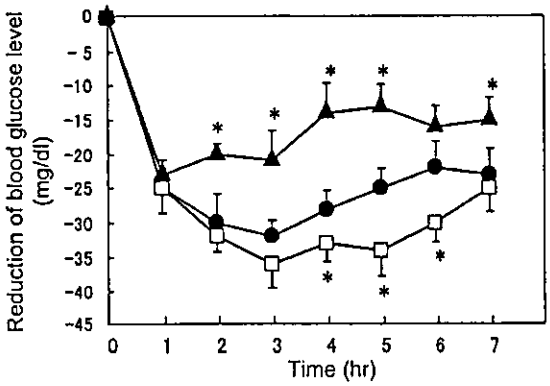

Fig. 1. Effects of simultaneous treatment and 5 day pretreatment with Ginkgo biloba extract (GBE) on the hypoglycemic effect of tolbutamide in aged rats. Aged rats (19 months old) were administered tolbutamide (40 mg/kg, p.o.) with or without GBE treatment. The GBE pretreated group was given feed containing 0.1% GBE for 5 days, and a simultaneous GBE treated group was given a single dose of GBE (100 mg/kg, p.o.) with tolbutamide. After the tolbutamide administration, blood was collected for the analysis of blood glucose concentrations. Each point represents the mean ± SD from six rats. ●, control group; ▲, GBE pretreated group; and □, a simultaneous GBE treated group. Significant difference from the control group is indicated by $*P<0.05$.

whereas the simultaneous GBE treatment at a single dose exhibited little significant interaction with tolbutamide (Fig. 2).

Competitive inhibition of GBE and tolbutamide on CYP2C9 activity in vitro

The direct interaction of tolbutamide and GBE toward (S)-warfarin 7-hydroxylase (CYP2C9) activity was examined using rat liver microsome in vitro. As shown in a Dixon plot, tolbutamide competitively

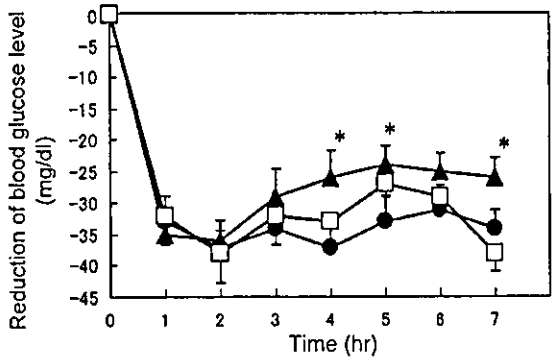

Fig. 2. Effects of simultaneous treatment and 5 day pretreatment with Ginkgo biloba extract (GBE) on the hypoglycemic effect of tolbutamide in young rats. Young rats (7 weeks old) were administered tolbutamide (40 mg/kg, p.o.) with or without GBE treatment. The GBE pretreated group was given feed containing 0.1% GBE for 5 days, and a simultaneous GBE treated group was given a single dose of GBE (100 mg/kg, p.o.) with tolbutamide. After tolbutamide administration, blood was collected for analysis of blood glucose concentrations. Each point represents the mean ± SD from six rats. ●, control group; ▲, GBE pretreated group; and □, a simultaneous GBE treated group. Significant difference from the control group is indicated by $*P<0.05$.

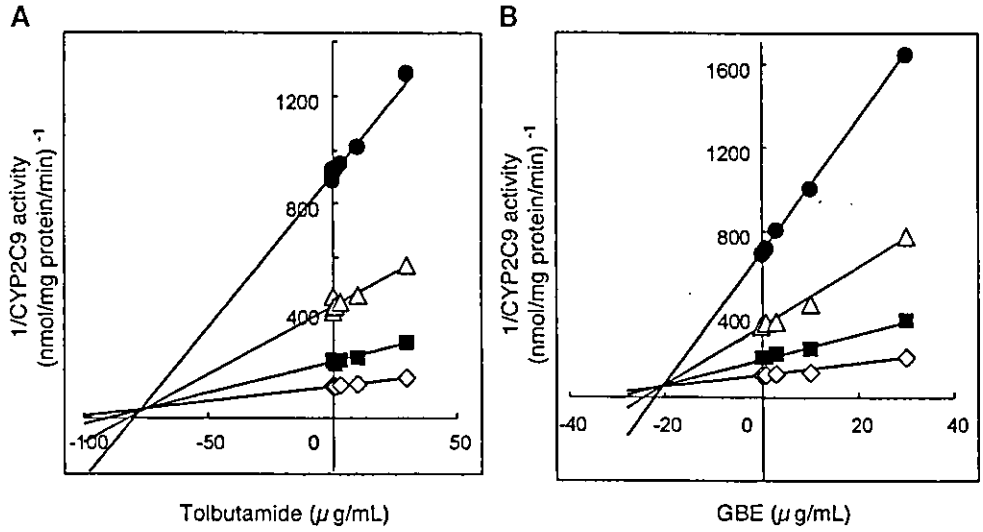

Fig. 3. Effects of tolbutamide (A) and Ginkgo biloba extract (B) on the (S)-warfarin 7-hydroxylase (CYP2C9) activity in rat liver microsome in vitro (Dixon plot). The (S)-warfarin 7-hydroxylase (CYP2C9) activities were measured in the presence of various concentrations of tolbutamide (0.1–30 μg/mL) or GBE (1.0–30 μg/mL) in rat liver microsome in vitro. Loaded (S)-warfarin concentrations were ●, 2 μM; △, 4 μM; ■, 8 μM and ◇, 16 μM. Each point represents the mean from five experiments.

inhibited the metabolism of (S)-warfarin by the (S)-warfarin 7-hydroxylase (Fig. 3A). Similarly, GBE decreased the (S)-warfarin 7-hydroxylase activity in vitro in a concentration-dependent manner, and showed competitive inhibition toward the enzyme activity (Fig. 3B). These results indicated that the hepatic metabolism of tolbutamide was competitively inhibited by GBE. The calculated Ki values were 19 μg/mL for GBE and 75 μg/mL for tolbutamide, respectively.

Discussion

Elderly people often suffer from functional impairments in their bodies and diabetes in these days. Considering these situations and the efficacy of GBE reported so far (Iliff and Auer, 1983; Lanthony and Cosson, 1988; Doly et al., 1992; Rapin et al., 1997), the simultaneous intake of GBE and anti-diabetic drug is most likely to occur in the elderly. For the safe use of both GBE and drugs, it is important to elucidate how GBE taken as a dietary supplement modifies the efficacy of therapeutic drugs. In the present study, we investigated the influence of GBE intake on the hypoglycemic efficacy of tolbutamide. We focused particularly on the elderly of advanced age and on the hepatic CYP-mediated interactions. The pharmacodynamic interaction was evaluated by inducing hypoglycemia with an excess administration of tolbutamide in aged (19-month-old) and young (7-week-old) rats.

As reported previously (Szabo et al., 1995; Umegaki et al., 2000), the intake of GBE did not change the basal blood glucose level in the aged and young rats with the pretreatment of GBE with diet (data not shown). However, the pretreatment and simultaneous treatment of GBE markedly influenced the hypoglycemic action of tolbutamide in the aged rats (Fig. 1). In the case of 5 days pretreatment of GBE, the hypoglycemic action of tolbutamide was attenuated both in the aged and young rats. As shown

in Table 2, the 5 day pretreatment of GBE markedly induced hepatic drug metabolizing enzymes, especially (S)-warfarin 7-hydroxylase, an enzyme corresponding to CYP2C9 which mainly metabolizes tolbutamide in the liver (Lee et al., 2003). Therefore, the induction by GBE pretreatment of hepatic CYP2C9 may reduce the plasma concentration of tolbutamide after oral administration, thereby leading to the attenuation of the hypoglycemic action of tolbutamide. In the present study, the plasma concentration of tolbutamide was not determined, due to the limited blood sample from the GBE-pretreated rats. However, the change in the blood glucose concentration was taken as the final outcome of interaction between GBE and tolbutamide, and the observed changes of CYP2C9 responsible for tolbutamide metabolism following the GBE pretreatment could well explain the mechanism of the pharmacodynamic interaction.

Kudolo (Kudolo, 2001) also reported that the ingestion of GBE by non-insulin-dependent diabetes mellitus subjects induced the hepatic metabolic clearance rate of insulin and hypoglycemic agents, suggesting an increase of blood glucose. In the previous paper (Shinozuka et al., 2002), we also showed that pretreatment of GBE significantly reduced the hypotensive action of nicardipine, which is metabolized by CYP3A2 isoform in rats. The mechanism of these interactions is similar to that in St John's Wort (Durr et al., 2000; Roby et al., 2000), a well-known inducer of CYP, indicating that the induction of CYP by pretreatment with GBE attenuates the efficacy of various drugs. Inasmuch as the beneficial effects of GBE in humans are expected after the continuous intake for more than 4 weeks (Gruenwald et al., 2000; Blumenthal, 1998), the induction of hepatic CYP would be taken into account for the interaction with drugs seen after a long-term intake of GBE as a dietary supplement.

In contrast to the GBE pretreatment, simultaneous treatment of tolbutamide with GBE as a single dose potentiated hypoglycemic action of tolbutamide in the aged rats (Fig. 1). In order to clarify the underlying mechanism of this interaction, a direct effect of GBE on the hepatic (S)-warfarin 7-hydroxylase (subtype of CYP2C9) activity was examined in vitro. As tolbutamide is mainly metabolized by (S)-warfarin 7-hydroxylase (Lee et al., 2003), tolbutamide competitively inhibited the hepatic metabolism of (S)-warfarin as shown in the Dixon plot analysis (Fig. 3A). Interestingly, GBE also showed the competitive inhibition toward the metabolism of (S)-warfarin (Fig. 3B), suggesting that GBE competitively inhibited the metabolism of tolbutamide by the CYP2C9 enzyme in the liver of rats. Thus, the pharmacodynamic interaction between GBE and tolbutamide after their single administration could be explained by the following mechanism. Following the simultaneous intake of GBE and tolbutamide, the hepatic metabolism of tolbutamide would be attenuated via the significant occupancy by GBE of the same active site on the CYP2C9 enzymes where this drug binds, resulting in an enhancement of hypoglycemic action of tolbutamide.

The induction of hepatic drug metabolizing enzymes and attenuation of hypoglycemic efficacy of tolbutamide were observed by the pretreatment of GBE both in the young rats and aged rats. However, the extent of these effects by GBE pretreatment was greater in the aged rats than in the young rats. This distinction may be explained by the difference between the two groups of rats in the induced level of CYP enzymes. In fact, the activities of pentoxyresorufin O-dealkylase and (S)-warfarin 7-hydroxylase were induced more (2 to 3-fold) by the GBE treatment in the aged rats than in the young rats (Table 2). This greater induction of CYP2B and CYP2C9 in the aged rats may be somehow related to the lower basal activities of these enzymes, which displayed about 23 and 41%, respectively, of the enzyme activity in the young rats. Interestingly, in the case of simultaneous treatment of GBE and tolbutamide, the potentiation of hypoglycemic action of tolbutamide was not detected in the young rats (Fig. 2). A clear explanation for the absence of enhancement of the pharmacological effect by tolbutamide in the young rats is lacking. Further

detailed experiments will be necessary to clarify pharmacokinetic and pharmacodynamic difference of tolbutamide between young and aged rats observed by the GBE treatment. In any event, it is evident that the aged rats may be more susceptible to the CYP-mediated interactions of GBE and tolbutamide when compared to the young rats. This finding may be applied for humans.

As a dietary supplement, 120 to 240 mg of GBE are generally taken in a day (Blumenthal, 1998; Gruenwald et al., 2000; Ernst, 2002), and this dose can be estimated as 2 to 4 mg/kg body weight. In this animal study, the intake of GBE was 30 ~ 100 mg/kg body weight per day both in dietary pretreatment and in a single dose treatment. In our previous study, a significant induction of (s)-warfarin 7-hydroxylase was detected even at the lower dose (10 mg/kg body weight) of GBE (Umegaki et al., 2002). Owing to the species difference in the susceptibility to GBE, it is still not clear whether the interaction of tolbutamide and GBE also occurs in humans. Although the extrapolation of pharmacokinetic and pharmacodynamic interaction of GBE and tolbutamide in the aged rats to the clinical situation should be interpreted with caution, it is anticipated, from our recent preliminary study with healthy volunteers, that the repeated oral intake of GBE may influence the pharmacokinetics and pharmacodynamics of tolbutamide in humans (unpublished observation).

In conclusion, the present study has shown that the intake of GBE significantly affects the efficacy of tolbutamide in the aged rats. Therefore, it is anticipated that the simultaneous and continuous intake of GBE as a dietary supplement with therapeutic drugs should be cautious, particularly in elderly people.

Acknowledgements

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トルブタミドおよびミダゾラムの体内動態に対するイチョウ葉エキスの影響

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【目的】近年健康食品が一般市場で容易に手に入る時代となり、その安全性、特に医薬品を併用した場合の相互作用が看過できない問題となっている。医薬品との相互作用を引き起こす健康食品には、人における主要な薬物代謝酵素であるチトクローム P450（CYP）に影響を与え作用するものがあり、西洋オトギリ草は CYP3A4 を誘導することが知られている。イチョウ葉エキス（*Ginkgo biloba extract*, GBE）は記憶や注意・集中などの認知機能の改善を目的として、わが国では栄養補助食品として市販されている。既に我々は GBE を投与したラット肝臓において CYP が誘導されることを報告した。本研究では GBE がヒトの CYP に及ぼす影響を明らかにする目的で、健常人に GBE を連日投与し CYP2C9 の基質であるトルブタミドおよび CYP3A4 の基質であるミダゾラムの体内動態について検討した。

【方法】健常男性 10 名より文書同意を取得した。対象患者の年齢は 24.9±2.6（平均値±SD）、体重は 68.6±7.1 であり、すべて非喫煙者であった。試験前日 22:00 より絶食とし、翌日 10:00 にグルコース(75g)を経口投与後 180 分まで経時的に血液中グルコース濃度を測定した。14 日後、同様に前夜より絶食下で、9:00 にトルブタミド(125 mg)を経口投与した。さらに 10:00 にミダゾラム(8 mg)およびグルコース(75g)を経口投与し、投与後 24 時間まで経時的に血漿中薬物濃度と血液中グルコース濃度を測定した。また 24 時間の蓄尿を行い、尿中薬物量を測定した。翌日より GBE (360 mg/日)を分 3 食後に投与し、GBE 投与 28 日後に同様にトルブタミド、ミダゾラムおよびグルコースを投与し血中濃度を測定した。なお血漿と尿中のトルブタミドおよびミダゾラム濃度は HPLC 法により、血液中グルコース濃度は自己血糖測定器（フリースタイルキッセイ）により測定した。

【結果】GBE 投与後において投与前に比べ、血漿中トルブタミド濃度は減少する傾向を示した（Fig. 1A）。GBE 投与後のトルブタミドの AUC は投与前に比べ 16%有意に減少した。他の薬物動態学的パラメータには有意な差異は認められなかった（Table 1）。また 4-OH トルブタミドのパラメータにおいては有意な変化を認めなかった。GBE 投与後における血漿中のトルブタミドと代謝物の AUC 比は投与前に比べ 17%有意に低値を示した。一方、尿中トルブタミドと代謝物の比には有意な差異は認められなかった。75g グルコース経口投与後の血液中グルコース濃度の 120 分までのトルブタミド投与前後の AUC 低下率は、GBE 投与前（18%低下）と比較し投与後（6%低下）で減少した。

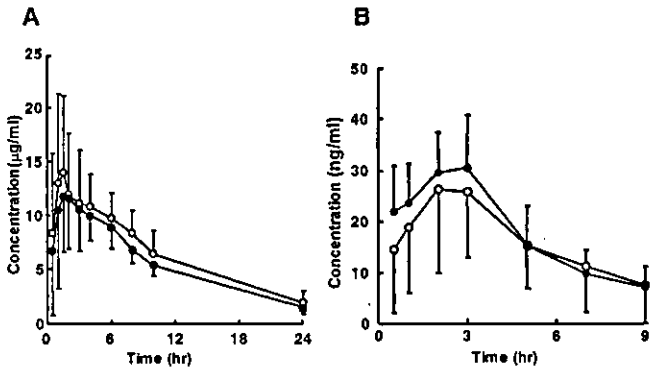

Fig. 1 イチョウ葉エキス投与前（○）および 28 日間投与後（●）におけるトルブタミド（A）およびミダゾラム（B）の血漿中濃度推移。データは Mean ±SD.

Table 1 イチョウ葉エキス投与前と 28 日間投与後におけるトルブタミドおよびミダゾラムの薬物動態学的パラメータ

Pharmacokinetic parameter	GBE 投与前	GBE 投与後
Tolbutamide		
$AUC_{0-\infty}$ (hr・mg/ml)	179 ± 65	150 ± 35*
CL_{tot}/F (ml/hr/kg)	11.1 ± 2.9	12.5 ± 1.8
MRT (hr)	11.2 ± 2.9	10.5 ± 1.9
Vd_{ss}/F (ml/kg)	120 ± 25	130 ± 28
Midazolam		
$AUC_{0-\infty}$ (hr・mg/ml)	167 ± 104	208 ± 101**
CL_{tot}/F (ml/hr/kg)	0.865 ± 0.357	0.639 ± 0.208**
MRT (hr)	5.33 ± 2.61	5.63 ± 1.69
Vd_{ss}/F (ml/kg)	4.34 ± 2.19	3.35 ± 0.70

Mean ± SD (n=10), *GBE 投与前との有意差、*P<0.05, **P<0.01
$AUC_{0-\infty}$: area under concentration-time curve, CL_{tot} : total clearance, F: bioavailability, MRT: mean residence time, Vd_{ss} : volume of distribution at steady-state

GBE 投与後において投与前に比べ、血漿中ミダゾラム濃度は増加し（Fig. 1）、その AUC は投与前に比べ 25%有意に増加した。また経口クリアランスは 26%有意に減少した（Table 1）。

【考察】GBE（360 mg）の 28 日間投与後において CYP2C9 の基質であるトルブタミドの血漿中濃度が減少したことから、GBE 投与により CYP2C9 が誘導されることが示唆された。さらに 75g グルコース経口投与後におけるトルブタミドの血液中グルコース濃度上昇抑制作用が GBE 投与により減弱する傾向が認められた。一方 CYP3A4 の基質であるミダゾラムの血漿中濃度は GBE の 28 日間投与により著明に上昇した。本研究の結果、CYP2C9 および 3A4 の基質薬物投与中の患者における GBE の使用は、注意を要すると考えられる。

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研究報告

特定保健用食品の組み合わせ摂取による安全性，有効性の検討II

ーエコナ油とヘルシア緑茶の併用ー

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はじめに

検診後，あるいは一般診療時に，食事療法の一環として生活習慣病予防，是正のため特定保健用食品を用いて指導することも多い．現在用いられている特定保健用食品については，個々の食品についての有効性，安全性は確認されている．しかし，時として組み合わせて摂取することもあり得るが，その有効性，安全性については確認されていない．

このような観点から，既に植物ステロール添加のジアシルグリセロール(エコナ油)と同様のヘルスクレイムを有する大豆蛋白を併用し，その有効性，安全性を検討し，発表してきた．その際，併用によるコレステロール低下は増強され，しかも安全性には問題がみられないことを確認した[1]．

今回は，"体に脂肪がつきにくい"ヘルスクレイムをもつエコナ油とカテキン添加緑茶の併用投与を臨床的に実施し，その有効性，安全性を検討した．

対象および方法

対象は平均年齢60歳(46～76歳)の男女15例(男性5例，女性10例)で総コレステロール(TC)値200 mg/dL以上，本試験の説明に対し了解し参加の同意を得た例である．

方法は図1に示すように普段の食生活などを変えないように指示した上で，植物ステロール添加ジアシルグリセロール(以下，エコナPS)を1日10 g添加して，1カ月後カテキン540 mgを含む緑茶(以下，ヘルシア)350 mLを1カ月間併用摂取し，次いでヘルシアを中止し，エコナPSのみを1カ月間継続摂取した．その間，図1に示す項目を定期的に検査した．

成　績

表1に体重，BMI，血圧の変化を示す．

体重の平均，BMIの平均は全例で全く変動はみられないが，一部には減少の傾向が併用時でみられた．収縮期ならびに拡張期血圧についても，有意の変動はみられていない．

表2に，脂質その他代謝パラメーターについて，その変動を示してある．TCについては，どの期間においても有意の変化はみられていないが，症例によってはエコナPSとヘルシア併用で200 mg/dL以下となった例が2例あり，エコナPS単独摂取にして再び230 mg/dL程度に復しており，症例によりTC低下を認めている．

トリグリセライド(TG)では，有意差はないが全般的に低下をみせる傾向にあった．LDL-コレステロール(LDL-C)では全く変動を認めていないが，HDL-コレステロール(HDL-C)はわずかに増加する例が多い．

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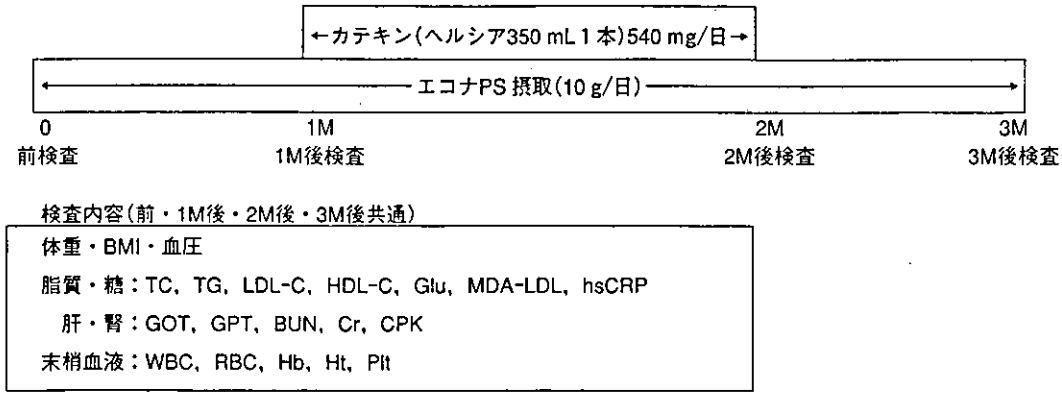

図1　方法と検査項目

表1　体重・BMI・血圧の数値表

	前	エコナPS 1M	エコナPS＋ヘルシア1M	エコナPS 1M
体重(kg)	60.0±13.2	59.9±9.8	59.8±13.5	60.0±13.3
BMI	24.3±3.4	24.2±3.3	24.1±3.4	24.2±3.3
収縮期血圧(mmHg)	130.8±4.6	131.9±2.9	131.1±3.9	129.7±4.6
拡張期血圧(mmHg)	83.7±2.4	84.0±1.6	83.5±4.3	82.5±2.4

表2　血清脂質・血糖・hsCRPに対する影響

	前	エコナPS 1M	エコナPS＋ヘルシア1M	エコナPS 1M
TC(mg/dL)	240.7±23.8	239.5±22.1	241.5±29.7	238.5±29.4
TG(mg/dL)	136.9±60.4	127.9±50.5	124.6±38.7	123.8±45.3
LDL-C(mg/dL)	148.9±23.4	148.9±14.6	151.1±24.5	147.5±20.5
HDL-C(mg/dL)	64.4±13.9	65.1±12.0	65.4±14.8	66.3±16.0
血糖(mg/dL)	99.2±10.8	99.0±9.4	96.9±10.9	100.1±10.8
MDA-LDL(U/L)	106.3±42.9	124.5±40.9	135.0±35.3*	120.1±34.1**
hsCRP(mg/L)	0.60±0.6	0.68±0.8	0.62±0.7	0.87±0.8

*：$p=0.063$　**：$p<0.05$

しかし，これもまた有意の変化ではない．血糖，hsCRPは全期間を通じて全く変化はみられていないが，MDA-LDLについてはエコナPSとヘルシア併用で有意に上昇し，エコナPS単独に戻しても，なお高値を示していた．

ヘルシアを併用することによりMDA-LDLの低下した症例6例，上昇した症例9例があり，この2群の層別検討をTC，TGについて行った(表3)．その結果，MDA-LDL下降群では，TC，TGに変化はみられず，MDA-LDL上昇群では，TCの変化は認められないがTGに上昇傾向を認めている．ヘルシア摂取による抗酸化作用の発現は，TGの上昇や，未測定ながらリン脂質の変動，特にその分子中のリノール酸，アラキドン酸などの増加により打ち消される可能性が考えられる．表として示していないが，一部で測定された血中カテキン濃度測定では，特にエピガロカテキンガレート濃度が明らかに上昇した例にMDA-LDLの低下がみられている点を考慮すると，カテキンの吸収低下あるいは摂取不足も関与するのかもしれない．

表4に，肝および腎機能などに対する影響を示してある．GOTは前値に比べエコナPS単独，エコナPSとヘルシアの併用でわずかながら有意に上昇を示したが，

表3　MDA-LDL・TC・TGとの関係

	MDA-LDL	エコナPS 1M	エコナPS＋ヘルシア1M
MDA-LDL(U/L)	上昇群	106.4±25.0	142.1±32.2
	下降群	151.6±44.8	124.3±36.8
TC(mg/dL)	上昇群	241.1±15.9	242.1±29.4
	下降群	237.1±28.7	240.3±30.1
TG(mg/dL)	上昇群	125.3±44.9	133.4±42.8
	下降群	131.8±57.6	111.3±26.3

表4　肝および腎機能に対する影響

	前	エコナPS 1M	エコナPS＋ヘルシア1M	エコナPS 1M
GOT(IU/L)	20.9±5.4	21.5±6.8*	25.1±7.7*	24.5±7.6*
GPT(IU/L)	23.3±11.8	22.9±9.5	25.1±13.4	24.6±12.8
BUN(mg/dL)	14.4±3.6	14.3±3.0	14.1±2.4	14.4±2.7
Cr(mg/dL)	0.7±0.2	0.7±0.1	0.7±0.2	0.7±0.2
CPK(IU/L)	122.5±48.9	102.1±30.3**	115.1±48.6	102.1±30.9***

*：$p<0.01$　**：$p=0.05$　***：$p=0.06$

表5　末梢血液に対する影響

	前	エコナPS 1M	エコナPS＋ヘルシア1M	エコナPS 1M
白血球数($\times10^2/\mu L$)	50.4±10.3	52.5±10.5	48.9±10.5	50.7±11.3
赤血球数($\times10^4/\mu L$)	460.7±35.3	455.5±43.1	456.3±40.8	450.7±44.3
ヘモグロビン(g/dL)	14.1±1.0	14.0±1.1	14.1±1.1	13.9±1.2
ヘマトクリット(%)	42.3±2.8	41.8±3.4	41.9±3.1	41.2±3.6
血小板数($\times10^4/\mu L$)	25.4±5.3	24.2±5.0	24.2±5.5	24.7±5.2

正常の範囲内の変動である．GPTには全く有意の変動はみられていない．BUN, Crについても全く変動を認めていない．CPKは，エコナPS単独時に減少しているが，これも正常範囲内の変動である．

表5に，末梢血液に対する影響をまとめて示してある．白血球数，赤血球数，ヘモグロビン，ヘマトクリット，血小板数などには，全く有意の変動はみられなかった．

考　察

体重は，併用により減少を示す例がみられるものの，全例の平均では有意の変動はみられなかった．BMI，血圧についても有意な変動はみられなかった．症例による個人差が大きいように思われた．

TC，TGも個々には変動を示したが，全体では有意の変化ではない．HDL-C，LDL-C，血糖，hsCRP，GPT，BUN，Cr，末梢血液所見は有意な変化はみられなかった．

最近，上海大学とナッシュビルのヴァンダービルト大学との共同研究[2]で，毎日375 mgのテアフラビン添加の緑茶を240例の男女に12週間飲用させた結果，TC11%，LDL-Cが16%の減少を認めている．茶カテキンにテアフラビンを加えたものであるが，有効性が確認されている．また，カテキン飲料を長期に摂取した土田らの成績[3]では，体重の平均1.6 kgの減少とともに

約4％のTCの減少を認めており，こうした面でのカテキンの効果は今後明らかにされるべきと考える．

ヘルシア併用でのGOTの上昇，CPKの減少はあくまでも正常範囲内の変動であり，特に副作用とは考えにくい．

また，ヘルシア摂取によりMDA-LDLの減少と増加を認めた例があり，特にMDA-LDLの上昇例ではTGも増加していた．ヘルシアの摂取により，エピガロカテキンガレートの明らかな増加のみられた例のあることは，ヘルシアの摂取量，吸収の問題や他の脂質の上昇に伴う脂肪酸の変化なども考えられ，今後，詳細な検討が必要であろう．安全性についても他の報告[2,3]とともに全く問題はなかった．

結　　論

エコナPSとヘルシア併用摂取による15例の高コレステロール血症臨床例での検討では，体重，血清脂質などに，併用による有効性は個々には変動がみられるものの，全体としては確認できなかった．肥満でなかったことが一因であろう．しかし，安全性には全く問題はなかった．

謝　　辞

最後に，血中カテキン濃度の測定に協力していただいた花王株式会社研究室の皆様に深謝致します．

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

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

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

Introduction

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

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

Health Canada has warned consumers not to use

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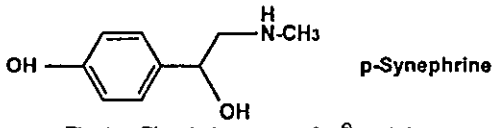

Fig. 1. Chemical structure of *p*-Synephrine.

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

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

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

Materials and Methods

Animals, diets and feeding trial

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

Assay procedures

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

Statistical analysis

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

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

Group (*Citrus aurantium* (mg/kg diet))	Group 1 (0)	Group 2 (40)	Group 3 (200)	Group 4 (1,000)	Group 5 (5,000)
Food intake (g/day)	18.8±1.0^{NS}	18.3±0.9^{NS}	18.8±1.1^{NS}	19.2±1.1^{NS}	18.8±1.1^{NS}
Citrus aurantium intake (mg/day)	0.0±0.0^{a}	0.7±0.03^{ab}	3.8±0.2^{b}	19.2±1.1^{c}	93.9±5.4^{d}
Synephrine intake (μg/day)	0±0^{a}	47±2^{ab}	244±15^{b}	1,228±71^{c}	6,008±344^{d}
Body weight gain (g)	271.8±18.2^{NS}	253.7±21.9^{NS}	266.6±27.6^{NS}	260.5±48.0^{NS}	241.3±31.0^{NS}
Initial body weight (g)	292.0±7.9^{NS}	292.7±9.2^{NS}	288.4±7.5^{NS}	293.3±10.9^{NS}	293.3±6.2^{NS}
Final body weight (g)	563.8±23.5^{NS}	546.3±22.3^{NS}	555.0±33.5^{NS}	553.8±51.9^{NS}	534.6±36.5^{NS}
Liver (g)	14.5±2.0^{NS}	15.0±1.1^{NS}	15.7±1.9^{NS}	14.8±2.8^{NS}	14.5±1.9^{NS}
Kidney (g)	2.73±0.19^{NS}	2.85±0.15^{NS}	2.89±0.11^{NS}	2.71±0.12^{NS}	2.65±0.10^{NS}
Heart (g)	1.38±0.09^{a}	1.32±0.03^{ab}	1.39±0.08^{a}	1.37±0.09^{a}	1.26±0.06^{b}
Testis (g)	3.78±0.14^{NS}	4.00±0.22^{NS}	3.72±0.53^{NS}	3.72±0.26^{NS}	3.92±0.14^{NS}
Spleen (g)	0.79±0.13^{ab}	0.85±0.10^{ab}	0.96±0.22^{a}	0.81±0.13^{ab}	0.76±0.08^{b}
Lung (g)	1.54±0.11^{NS}	1.66±0.17^{NS}	1.63±0.18^{NS}	1.69±0.13^{NS}	1.70±0.13^{NS}
Perirenal fat pad (g)	22.4±2.5^{a}	18.9±3.4^{ab}	22.8±4.2^{a}	18.2±4.4^{ab}	18.0±2.9^{b}
Epididymal fat pad (g)	15.3±1.7^{NS}	14.6±2.9^{NS}	15.7±2.9^{NS}	14.1±4.1^{NS}	12.5±3.5^{NS}

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

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

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

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

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

Results

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

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

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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High dose of *Garcinia cambogia* is effective in suppressing fat accumulation in developing male Zucker obese rats, but highly toxic to the testis

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Abstract

We investigated the ability of *Garcinia cambogia* extract containing (−)-hydroxycitric acid (HCA) to suppress body fat accumulation in developing male Zucker obese (fa/fa) rats. We also examined histopathologically the safety of its high doses. Diets containing different levels of HCA (0, 10, 51, 102 and 154 mmol/kg diet) were fed to 6-week-old rats for 92 or 93 days. Each diet group was pair-fed to the 154 mmol HCA/kg diet group. Epididymal fat accumulation and histopathological changes in tissues were observed. The highest dose of HCA-containing *Garcinia cambogia* (154 mmol HCA/kg diet) showed significant suppression of epididymal fat accumulation in developing male Zucker obese rats, compared with the other groups. However, the diets containing 102 mmol HCA/kg diet and higher (778 and 1244 mg HCA/kg BW/d, respectively) caused potent testicular atrophy and toxicity, whereas diets containing 51 mmol HCA/kg diet (389 mg HCA/kg BW/d) or less did not. Accordingly, 51 mmol HCA/kg diet (389 mg HCA/kg BW/d) was deemed to be the no observed adverse effect level (NOAEL).

Keywords: *Garcinia cambogia*; (−)-hydroxycitric acid; Zucker obese rat; Epididymal fat accumulation; Testicular toxicity; Testis

1. Introduction

Obesity, particularly with visceral fat accumulation, is a serious risk factor for so-called lifestyle-related diseases such as diabetes, cardiovascular disease and hypertension (Jebb, 1999; Nakamura et al., 1994). Therefore, antiobesity foods and food ingredients may avert obesity, possibly leading to prevention of lifestyle-related diseases, if they are effective in reducing body fat accumulation.

Garcinia cambogia, also known as *Malabar tamarind*, is a plant native to Southeast Asia. The dried rind has been extensively used for centuries throughout Southeast Asia as a food preservative, flavoring agent and carminative, and is now popularly used as an ingredient of dietary supplements for weight loss in developed countries. (−)-Hydroxycitric acid (HCA), the primary acid in the fruit rinds of *Garcinia cambogia* (Lewis and Neelakantan, 1965), has been shown to be active in suppressing appetite and body fat accumulation in experimental animals (Greenwood et al., 1981; Ishihara et al., 2000;

Abbreviations: ALP, alkaline phosphatase; ALT, L-alanine:2-oxoglutarate aminotransferase; AST, L-aspartate:2-oxoglutarate aminotransferase; BUN, blood urea nitrogen; FSH, follicle-stimulating hormone; γ-GTP, γ-glutamyltranspeptidase; H.E., hematoxylin-eosin; HCA, (−)-hydroxycitric acid; LH, luteinizing hormone; NEFA, non-esterified fatty acid; NOAEL, no observed adverse effect level

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

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

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

2. Materials and methods

2.1. Animals and dietary treatment

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

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

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

2.2. Assay procedures

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

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

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

HCA, (−)-hydroxycitric acid.

[a] The diet composition was based on the AIN-93G (Reeves et al., 1993) with a slight modification. The vitamin mixture (AIN-93-VX) contained choline bitartrate at 2.5 g/kg diet.

[b] *Garcinia cambogia* powder S® supplied by Nippon Shinyaku Co. Ltd. was used. The HCA content was 41.2% and the ratio of its free to lactone form was 36.6 to 63.4.

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

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

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

2.3. Statistical analysis

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

3. Results

3.1. Food intake and growth

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

3.2. Organ and epididymal fat pad weights

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