (900 mg/day) for 6 weeks with exercise and mild caloric restriction lost body weight and body fat significantly. In addition, Gougeon *et al.* [*23*] reported that human subjects received single dose of CA capsules (containing 26 mg synephrine and 10.5 mg other adrenergic amines) were significantly higher in energy expenditure compared with the baseline. Carpéné *et al.* [*24*] showed that adrenergic agonists stimulated lipolysis in white fat cells through the activation of β_3-adrenergic receptor in rats, but through the activation of β_1- and/or β_2-adrenergic receptor in humans, while synephrine activated β_1- and/or β_2-adrenergic receptor rather

Table 4. Influence of dietary *Citrus aurantium* (CA) combined with caffeine (Caf) and/or tea catechins (Cat) on serum biochemical indicators in rats.

Group	Control	CA	Caf	CA + Caf	Cat	CA + Cat	CA + Caf + Cat
Total protein (g/liter)	60.8 ± 2.2^{NS}	58.5 ± 1.5	60.8 ± 1.9	60.6 ± 2.1	59.3 ± 1.5	59.8 ± 0.4	59.3 ± 1.7
Albumin (g/liter)	29.5 ± 1.0^{ab}	28.8 ± 0.8^{a}	30.5 ± 0.5^{b}	30.3 ± 1.3^{b}	29.3 ± 0.5^{ab}	29.8 ± 0.8^{ab}	28.6 ± 0.5^{a}
Ratio of albumin/globulin	0.95 ± 0.05^{NS}	0.97 ± 0.05	1.00 ± 0.06	1.01 ± 0.04	0.97 ± 0.05	0.98 ± 0.08	0.94 ± 0.05
Aspartate aminotransferase (U/liter)	87.7 ± 11.9^{NS}	86.3 ± 11.6	85.3 ± 7.9	79.1 ± 11.8	91.7 ± 12.4	91.7 ± 7.7	86.3 ± 12.8
Alanine aminotransferase (U/liter)	28.3 ± 4.6^{NS}	30.3 ± 8.3	24.8 ± 5.5	26.4 ± 6.1	28.3 ± 6.0	25.5 ± 1.9	23.4 ± 2.3
Alkaline phosphatase (U/liter)	405 ± 40^{NS}	367 ± 66	361 ± 53	417 ± 114	349 ± 60	461 ± 79	418 ± 68
Lactate dehydrogenase (U/liter)	655 ± 275^{NS}	747 ± 290	705 ± 215	594 ± 155	972 ± 351	608 ± 131	798 ± 367
Creatinine (μmol/liter)	29.3 ± 1.5^{NS}	30.1 ± 3.7	26.1 ± 1.5	26.1 ± 3.3	27.8 ± 0.9	27.0 ± 4.9	28.3 ± 1.0
Urea nitrogen (mmol/liter)	4.9 ± 0.9^{NS}	4.7 ± 0.5	4.6 ± 0.6	4.4 ± 0.7	4.9 ± 0.4	4.5 ± 0.4	4.5 ± 0.5
Glucose (mmol/liter)	13.7 ± 1.4^{NS}	13.5 ± 1.7	13.1 ± 1.4	12.7 ± 1.8	12.6 ± 0.4	13.5 ± 1.5	1.30 ± 1.3
Glycosylated albumin (%)	8.47 ± 0.48^{NS}	7.45 ± 1.28	6.22 ± 1.96	6.54 ± 1.57	7.55 ± 1.02	6.72 ± 1.73	7.34 ± 1.17
Triacylglycerol (mmol/liter)	1.79 ± 0.57^{NS}	1.51 ± 0.50	1.53 ± 0.59	1.74 ± 1.07	1.48 ± 0.61	1.44 ± 0.73	1.62 ± 1.24
Phospholipids (mmol/liter)	1.98 ± 0.28^{NS}	1.61 ± 0.25	1.93 ± 0.37	1.97 ± 0.49	1.64 ± 0.38	1.69 ± 0.36	1.69 ± 0.40
Free fatty acids (mmol/liter)	0.72 ± 0.16^{NS}	0.64 ± 0.16	0.66 ± 0.13	0.64 ± 0.20	0.69 ± 0.20	0.60 ± 0.21	0.58 ± 0.14
Total cholesterol (mmol/liter)	1.85 ± 0.28^{NS}	1.48 ± 0.22	1.80 ± 0.45	1.91 ± 0.33	1.44 ± 0.29	1.53 ± 0.18	1.61 ± 0.33
HDL-cholesterol (mmol/liter)	0.72 ± 0.07^{NS}	0.63 ± 0.08	0.70 ± 0.10	0.71 ± 0.12	0.61 ± 0.11	0.65 ± 0.10	0.62 ± 0.07
Total bilirubin (μmol/liter)	2.0 ± 0.7^{NS}	1.7 ± 0.0	2.9 ± 0.9	1.7 ± 0.0	2.3 ± 0.9	2.3 ± 0.9	2.0 ± 0.6
Triiodothyronine (nmol/liter)	1.08 ± 0.12^{NS}	1.01 ± 0.16	1.03 ± 0.17	1.06 ± 0.17	1.07 ± 0.11	1.15 ± 0.16	1.01 ± 0.14
Thyroxine (nmol/liter)	59.0 ± 3.6^{NS}	50.4 ± 9.3	57.7 ± 7.3	57.7 ± 9.4	54.5 ± 3.4	57.3 ± 5.1	53.7 ± 9.8
Inhibin-B (ng/liter)	32.8 ± 11.2^{NS}	35.7 ± 9.8	30.3 ± 10.9	33.2 ± 10.3	41.2 ± 11.8	34.4 ± 10.2	34.2 ± 12.3

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

than β_3-adrenergic receptor in rats. Thus, the suppressive effect of synephrine against body fat accumulation in rats may not directly apply to that in humans, and *vice versa*. However, there is still little evidence that CA itself would be effective for weight loss in humans, although many products for weight loss containing CA have been widely marketed. In addition, Jordan *et al.* [*9*] reported that synephrine was more than 4 orders of magnitude less active β_1- and β_2-antagonist compared to noradrenaline. Therefore, CA seems not to be effective for weight loss and body fat accumulation in humans.

On the other hand, the intake of CA even in combination with caffeine and tea catechins did not induce cardiotoxicity. In addition, serum and plasma biochemical parameters including plasma adrenaline and dopamine, which were increased by the large excess intake of CA in our previous study [*14*], did not change in any of the treatment groups. We also measured inhibin-B as a marker of spermatogenesis, which we found to be decreased in male rats by an excess intake of *Garcinia cambogia* contained also in weight loss products [*25*, *26*]. But the concentration did not change even after the intake of CA combined with caffeine and tea catechins.

Urinary excretion of adrenaline in the CA groups was markedly increased, but not synergistic with caffeine and/or tea catechins. Prolonged elevation of catecholamines in the circulation begins to initiate deleterious effects, particularly on the heart [*27*]. Elevated circulating adrenaline due to the intake of CA seems to be excreted promptly into urine, and usual levels of caffeine and tea catechins did not affect the circulating level of adrenalin and its excretion, and thereby no cardiotoxicity may occur under the current experimental

Table 5. Influence of dietary *Citrus aurantium* (CA) combined with caffeine (Caf) and/or tea catechins (Cat) on 24-h urinary excretion of catecholamines and homovanillic acid in rats.

Group	Control	CA	Caf	CA + Caf	Cat	CA + Cat	CA + Caf + Cat
				13th day of experiment			
Adrenaline (nmol/day)	1.27 ± 0.39^{a}	3.85 ± 0.94^{b}	1.09 ± 0.34^{a}	6.98 ± 5.11^{b}	1.54 ± 0.25^{a}	5.16 ± 2.02^{b}	5.51 ± 1.07^{b}
Noradrenaline (nmol/day)	7.66 ± 1.01^{ab}	7.17 ± 1.42^{a}	8.50 ± 0.51^{ab}	8.08 ± 1.22^{ab}	8.89 ± 1.15^{ab}	9.13 ± 2.36^{ab}	10.04 ± 1.94^{b}
Dopamine (nmol/day)	21.8 ± 6.26^{NS}	19.0 ± 3.87	20.8 ± 1.03	26.1 ± 7.16	26.3 ± 4.98	25.2 ± 7.08	24.6 ± 3.13
Homovanillic acid (nmol/day)	129 ± 27^{a}	136 ± 15^{a}	149 ± 12^{ab}	149 ± 24^{ab}	139 ± 16^{ab}	170 ± 11^{b}	147 ± 17^{ab}
				28th day of experiment			
Adrenaline (nmol/day)	1.43 ± 0.53^{a}	4.23 ± 1.15^{b}	1.15 ± 0.31^{a}	3.99 ± 0.90^{b}	1.52 ± 0.28^{a}	3.17 ± 0.54^{b}	3.83 ± 1.30^{b}
Noradrenaline (nmol/day)	9.19 ± 2.10^{NS}	8.00 ± 0.96	9.30 ± 1.20	7.86 ± 0.88	8.91 ± 1.22	9.02 ± 1.63	8.37 ± 2.40
Dopamine (nmol/day)	25.0 ± 4.59^{NS}	23.6 ± 2.53	24.9 ± 3.98	20.5 ± 2.42	22.6 ± 4.58	21.8 ± 3.76	20.7 ± 4.48
Homovanillic acid (nmol/day)	137 ± 14^{NS}	155 ± 22	151 ± 13	139 ± 11	143 ± 11	152 ± 21	137 ± 18
				39th day of experiment			
Adrenaline (nmol/day)	1.59 ± 0.68^{a}	3.61 ± 1.27^{b}	1.18 ± 0.43^{a}	3.32 ± 0.77^{b}	1.49 ± 0.33^{a}	3.34 ± 0.52^{b}	3.97 ± 1.38^{b}
Noradrenaline (nmol/day)	10.17 ± 2.84^{NS}	8.86 ± 1.33	9.95 ± 1.70	8.52 ± 0.97	9.93 ± 0.80	10.26 ± 2.15	9.77 ± 2.04
Dopamine (nmol/day)	28.4 ± 8.19^{NS}	25.8 ± 4.06	25.2 ± 2.90	21.4 ± 3.28	24.5 ± 3.19	23.2 ± 4.35	23.4 ± 3.77
Homovanillic acid (nmol/day)	152 ± 25^{NS}	161 ± 16	154 ± 10	146 ± 15	148 ± 16	167 ± 24	151 ± 18

Each value is the mean ± SD of 5–7 rats in each group. Means in a row that are not followed by a common superscript letter are different, $p<0.05$. NS, not significant.

conditions. Therefore, if the instructions on the labels of CA products are properly followed, no safety problems may occur even in combination with habitual intakes of caffeine and tea catechins.

Haller *et al.* [*28*] have reported that single dose of Xenadrine EFX, a product containing 5.5 mg synephrine in combination with other many stimulants such as green tea extract, yerba mate, tyramine, *etc.*, induced more intense cardiovascular stimuli compared with Advantra Z, a product containing 46.9 mg synephrine alone, in a clinical trial; stimulants included in Xenadrine EFX may increase cardiovascular effect of synephrine. Furthermore, Dulloo *et al.* [*29*] showed that a green tea extract containing 50 mg caffeine and 90 mg epigallocatechin gallate increased the 24-hr energy expenditure and 24-h urinary noradrenaline excretion in humans; caffeine and tea catechins seem to affect the sympathetic nervous system. In addition, there are some case reports of adverse effects associated with use of ephedra-free dietary supplements containing CA (see Introduction). Therefore, the safety of CA with higher levels of caffeine and tea catechins needs to be examined further.

The characteristic observations in this and our previous studies are the elevated urinary excretion of adrenaline, but not noradrenaline, in rats fed CA. In addition, an intake of a CA product also increased excretion of adrenaline in humans [*23*]. As mentioned above, elevated adrenaline exceeding physiological concentrations may cause cardiotoxicity, and adrenochrome, an oxidative metabolite of adrenaline, is postulated to be the biochemical initiators of cardiotoxicity, rather than adrenaline *per se* [*27*]. It is noteworthy that synephrine was reported to be oxidized to adrenochrome by mushroom tyrosinase, *via* adrenaline as an intermediate [*30*]. If the increased adrenaline is a metabolite of synephrine catalyzed by mammalian tyrosinase, adrenochrome may be formed at the same time. Hence, the long-term safety evaluation of CA is left to be investigated further.

The results obtained here show that the intake of CA combined with a usual level of caffeine and tea catechins failed to suppress body fat accumulation, whereas no deleterious effect was observed in this short-term study in rats. Therefore, as far as recommended daily intakes designated on the labels of CA products are followed, no safety problems may occur even in people who habitually consume coffee and tea. However, the safety evaluation of CA intake along with high levels of caffeine and tea catechins, especially in people at risk for heart disease, needs to be elucidated further.

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