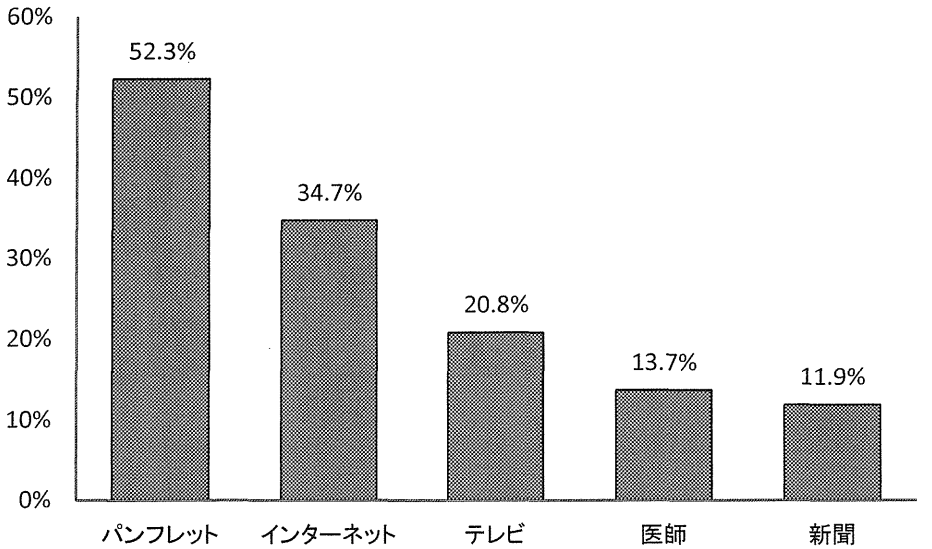

図 16 健康食品の正しい知識を受け取りたい媒体(自由記述)

表 健康食品が薬の代わりになると思っている人の健康食品に対するイメージの特徴(一部抜粋)

	薬の代わりになる % (n)	薬の代わりにならない % (n)	分からない % (n)	p 値[a]
総計	9.0 (283)	66.3 (2093)	24.8 (783)	
1.健康食品のイメージについて				
①安全である				< 0.01
思う	77.5 (213)	37.7 (781)	58.9 (229)	
思わない	4.0 (11)	30.7 (635)	6.9 (27)	
分からない	18.5 (51)	31.6 (655)	34.2 (133)	
②効果が期待できる				< 0.01
思う	84.5 (234)	29.4 (609)	52.6 (204)	
思わない	2.5 (7)	41.9 (869)	5.7 (22)	
分からない	13.0 (36)	28.7 (595)	41.8 (162)	
③薬と併用しても大丈夫				< 0.01
思う	79.1 (220)	24.4 (506)	31.4 (122)	
思わない	7.6 (21)	56.3 (1166)	21.2 (82)	
分からない	13.3 (37)	19.2 (398)	47.6 (185)	
2.健康食品の利用状況について				< 0.01
現在も利用している	44.9 (122)	28.9 (585)	39.4 (149)	
過去に利用していた	26.8 (73)	34.1 (689)	26.5 (100)	
利用していないが今後は利用してみたい	15.1 (41)	8.2 (165)	11.9 (45)	
利用していないし今後も利用しない	13.2 (36)	28.8 (582)	22.2 (84)	
3.健康食品の利用目的				
①病気の予防	32.8 (63)	23.9 (297)	28.0 (69)	< 0.05
②病気の治療	17.7 (34)	7.6 (94)	8.9 (22)	< 0.01
4.健康食品利用による効果の実感				< 0.01
効果があった	36.7 (69)	22.5 (278)	30.0 (73)	
効果がなかった	4.3 (8)	10.6 (131)	6.2 (15)	
分からない	59.0 (111)	67.0 (829)	63.8 (155)	

[a] χ^2 検定を行なった。
%は欠損値を除いて算出した。

別紙　研究成果の刊行に関する一覧表

雑誌

発表者名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kagawa Y, Maeda T, Kato Y, Ueda I, Kudo T, Watanabe N, Kimura M, Minami T, Sakamoto T, Yamada H, Takagi M.	Influence of the slow infusion of a soybean oil emulsion on plasma cytokines and ex vivo T cell proliferation after an esophagectomy.	*J Parenter Enteral Nutr.*	37(1)	123-128	2013
山田浩、一丸佳代、小野彩奈、高橋光明、松本圭司、小菅和仁、藤本和子、脇昌子、中島光好、梅垣敬三.	健康食品の摂取に伴う有害事象の因果関係評価のための樹枝状アルゴリズムの構築.	臨床薬理	43(6)	399-402	2012
Nantiga Virgona, Yuko Taki, Shizuo Yamada, Keizo Umegaki.	Dietary *Coleus forskohlii* extract generates dose-related hepato- toxicity in mice.	*J Appl Toxicol.* (in press)	53 (4)	15-19	2011
横谷馨倫，千葉剛，佐藤陽子，窪田洋子，渡邉泰雄，村田容常，梅垣敬三.	*Coleus forskohlii* エキス中の肝シトクローム P450 誘導物質の推定.	応用薬理	82(5/6)	67-73	2012
Kaori Yokotani Tsuyoshi Chiba, Yoko Sato, Yuko Taki, Shizuo Yamada, Kazumasa Shinozuka, Masatsune Murata, Keizo Umegaki.	Hepatic cytochrome P450 mediates interaction between warfarin and *Coleus forskohlii* extract *in vivo* and *in vitro*.	J Pharm Pharmacol.	64(12)	1793-801	2012
Kaori·Yokotani Tsuyoshi Chiba, Yoko Sato, Tomoko Nakanishi, Masatsune Murata, Keizo Umegaki.	Influence of dietary macronutrients on induction of hepatic drug metabolizing enzymes by *Coleus forskohlii* extract in mice.	*J Nutr Sci Vitaminol*	59	37-44	2013

Original Communication

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Influence of the Slow Infusion of a Soybean Oil Emulsion on Plasma Cytokines and Ex Vivo T Cell Proliferation After an Esophagectomy

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Abstract

Background: Lipid emulsions have been suggested to reduce immune responses, particularly in severely stressed patients. The authors investigated the influence of the slow intravenous infusion of a soybean oil–based lipid emulsion on some immune parameters in patients who had undergone an esophagectomy for esophageal cancer. *Methods:* Thirty-two patients who had undergone an esophagectomy were randomly divided into a lipid emulsion (LPD)–treated group and a control group. All patients received parenteral feeding with a glucose-based solution. Patients in the LPD group received 100 mL of a 20% soybean oil emulsion for 7 days after the esophagectomy in addition to the glucose-based feeding. A slow infusion rate (0.09–0.12 g/kg/h) was adopted to take account of the intrinsic degradation of infused lipids. Immune responses were measured based on lymphocyte proliferation and serum concentrations of monocyte chemoattractant protein–1 (MCP-1), interleukin-6 (IL-6), and tumor necrosis factor–α (TNF-α). The authors also measured levels of rapid turnover proteins (ie, transferrin, prealbumin, and retinol-binding protein). *Results:* Phytohemagglutinin- and concanavalin A–stimulated lymphocyte proliferation significantly decreased after the esophagectomy, but no significant difference was seen between the LPD and control groups. No significant difference in changes in plasma concentrations of MCP-1, IL-6 and TNF-α occurred between the 2 groups either. Plasma concentrations of rapid turnover proteins did not differ between the groups. *Conclusions:* These results indicate that the lipid emulsion did not affect the immune parameters measured in patients who had undergone an esophagectomy when administered at a slow rate. (*JPEN J Parenter Enteral Nutr.* 2013;37:123-128)

Keywords

lipid emulsion; lymphocyte proliferation; monocyte chemoattractant protein–1; interleukin-6; tumor necrosis factor–α; rapid turnover protein; esophagectomy

Malnutrition is associated with mortality in hospitalized patients.[1] Parenteral nutrition (PN) is provided after an esophagectomy. The supply of enough energy through an intravenous (IV) catheter is essential for a full recovery and to avoid malnutrition. Lipid emulsions are frequently used as an IV infusion to provide energy to patients who need high-calorie intake because lipids have more calories per weight than carbohydrates. A soybean oil emulsion is commercially available and frequently used for IV injections in many countries.

The fatty acids in lipid emulsions administered intravenously attract apoprotein from high-density lipoprotein (HDL) and form lipoproteins. The lipoproteins are converted back to fatty acids by lipoprotein lipase (LPL) and used for energy.[2] The infusion of a soybean oil emulsion, however, was reported to reduce immune function in severely stressed patients[3]: concanavalin A (Con A)– and phytohemagglutinin (PHA)–stimulated lymphocyte proliferation 7 days after esophagectomy was significantly lower in patients given the soybean oil emulsion than in those given a glucose-based solution alone. The intrinsic breakdown of lipid emulsions administered intravenously has a saturable level because the supply of apoprotein from HDL has limitations. Therefore, an infusion rate greater than the intrinsic degradation capacity may cause hyperlipidemia. Macrophages phagocytize excess lipid particles in the bloodstream of patients with hyperlipidemia. These foamy macrophages affect cellular immunity and immune responses. In turn, soybean oil emulsions contain a lot of linoleic acid, including ω-6 polyunsaturated fatty acids (PUFAs). ω-6 PUFAs are a precursor of arachidonic acid, which in turn gives

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rise to leukotrienes and dienoic prostaglandins. It is known that dienoic prostaglandins, such as prostaglandin E_2, suppress host immune responses.[4] Lipid emulsions with a high proportion of linoleic acid promote the apoptosis or necrosis of lymphocytes in vitro.[5] Therefore, immune function is reported to be compromised by lipid emulsions rich in ω-6 PUFAs.[6] Although a decrease in immune response is predicted to be associated with the infusion rate of lipid emulsions,[7,8] no study has focused on the infusion rate of soybean oil emulsions.

Monocyte chemoattractant protein–1 (MCP-1), an inflammatory cytokine, attracts monocytes, memory T lymphocytes, and natural killer cells.[9,10] Interleukin-6 (IL-6) is a pleiotropic cytokine that plays an important role in host defense due to its wide range of immune and hematopoietic activities and its ability to induce an acute phase response.[11] Production of IL-6 is induced in response to tumor necrosis factor–α (TNF-α).[12] MCP-1 production is also induced in response to TNF-α.[13,14] Serum levels of MCP-1, IL-6, and TNF are known to increase in association with defects in cell-mediated immunity.[3,15]

In the present study, we evaluated the influence of the slow IV infusion of a lipid emulsion on plasma cytokines and ex vivo T cell proliferation in patients who had undergone an esophagectomy for esophageal cancer. We also investigated changes in plasma concentrations of rapid turnover proteins—transferrin (TF), prealbumin (PA), and retinol-binding protein (RBP)—to assess changes in nutrition status after an esophagectomy.

Materials and Methods

Patients and Clinical Protocol

Thirty-two patients who underwent an esophagectomy for esophageal cancer at Shizuoka Prefectural General Hospital from September 2006 to March 2009 were prospectively enrolled into the study. The protocol was approved by the institutional review board at the Shizuoka Prefectural General Hospital. All patients provided written informed consent before entry into the study.

Study Design

This study was conducted as a prospective randomized open-label trial. Patients fed exclusively PN were divided into 2 groups: a lipid emulsion (LPD) group and a control group. The LPD group received daily 100 mL of a 20% soybean oil emulsion (Intralipos; Otsuka Pharmaceutical Factory, Inc, Tokushima, Japan), 20 g as soybean oil, with an infusion rate of 0.09–0.12 g/kg/h in addition to PN consisting of glucose from 1 day (day 1) to 7 days (day 7) after the operation. The soybean oil emulsion contained 51.6% linoleic acid, 7.7% α-linolenic acid, and 1.6% arachidonic acid as essential fatty acids. The control group received 1120 kcal/d with fat-free PN and approximately 200 kcal less energy than the LPD group. Patients in the LPD group received 24% (days 1 and 7) and 17% (days 2–6) of this total daily calorie intake from the soybean oil emulsion. Every patient intravenously received a bolus (250 mg) of methylprednizolone sodium succinate (Solu Medrol) just before the esophagectomy.

Laboratory Analyses

Serum concentrations of IL-6, MCP-1, and TNF-α were determined using the Human IL-6 ELISA Kit (Endogen, Inc, Woburn, MA), Human MCP-1 ELISA Kit (Endogen, Inc), and Quantikine HS Human TNF-α/TNFSF1A Immunoassay (R&D Systems, Inc, Minneapolis, MN), respectively. Serum concentrations of RBP, TF, and PA were determined using N-Assay LA RBP, N-Assay TIA Tf-H, and N-Assay TIA Prealbumim (Nittobo Medical Co, Ltd, Tokyo, Japan).

Con A– or PHA-stimulated lymphocyte proliferation was measured based on the incorporation of [^{3}H]thymidine into DNA of lymphocytes at the SRL Laboratory (Tokyo, Japan). Results were evaluated using a stimulated index (SI). The SI values were estimated by the dividing radioactivity with mitogen by that without mitogen.

Serum concentrations of MCP-1, IL-6, TNF-α, RBP, PA, and TF were measured 1 day before, 2 hours after, and 1, 2, and 9 days after surgery. Blood samples were centrifuged at 1000 *g* for 15 minutes, and the sera were stored at –80°C until assayed.

Statistical Analyses

All values are expressed as the mean ± SD. Statistical analyses for temporal changes in immune cytokines and rapid turnover proteins were performed using the repeated-measure analysis of variance (repeated-ANOVA). If a significant difference was detected with the repeated-ANOVA, the Tukey test was performed to compare multiple groups. Comparisons of parameters between the LPD and control groups were performed using the Mann-Whitney *U* test. Demographic, preoperative, and surgical data were analyzed using the Mann-Whitney *U* test or Fisher exact test. All statistical analyses were carried out with the use of SPSS software, version 17.0 (SPSS, Inc, an IBM Company, Chicago, IL). $P < .05$ was considered significant.

Results

Demographic, preoperative, and surgical data are presented in Table 1. The LPD group had more frequently received preoperative chemotherapy. There were no significant differences in demographic data between the groups. Operative blood loss and volume of fluid infusion during surgery were significantly larger in the LPD group. SI values for Con A–stimulated lymphocyte proliferation before the operation were significantly lower in the LPD group. Blood cell counts and renal and hepatic function test results from before the operation were comparable between the groups with 1 exception: platelet counts were significantly lower in the LPD group.

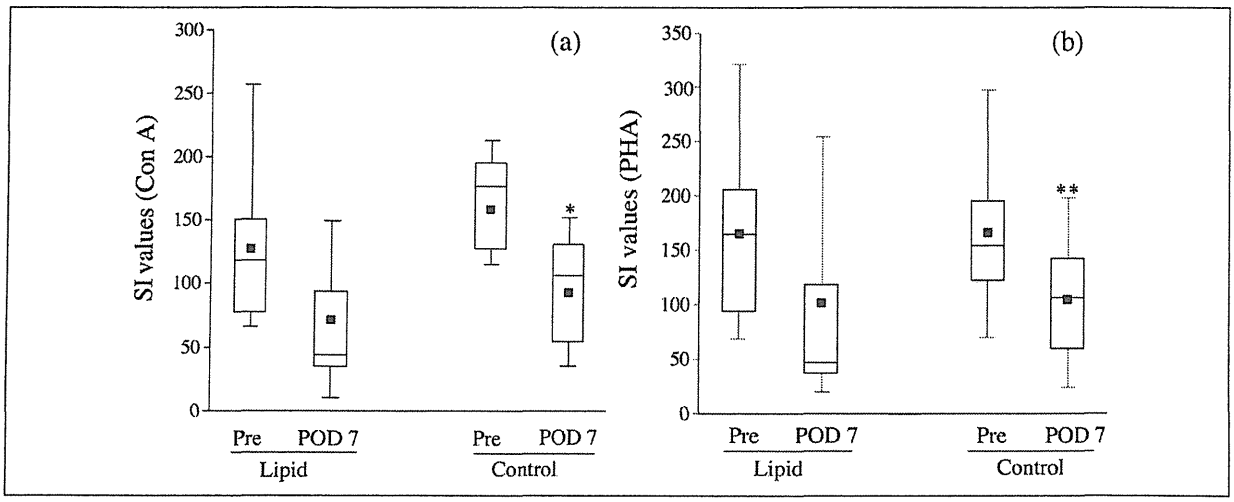

Figure 1. Postoperative changes in PHA or Con A–stimulated lymphocyte proliferation. The SI values obtained by PHA or Con A stimulation in the lipid and control groups are shown in (a) and (b), respectively. *$P < .05$, **$P < .005$ as compared with preoperative levels. Con A, concanavalin A; PHA, phytohemagglutinin; POD, postoperative day; SI, stimulated index.

Table 1. Patient Characteristics.

Group	Lipid Infusion	Control	*P* Value
Number of patients (M/F)	15 (15/0)	17 (15/2)	.274[a]
Age, y	59.6 ± 7.1	63.9 ± 6.0	.289[b]
Body weight, kg	54.6 ± 7.8	51.1 ± 10.3	.168[b]
BMI	19.9 ± 2.0	19.7 ± 3.3	.558[b]
Stage of carcinoma			.097[a]
I	0	3	
II	8	5	
III	1	5	
IV	6	4	
Lymph node dissection			.366[a]
2 fields	10	8	
3 fields	3	7	
4 fields	2	2	
Operation time, min	413 ± 125	341 ± 46	.234[b]
Anesthesia time, min	466 ± 47	432 ± 60	.439[b]
Operative blood loss, mL	603 ± 99	333 ± 174	.005[b]
Volume of infusion during operation, mL	3048 ± 674	2297 ± 793	.041[b]
Blood transfusion, mL	452 ± 100	267 ± 195	.105[b]
Stimulated index values for lymphocyte proliferation			
PHA stimulated	162 ± 116	165 ± 82	.720[b]
Con A stimulated	130 ± 86	161 ± 50	.027[b]
Treatment before surgery			
Preoperative chemoradiotherapy	2	4	.392[a]
Preoperative chemotherapy	8	3	.040[a]

Values are the mean ± SD or number of patients. BMI, body mass index; Con A, concanavalin A; PHA, phytohemagglutinin.
[a]Fisher exact test.
[b]Mann-Whitney *U* test.

Figure 1 shows that the SI values for PHA and Con A in the control group significantly decreased at 7 days after the esophagectomy compared with those before surgery ($P = .012$ and .002, respectively), whereas in the LPD group, they tended to decrease in the same period ($P = .11$ and .055, respectively). The mean decrease of SI values for PHA in the LPD and control groups was 66.1% and 56.4%, respectively. Similarly, the mean reduction in SI values for Con A in the 2 groups was 54.0% and 58.2%, respectively. There was no difference in SI values and these reduction rates between the groups. SI values after the operation did not differ between the groups either.

Because we did not measure simultaneous changes in serum triglyceride levels during the infusion of soybean oil emulsion, it was unclear whether their serum concentrations in patients were constant during the infusion. However, no significant changes were seen in serum triglyceride or cholesterol levels 1, 2, and 4 days after the esophagectomy between the LPD group and the control group (data not shown).

Changes in serum MCP-1 concentrations are shown in Figure 2. There was a significant difference between sampling points within the control group but not the LPD group. In the control group, serum MCP-1 concentrations were significantly higher at 2 days after surgery than at other time points. There was no significant difference in serum MCP-1 concentrations at any sampling points between the LPD group and the control group.

Serum IL-6 concentration profiles are shown in Figure 3. In the control group, the concentrations were significantly higher 2 hours after surgery than at other sampling points. No significant difference between the sampling points was seen in the LPD group. One patient in the LPD group showed an extremely high concentration (686 pg/mL) at 2 hours after the operation.

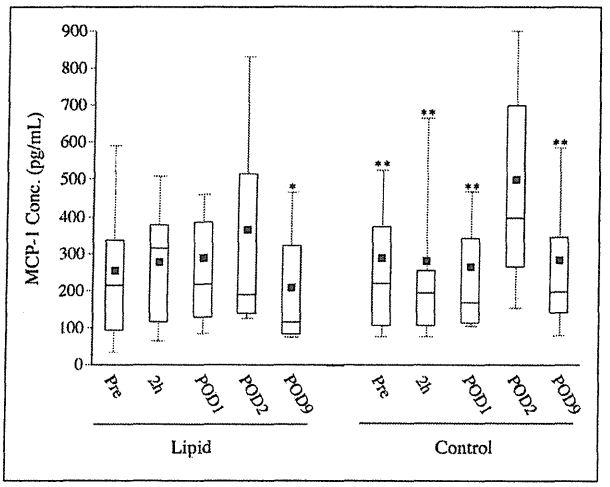

Figure 2. Changes in serum monocyte chemoattractant protein–1 (MCP-1) concentrations in patients after esophagectomy. Vertical bars in box plots indicate the 10th percentile, lower quartile, median, upper quartile, and 90th percentile, and closed squares indicate the mean. $*P < .05$, $**P < .005$ as compared with levels 2 days after the operation in each group. POD, postoperative day.

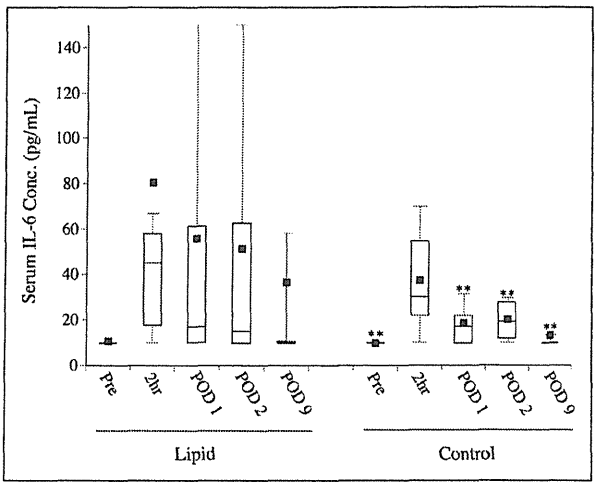

Figure 3. Changes in serum interleukin-6 (IL-6) concentrations in patients after esophagectomy. Vertical bars in box plots indicate the 10th percentile, lower quartile, median, upper quartile, and 90th percentile, and closed squares indicate the mean. $**P < .005$ as compared with levels 2 hours after the operation in each group. POD, postoperative day.

There was, however, no significant difference in serum IL-6 concentrations between the LPD and control groups.

Serum TNF-α concentrations significantly decreased in the control group 2 hours after surgery as compared with those before the operation, whereas no significant decrease was seen in the LPD group at the same time points (Figure 4). No significant difference was seen in serum TNF-α concentrations between the 2 groups.

Serum TF, PA, and RBP concentrations after the esophagectomy significantly decreased in both the LPD group and the control group until 2 days after surgery and had nearly recovered to preoperative levels at 9 days (Figure 5). There was no significant difference in serum RTP profiles between the groups.

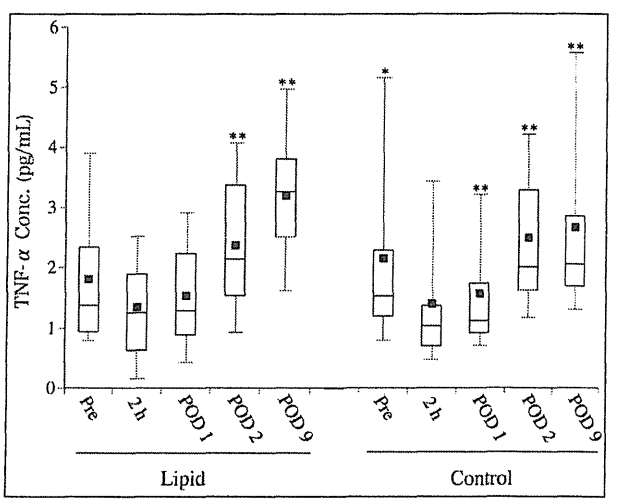

Figure 4. Changes in serum tumor necrosis factor–α (TNF-α) concentrations in patients after esophagectomy. Vertical bars in box plots indicate the 10th percentile, lower quartile, median, upper quartile, and 90th percentile, and closed squares indicate the mean. $*P < .05$, $**P < .005$ as compared with levels 2 hours after the operation in each group. POD, postoperative day.

Discussion

The present study showed that when 20 g of soybean oil was intravenously infused as a lipid emulsion at a rate of 0.09–0.12 g/kg/h, there was no significant change in the immunological parameters measured in patients who had undergone an esophagectomy. Battistella et al[6] reported that the IV infusion of a soybean oil–based emulsion caused an increase in susceptibility to infection in trauma patients. We, however, did not find an increased infection rate in the LPD group compared with the control group (data not shown). They administered 16% higher daily total calories and twice as many calories as we provided as a soybean oil emulsion. Other differences were that the patients in their study were younger and suffered from trauma but not malignancies. Those differences may explain the different results. Furukawa et al[3] showed that the administration of a lipid emulsion to patients after an esophagectomy impeded lymphocyte proliferation and raised serum IL-6 concentrations 2-fold as compared with levels in patients who received fat-free PN. They suggested that the excessive rise in IL-6 levels reflected decreased immune function but did not

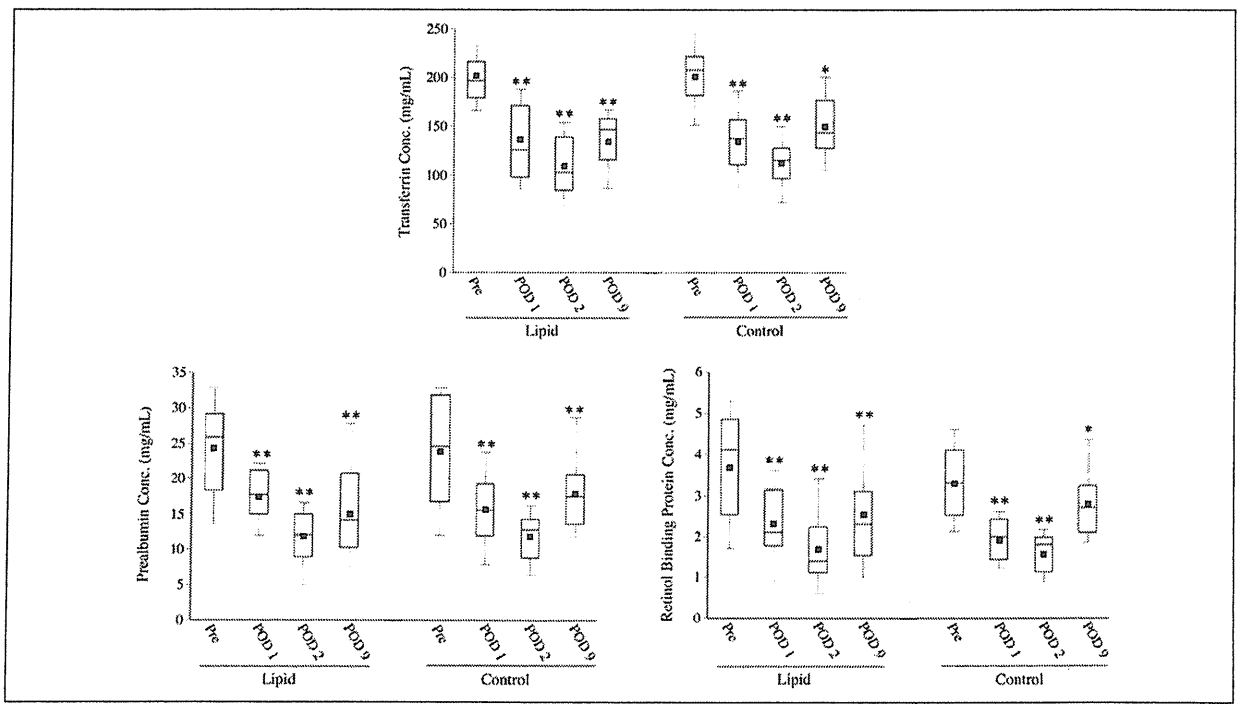

Figure 5. Changes in serum transferrin, prealbumin and retinol binding protein concentrations in patients after esophagectomy. Vertical bars in box plots indicate the 10th percentile, lower quartile, median, upper quartile, and 90th percentile, and closed squares indicate the mean. $*P < .05$, $**P < .005$ as compared with preoperative levels in each group. POD, postoperative day.

mention the rate of infusion. The rate at which an oil emulsion is infused is thought to be very important because the rate of intrinsic breakdown of lipid particles has limitations. Some experts have recommended that infusions of parenteral lipid emulsions at rates of 0.8–1.5 g/kg/d are safe but should not exceed 2.6 g/kg/d (0.11 g/kg/h).[16,17] The study of Intralipid shows that the infusion rate can be increased up to 0.2 g fat/min, which converts to 0.24–0.15 g/kg/h in patients whose body weight is 50–80 kg. This rate is higher than that recommended by experts. In Japan, soybean oil–based emulsions (20%) are generally infused at a rate of 83 mL/h (≈ 0.3 g/kg/h) as recommended by their manufacturers. This rate is 3 times the one used here and exceeds the upper limit of the infusion rate proposed by experts. The infusion rate of 0.09–0.12 g/kg/h in the present study was almost within the recommended limit. The slow infusion of the lipid emulsion in this study may have contributed to the lack of deterioration in host immune function.

Differences between the study by Furukawa et al[3] and our study are that we clearly stated the rate of infusion of the lipid emulsion, and corticosteroids were administered before esophagectomy. The preoperative administration of corticosteroids might affect immune responses. Although no corticosteroid was administered to the patients in the previous study, preoperative corticosteroid treatment is generally used in current clinical settings. Therefore, our study adopted the slow infusion of a soybean oil–based emulsion and administration of corticosteroids before the esophagectomy. Under our study conditions, no significant difference in immune response parameters, such as PHA and Con A–stimulated lymphocyte proliferation, in patients after esophagectomy was seen between infusion of the soybean oil emulsion in addition to the glucose-based fluid and the glucose-based fluid alone.

Despite the random allocation, the total volume of blood loss, volume of infusion, and volume of blood transfusion during the operation were significantly larger in the LPD group than in the control group. The differences indicated that operative stress was more severe in the LPD group and might influence host immune activity because severe stress such as hemorrhage is known to cause a significant elevation in serum IL-6 levels and decrease in lymphocyte proliferation.[3,18] Platelet counts were significantly lower in the LPD group. Lower platelet counts before the operation might have caused more bleeding in the LPD group. Moreover, the mean SI value for Con A–stimulated lymphocyte proliferation was significantly lower in the LPD group before the operation. Despite this disadvantage, there was no significant difference in lymphocyte proliferation or serum concentrations of MCP-1, IL-6, and rapid turnover proteins between the groups. One patient in the LPD group showed extremely high concentrations of IL-6

in serum after the operation, which led to a large deviation value and might explain the lack of a significant temporal change in the group. Although an elevation in serum IL-6 levels is reported to be associated with hemorrhage,[18] the blood loss by this patient during the operation was less than the average for the LPD group.

We measured nutrition changes in patients after the esophagectomy. Patients in the LPD group received 200 kcal more than those in the control group. There was, however, no significant difference in plasma profiles of rapid turnover proteins (ie, PA, TF, and RBP) between the groups. The amount of bleeding was significantly greater in the LPD group. It is well established that severe bleeding causes protein catabolism and a deterioration in rapid turnover proteins. That there was no difference in the plasma concentrations of rapid turnover proteins between the LPD and control groups until 9 days after the surgery indicates that the lipid emulsion did not have a harmful influence on host nutrition after the esophagectomy. Our results did not show a nutrition advantage from the lipid emulsion in those patients, probably because of a difference in the extent of bleeding between the groups and the small amount (200 kcal/d) of lipid emulsion used. A larger amount of lipid emulsion may be required to obtain an obvious nutrition advantage in severely stressed patients.

Limitations of this study include an imbalance in the extent of bleeding between the LPD and control groups, as well as a small sample size. We should also mention that the lack of difference in the immune-related parameters examined does not mean that immune function was equivalent between the 2 groups.

In conclusion, this study indicated that the slow infusion of a lipid emulsion did not affect the immune response of severely stressed patients who need hyperalimentation. Further study is needed to assess the advantages of lipid emulsions in addition to glucose-based alimentation.

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短 報

健康食品の摂取に伴う有害事象の因果関係評価のための樹枝状アルゴリズムの構築

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Application of a Dendritic Algorithm for the Evaluation of Causal Relationships of Adverse Events with Health Food

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緒 論

近年，健康志向の高まりからサプリメント等の栄養補助食品や，いわゆる健康食品（以下，健康食品と略す）の需要が増加し，それとともに健康食品の摂取に伴う健康被害事例が報告されるようになってきている[1]．健康食品の摂取に伴う健康被害の報告は，ドラッグストアや製造販売元への利用者からの問い合わせ，あるいは医療機関で治療を受けた場合の診療記録等の情報を基に，保健所を介して厚生労働省に集約されていく．また，消費者庁を介しての被害情報の集積も行われている．しかし，これらの情報は種々雑多であり，正確に因果関係評価を行うことは極めて難しく，また科学的に吟味するための臨床上有用な方法論も十分には確立していない．

すでに我々は，健康食品と医薬品が共に機能的に生体に作用するという類似性に着目し，医薬品の投与に伴う有害事象の因果関係評価において種々開発されている評価法[2]のうち，比較的汎用性の高いアルゴリズムを改変することで，健康食品の摂取に伴う有害事象の因果関係評価法の構築を試みてきた[3~5]．その過程で，まず始めに，評価票形式で質問項目ごとの点数の重み付けを行い加算するNaranjoらの評価票[6]を基にして，健康食品の特性を考慮した改変を重ねてきた[4]．本研究ではNaranjoらの評価票と並び，医薬品の有害事象の因果関係評価で汎用されているJonesの樹枝状アルゴリズム[7]の内容を再検討し，質問項目や分枝形式ならびにカテゴリー分類を改変することで，健康食品に適した，より臨床応用可能な樹枝状アルゴリズムの構築を試みた．

方 法

すでに作成したJonesの樹枝状アルゴリズムの改変[3]を，健康食品の有する情報の特性を考慮して再検討し，健康食品の摂取に伴い生じた有害事象の因果関係判定に重要と考えられる質問項目や分枝方式ならび

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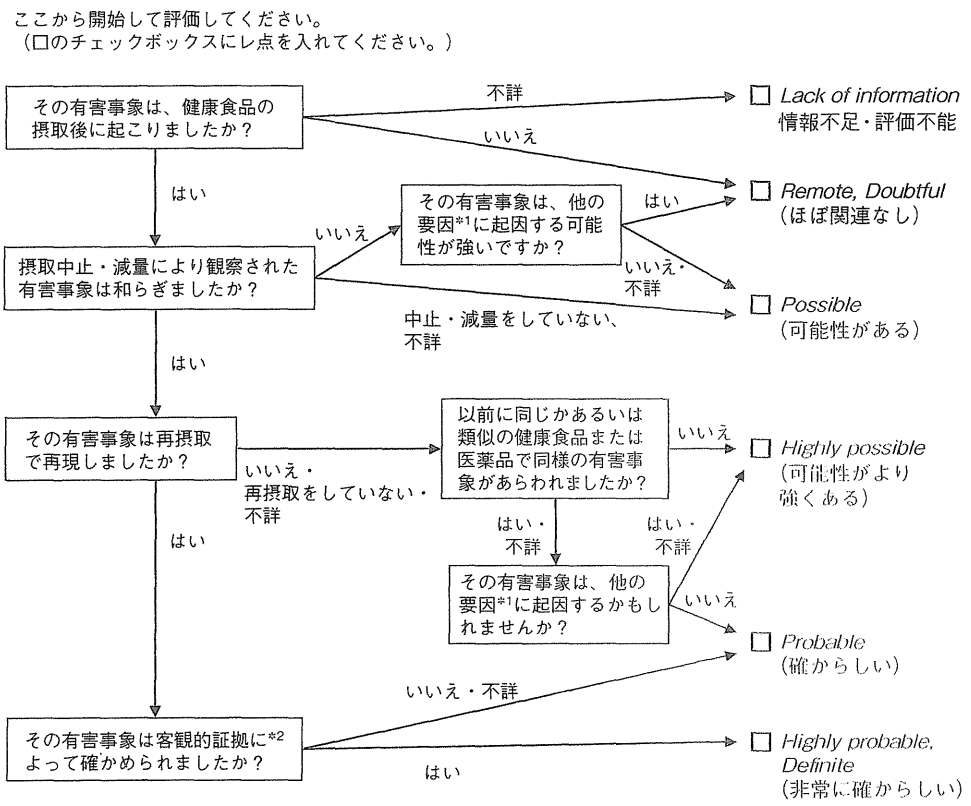

*1 他の要因としては、基礎疾患や合併症の病態、併用薬やほかの健康食品の摂取、年齢などを考慮します。
*2 客観的証拠とは、当該健康食品に含まれる成分に関してDLST、パッチテストなどの特異的な検査によって確認されたものです。

Fig. 1 健康食品の摂取に伴う有害事象の因果関係評価のために開発した改変樹枝状アルゴリズム

にカテゴリー分類を吟味し，改変を加えた（Fig. 1）．改変は，以下の8項目について行った（変更点1～5は質問項目，6は分枝方式，7～8はカテゴリー分類における変更点である）．

1）評価開始時の質問項目において，「時間との関連」という表現は曖昧であるため，「摂取後に起こりましたか？」と前後関係を明確にした．
2）摂取中止による症状の変動を問う質問項目において，「摂取中止・減量」に変更することで，減量による変動も評価に加えた．
3）再摂取後の症状の出現を問う質問項目において，症状の再現が見られなかった場合や再摂取をしていない場合には，次に進む質問項目として，「以前に同じかあるいは類似の健康食品または医薬品で同様の有害事象があらわれましたか？」を追加した．
4）再摂取後に症状の再現があった場合，次に進む質問項目として，客観的な検査の有無を問う項目を追加し，注釈「客観的証拠とは，当該健康食品に含まれる成分に関してDLST，パッチテストなどの特異的な検査によって確認されたものです．」を加えた．
5）他の要因を問う質問項目を追加・修正し，「既存の臨床症状」という表現は曖昧であるため，「他の要因」という表現に変え，注釈「他の要因としては，基礎疾患や合併症の病態，併用薬やほかの健康食品の摂取，年齢などを考慮します．」を加えた．
6）分枝方式を，「はい」，「いいえ」の2分枝のみから，「不詳」を加え3つに細分化した．
7）時間的な関連性が不詳の事例は，情報不足「lack of information」のカテゴリー分類とした．
8）すでに作成したNaranjoら調査票の改変によるカテゴリー分類[4]と同様，可能性がある「possible」を，因果関係が強い順に，可能性がより強くある「highly possible」，可能性がある「possible」の2つに細分化した．

次いで今回，改変を加えた樹枝状アルゴリズム（以下，改変樹枝状アルゴリズムと略す）およびNaranjoら評価票の改変を重ねた改変評価票[4]（以下，改変評価票と略す）を用い，健康食品販売業者のお客様センターに寄せられた保健機能食品（特定保健用食品，栄

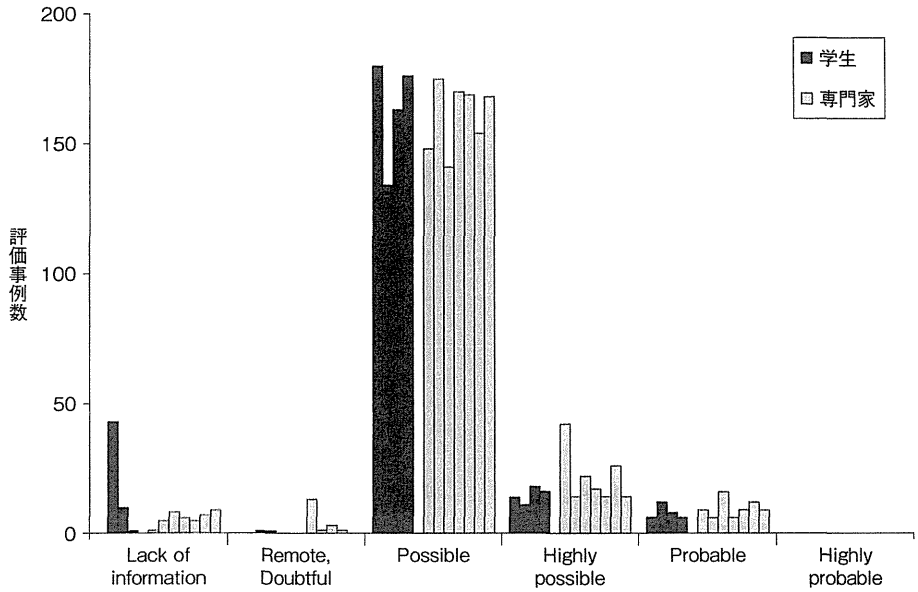

Fig. 2　改変樹枝状アルゴリズムにおける学生および専門家の評価結果の分布
評価者 11 名（薬学系学生 4 名；黒色地，専門家 7 名：灰色地），評価した有害事象事例 200 例

食機能食品）および保健機能食品以外のいわゆる健康食品の摂取に伴う健康被害相談事例 200 例に対して，薬学系学生 4 名および専門家 7 名（医師 3 名，健康食品関連の情報を扱っている専門家 3 名，大学薬学部教員 1 名），計 11 名の評価者により，それぞれ独立に因果関係を評価した．改変樹枝状アルゴリズムにおける評価判定は，因果関係が強い順に，非常に確からしい（highly probable, definite），確からしい（probable），可能性がより強くある（highly possible），可能性がある（possible），ほぼ関連なし（remote, doubtful），情報不足・評価不能（lack of information）の 6 段階にカテゴリー分類した．改変評価票に関しては合計点をスコア化し，改変樹枝状アルゴリズムと同様にカテゴリー分類した．次いで，多評価者間 κ 係数を Fleiss の方法により算出し，両評価法の信頼性を評価した．統計解析は，R ver. 2.15.1（R Development Core Team, 2012）を用いて行った．なお，本研究で利用した健康被害相談事例の個別内容については，機微情報を含むことから提示しないこととした．

結　　果

改変樹枝状アルゴリズムによる 200 事例の評価は，薬学系学生および専門家共に，possible に多く集中し，highly probable はなかった（Fig. 2）．薬学系学生および専門家における改変樹枝状アルゴリズムによる多評価者間 κ 係数は，それぞれ 0.50 と 0.52 であった．一方，薬学系学生および専門家における改変評価票による多評価者間 κ 係数は，それぞれ 0.21 と 0.44 であった．

考　　察

今回の研究では，医薬品の有害事象の因果関係評価に汎用されている Jones の樹枝状アルゴリズムに改変を重ねることで，健康食品の摂取に伴う有害事象の因果関係評価法の構築を試みた．改変樹枝状アルゴリズムと，その基となった Jones の樹枝状アルゴリズムの最大の相違は，健康食品においては有害事象評価に必要な情報が非常に少ないという状況を考慮した点にある．実際，健康食品から得られる情報は医薬品と比べ不十分で，判断が難しいことが多い[1]．この点を考慮し改変樹枝状アルゴリズムでは，選択肢に「はい」，「いいえ」のみでなく「不詳」を加え，カテゴリー分類に，情報不足・評価不能（lack of information）を加えた．さらに，Jones の樹枝状アルゴリズムが Naranjo ら調査票と比べ質問項目が少なく簡略化されていることにも着目し，因果関係の評価に重要と考えられる質問項目を加えることで，より正確な評価を行えるようにした．

このようにして構築した改変樹枝状アルゴリズムを，薬学系学生と専門家を評価者として信頼性を評価した結果，学生，専門家共に κ 係数は良好な値を示した．一方，改変評価票においては，専門家では改変樹枝状アルゴリズムとほぼ同様の κ 係数を示したものの，学生による κ 係数は低かった．改変評価票で学生の信頼性評価が低かった理由としては，評価票が樹枝

状アルゴリズムと比べ簡便性において劣るためと考えられた．この改変評価票の信頼性を向上させるためには学生等，評価に不慣れな評価者に対しては事前に専門的トレーニングが必要であった可能性がある．すなわち，今回構築した改変樹枝状アルゴリズムは改変評価票と比較し，熟練性を要さずに精度よく使用できる簡便性があり，一般消費者から報告される有害事象報告を因果関係の確からしさに基づいて篩い分ける際の，ドラッグストア，医療機関，製造販売元，保健所等，職種の異なる臨床現場での有害事象評価におけるスクリーニングとしての活用が期待されると考えられた．

今回の研究では，新たに構築した改変樹枝状アルゴリズムの信頼性評価を，すでに構築した改変評価票との比較により検討した．その理由は，高い多評価者間 κ 係数を報告した改変評価票[4]が，その後の追試験で異なる職種間に適用した場合，必ずしも満足のいく信頼性を示さなかったことによる[8]．そのため今回，異なる職種においても信頼性を保つ評価法を目指して改変樹枝状アルゴリズムの構築を行い，改変評価票との比較を行った．

評価者により評価された健康食品の摂取に伴う有害事象の多くは，すでに改変評価票で報告した結果[4]と同様，因果関係の弱いカテゴリーである“possible”に集中した（Fig. 2）．この傾向が生じる理由としては，健康食品の情報のもつ不確かさ（曖昧性）が影響していると考えられる．そのような健康食品の有害事象の特殊性を踏まえ，今回の研究では，改変評価票のカテゴリー分類に準じて，Jones 改変アルゴリズムの“possible”を，“highly possible”と“possible”の2つに細分類した．さらに「時間的関連はあるが，他の要因による可能性が強いケース」を，他の要因が考えにくい他の“possible”のケースと区別するために“remote”に篩い分けた．その結果，「時間的関連はあるが，他の要因の可能性が強いケース」と「時間的関連がないケース」のいずれもが“remote”に含まれたことに関して議論の余地が生じた．また，健康食品の多くは均一な物質ではなく，さまざまな物質を含んでおり，その割合は生産ロットによって異なると予想されるが，本改変樹枝状アルゴリズムでは，製品間の不均一性に関する情報は含まれていない点，因果関係評価における限界を有している．これらの点に関しては，今後，改良の余地が残された．

健康食品の摂取に伴う有害事象には，たとえば“摂取しない状態でも一定頻度で起こるような有害事象があり，そのような場合は頻度の増加を検出する必要性から，個別症例ではなく，同様の症例を集積して評価することが有用である．最近，自発報告等による大量の有害事象報告についてデータマイニング手法で安全性シグナルを検出し，その妥当性の検証や優先順位付けを行うことが，リスク管理の観点から重要視されつつある．そのような流れの中で，安全性シグナルの基となる情報の確からしさの評価として，本アルゴリズムの必要性は高いと考える．

結　論

今回構築した改変樹枝状アルゴリズムは，改変評価票と同様の信頼性を有し，さらに臨床現場でのスクリーニングとして，健康食品の摂取に伴う健康被害の因果関係判定法として使用が可能であると考えられた．今後，実際に医療現場で使用する職種における評価での臨床的な有用性を検討する必要がある．

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Dietary *Coleus forskohlii* extract generates dose-related hepatotoxicity in mice

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ABSTRACT: *Coleus forskohlii* root extract (CFE) represented by its bioactive constituent 'forskolin' is popularly used as a natural weight-lowering product, but the association of its use with liver-related risks is very limited. In the present study, the effect of standardized CFE with 10% forskolin on liver function of mice was examined. Mice were given 0–5% CFE in an AIN93G-based diet for 3–5 weeks. Food intake, body weights, relative organ weights and liver marker enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP)] combined with histophatological analysis were assessed. CFE (0–0.5%) only had minimal effects on food intake and body weight whereas a significant difference was observed in mice receiving the highest dose (5% CFE). The extract 0.05–5% dose-dependently decreased visceral fat weight by between 16% and 63%, and a dose-dependent several folds increase was observed in liver weights and plasma AST, ALT and ALP activities with quick onset apparent after only 1 week of 0.5% CFE intake. The hepatic effect persisted throughout the 3-weeks course but was restored towards normalization within 1 week after withdrawal of treatment. Liver histology of mice fed 0.5% CFE for 3 weeks showed hepatocyte hypertrophy and fat deposition. In contrast, none of the hepatic responses measured were altered when mice were given a diet containing pure forskolin alone at the dose corresponding to its content in 0.5% CFE. The present study clearly indicated that forskolin was not involved in the CFE-induced hepatotoxicity and was caused by other unidentified constituents in CFE which warrants further studies. Copyright © 2012 John Wiley & Sons, Ltd.

Keywords: forskolin; *Coleus forskohlii*; hepatotoxicity; liver marker enzymes; fatty liver; visceral fat

Introduction

Obesity is a chronic metabolic disorder and is associated with the genesis or development of various diseases, such as type 2 diabetes, cardiovascular disease, hypertension, fatty liver disease and certain forms of cancer (Grundy, 2004). The rise in obesity has caused an increasing demand for effective and safe anti-obesity agents, including herbal products (Egras *et al.*, 2011). Over the years, a variety of medicinal plants and their extracts have been reported to have beneficial effects in reducing the risk of obesity (Kamisoyama *et al.*, 2008, Stewart *et al.*, 2008). These natural compounds ameliorate obesity either by increasing energy expenditure or by inhibiting adipocyte differentiation.

The rhizome part of the perennial plant *Coleus forskohlii*, native to India, has been traditionally used in Ayurvedic medicine as a remedy for heart disease, respiratory, gastrointestinal and central nervous systems disorders (Ammon and Muller, 1985). Many of the beneficial effects of *C. forskohlii* consumption have been attributed to the pharmacological actions of forskolin, a major diterpene isolated from the root of *C. forskohlii*. Forskolin increases cyclic adenosine monophosphate (cAMP) via activation of the enzyme adenylate cyclase by binding to the glucose transporter owing to the right ring of the Decalin portion of forskolin having structural similarity with alpha-D-galactose (Abbadi and Morin, 1999; Laurenza *et al.*, 1989). Enhanced lipolysis as a result of elevation of cAMP by forskolin resulted in the breakdown of stored fats in animal cells (Okuda *et al.*, 1992) and human fat cells (Allen *et al.*, 1986). Anti-obesity effects have also been attributed to *C. forskohlii* extract (CFE) by reducing fat accumulation in ovariectomized rats (Han *et al.*, 2005), overweight females (Henderson *et al.*, 2005) and males (Godard *et al.*, 2005). Based on these findings, there are an increasing number of commercial dietary supplement products in which CFE is used as an herbal ingredient to promote weight loss.

There are examples of hepatotoxicity induced by many types of herbal remedies used as weight loss agents (Egras *et al.*, 2011). Considering the widespread use of CFE in herbal weight loss products there are only a limited number of studies on the involvement of CFE or its constituents regarding toxicity and the detrimental effects of this extract. Earlier, an *in vitro* study demonstrated that forskolin and its analog induced cytochrome P450 (CYP) family, CYP3A gene expression in primary hepatocytes (Ding and Staudinger, 2005). We recently reported that both CFE and pure forskolin induced CYP3A and glutathione *S*-transferase (GST) activities. However, only CFE showed a significant increase in liver weight with dose-related responses in mice (Virgona *et al.*, 2012). Therefore, whether the dose or duration of use may be correlated with the risk of liver damage remains unknown. It is also unclear if the safety profile of CFE is similar to other agents used in the management of body weight without hepatotoxicity effect. Thus, in the present study, we aimed to investigate the extent to which hepatic function of mice is affected by consumption of CFE. Four dose levels of a standardized CFE extract, and its main constituent forskolin were used to assess possible hepatotoxicity. Plasma marker enzymes

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for liver damage were measured to monitor treatment-related adverse effects. The extent of CFE treatment-related changes in liver tissues were assessed with histopathology.

Materials and Methods

Materials

Powdered CFE standardized with 10% forskolin used in the present study was extracted and prepared by Tokiwa Phytochemical Co., Ltd (Chiba, Japan). In brief, dried roots of *C. forskohlii* were crushed and applied to supercritical extraction with CO_2 gas. The obtained forskolin rich extract (20–30%) was incorporated into powdered dextrin to yield dry CFE powder with a forskolin concentration of 10%. The components of the CFE material were: water, 5.6%; protein, 0.3%; lipids, 22.7%; ash, 2.2%; and carbohydrates, 69.2%. The quality of the standardized CFE was determined by a validated HPLC method with an evaporative light scattering detector (ELSD) (Virgona *et al.*, 2010) using forskolin (purity > 99%; Biomol, Plymouth Meeting, PA, USA) as a standard. All other reagents were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Animals

In all the experiments, male ICR mice, 5 weeks old (CLEA Japan, Inc., Tokyo, Japan), were kept at a constant temperature (23 ± 1 °C) with a 12-h light–dark cycle with free access to water and the assigned diets for the length of each experiment. The mice were housed in individual polypropylene cages after a 7-day acclimatization period, maintained on an AIN93G semi-purified diet (Oriental Yeast Co., Ltd., Japan). On the seventh day of the acclimatization, mice were divided into groups of six and were given the experimental diets described later in this text. For all mice clinical observations, body weights and food consumptions were monitored and recorded every 2 days throughout the entire study. Mice were fasted overnight and were sacrificed under pentobarbital anesthesia during the next day. Blood was taken from the inferior vena cava with heparin as an anticoagulant. The plasma obtained from each mouse was frozen at −20 °C until measurement (within 24 h). After blood collection, the liver, kidney and visceral fat tissues were quickly removed from each mouse and weighed. The excised livers samples were fixed in 10% neutral-buffered formalin for histopathological examination. All procedures were in accordance with the National Institute of Health and Nutrition guidelines for the Care and Use of Laboratory Animals, and approved by an ethical committee. Efforts were also made to minimize the number of animals as well as their suffering.

Diets

The experimental diets were formulated on the basis of the abundance of a marker compound in CFE which is composed of 10% forskolin. The experimental diets consisting of: (a) control diet: AIN93G diet, composition of the diet was as in Reeves *et al.* (1993); (b) CFE diets: consisting of a control diet supplemented with 0.005–5% CFE, composition of the CFE diets were the same as the control diet except that a portion of dextrin (0.005–5%) was replaced with the corresponding amount of CFE; and (c) forskolin diet: the control diet supplemented with 0.05% pure forskolin (Biomol) replacing an equal amount of dextrin. The dose formulations were stored at approximately 4 °C in a refrigerator and were stable throughout the period of study.

Experimental Design

Three separate dietary CFE experiments were designed to examine the dose, duration and role of its main constituent on the hepatic function of mice.

Experiment 1: dose response effect of dietary CFE

Mice were randomly divided into five groups ($n = 6$ in each group) and were given either the control diet (AIN 93 G) or a control diet supplemented with 0.005%, 0.05%, 0.5% and 5% CFE (nominally containing 5, 50, 500 or 5000 mg forskolin per kg diet) for 3 weeks.

Experiment 2: time response and post treatment effect of CFE

Mice were given the control diet for up to 5 weeks, or the 0.5% CFE diet for 3 weeks (CFE groups) then switched to the control diet for up to 2 weeks (post-CFE fed groups). Time response to diet measurements ($n = 6$ mice in each group) of liver marker enzymes were performed at weeks 3, 4 and 5 for the control groups, weeks 1, 2 and 3 for the CFE treatment groups, and at weeks 4 and 5 for the post-CFE fed groups (post-CFE fed weeks 1 and 2, respectively).

Experiment 3: differential hepatic effects between intake of CFE and its principal constituent

Mice were randomly divided into three groups ($n = 6$) and were given free access to either the control diet, 0.5%CFE diet or 0.05% forskolin diet for 3 weeks.

HPLC Analysis

Forskolin content in the CFE and experimental diets was quantified by validated HPLC–ELSD. Briefly, samples of CFE and diets were accurately weighed into centrifuge tubes and sonicated in 3 ml of acetonitrile for 15 min. After centrifugation the supernatant was then transferred to a 25-ml volumetric flask. The procedure was repeated two more times and the respective supernatants combined. The sample supernatant (10 µl) was injected into a L column ODS 4.6 × 150 mm, 5-µm particle size (Chemical Inspection & Testing Institute, Tokyo, Japan) with a linear gradient elution system of water/acetonitrile. The temperature of the column was adjusted to 40 °C and a flow rate of 1.0 ml min^{-1}. Column effluent was monitored by UV absorption at 210 nm. The evaporator tube temperature of ELSD was set at 35 °C and a nebulizing gas flow-rate of 3.0 bars [Shimadzu HPLC-VP system, (Kyoto, Japan) coupled with double detectors of UV (SPD-10A) and ELSD (ELSD-LT)].

Biochemical Analysis

Plasma biochemistry measurements of the following parameters were determined according to the standard procedure (SRL Inc., Tokyo, Japan); aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), calcium (Ca), phosphorus (P), sodium (Na), potassium (K) and chlorine (Cl).

Histopathological Examination of Liver Sections

The fixed liver samples were embedded in paraffin and sections of 3 μm were subjected to haematoxylin and eosin (H&E) staining or sectioned at 10 μm for staining with oil red O according to standard procedures (Biosafety Research Center, Foods, Drugs and Pesticides, Shizuoka, Japan). Each liver section stained with H&E was microscopically examined for distribution of lesions and fatty change, and oil red O for the evaluation of fatty droplets. The lesion and fatty change (macro/microvesicular) were graded semi-quantitatively 0–3, based on percent of hepatocytes in sections which were affected as, 0 = none, 1 = slight (up to 33%), 2 = moderate (up to 66%) and 3 = marked (> 66%). The histological evaluation of the liver sections was performed blindly.

Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey's multiple comparison tests where appropriate. Results are presented as the mean ± standard error (SE) for the individual groups. Differences with $P < 0.05$ were considered to be significant. Statistical analyses were performed with Prism 5.04 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

HPLC Analysis of Forskolin

Concentration of forskolin in both CFE and experimental diets were calculated on the basis of the standard curve. The standardized CFE contained 10.88% forskolin w/w. The forskolin levels in CFE diets reflected the differences in dietary CFE supplementation (Table 1) ranging from 0.47 to 556 mg per 100-g diet. The pure forskolin diet was found to contain 55.2 ± 0.3 mg of forskolin per 100-g diet.

Dose Response Effect of Dietary CFE

Both food consumption values and estimated daily intakes of CFE in each group of mice were presented in Table 1, and their growth indexes are depicted in Fig. 1A. A slight but non-significant decrease in daily food intake was observed in treated mice (0.05–0.5% CFE groups); however, at the highest dose (5% CFE group) significantly suppressed food intake and body weight were noted. There were no statistically significant differences in the relative kidney weight and serum electrolytes such as calcium, potassium, sodium, chloride or phosphorus considered attributable to CFE treatment (data not shown). However, there were significant dose-related increases of 92% and 210% in the relative liver weight of the 0.5% and 5% CFE groups, respectively, compared with the control (Fig. 1B). A significant reduction of body fat was noted in the 0.05–5% CFE groups; the visceral fat weight was reduced by over 60% at the highest treatment dose of CFE. Similar to the results of substantial high liver weight, there were multiple-fold increases in plasma AST, ALT and ALP enzyme activities (Fig. 1C–E); these increases were statistically significant in the mice receiving 0.5% CFE or greater.

Time Response and Post-Treatment Effect of CFE

As there were apparent treatment-related hepatotoxicity effects of the 0.5% CFE dose as demonstrated by the significant elevation of liver marker enzymes, the same dose was then selected for a 5-week time-course study. After 1 week of CFE treatment, relative liver weight and visceral fat were approximately 75% higher and 30% lower, respectively, compared with the baseline (3 weeks) control group (Fig. 2A, B). These values remained at similar levels for weeks 2 and 3; however, they were largely corrected to the level of control by only 1 week of post-CFE treatment. At 2 weeks post-CFE, the relative liver and visceral fat weights had recovered to be almost identical to the corresponding control 5-weeks group. Elevation in the level of liver marker enzymes were immediately evident after 1 week of CFE treatment and remained higher than in controls for the entire 3 weeks of CFE feeding. The effect of CFE on plasma AST and ALT were maximal at week 2 (Fig. 2C, D) which resulted in increases of approximately 130% and 375%, respectively, compared with the week 3 control. Plasma ALP (Fig. 2E) was moderately (but non-significantly) increased by approximately 65% compared with the control group throughout the 3-week CFE diet period. After 1 week of post-CFE treatment, plasma AST, ALT and ALP levels were substantially restored towards normal.

Differential Hepatic Effects Between Intakes of CFE and its Principal Constituent (Forskolin)

Although all the constituents of the tested CFE involved in hepatotoxicity are not known, forskolin is the quantitatively important active component of CFE. The chromatographic

Table 1. Body weight and food intake of mice fed a diet containing *Coleus forskolii* extract (CFE) for 21 days

CFE % (w/w)	Analysed forskolin content (mg per 100-g diet)	Body weight (g)		Food intake (g mouse^{-1} day^{-1})	CFE intake (mg kg^{-1} BW day^{-1})
		Initial	Final		
0.0	0.0	33.9 ± 0.8	38.7 ± 1.6	4.97 ± 0.18	0
0.005	0.49 ± 0.05	33.3 ± 0.4	39.5 ± 1.0	4.57 ± 0.18	6.09
0.05	5.13 ± 0.12	33.3 ± 0.5	37.5 ± 1.2	4.47 ± 0.16	61.2
0.5	54.6 ± 0.8	33.6 ± 0.6	37.2 ± 1.5	4.50 ± 0.16	612
5.0	556 ± 1	33.3 ± 0.3	33.8 ± 0.6*	4.03 ± 0.19**	5915

Values are expressed as means ± standard error (SE).
*,
** Denote significant differences from the control (0% CFE), $P < 0.05$ and $P < 0.01$, respectively.

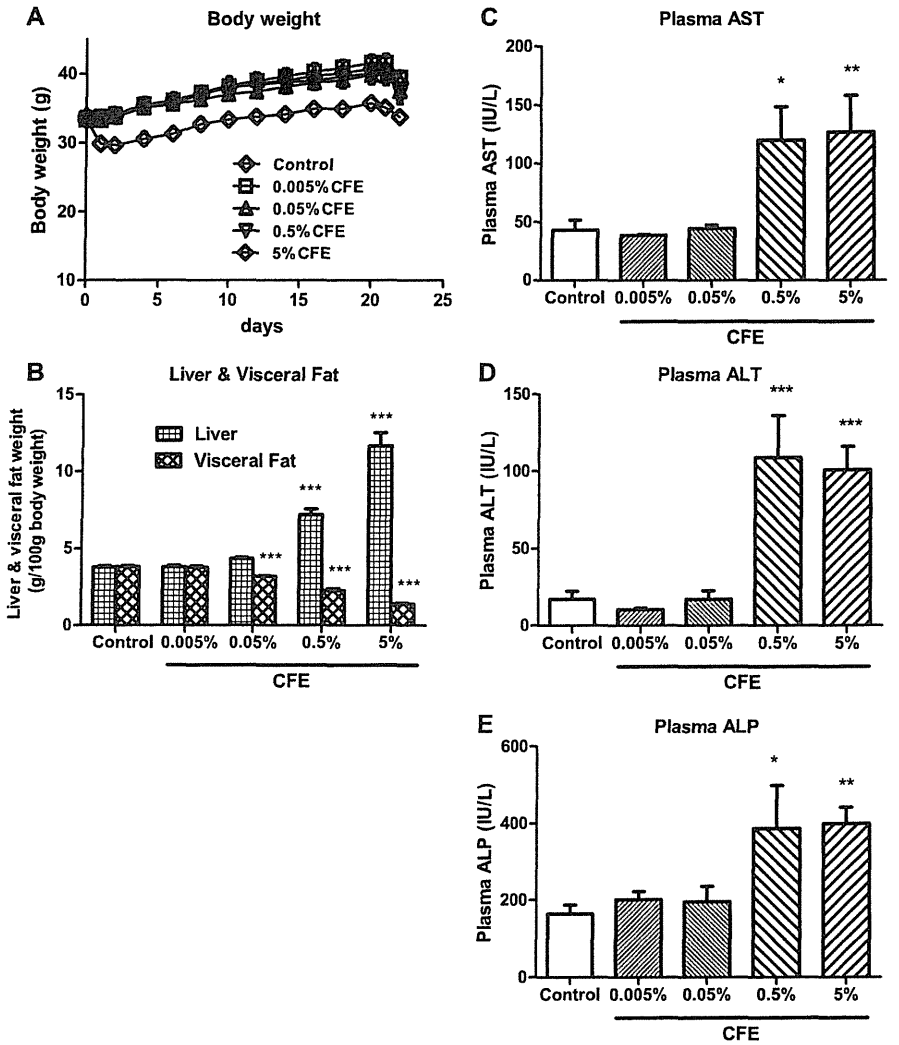

Figure 1. *Dose-dependent effects of Coleus forskohlii extract (CFE) on: body weight in mice fed the experimental diets (A); relative liver and visceral fat weight (B); plasma markers of liver damage, aspartate aminotransferase (AST) (C), alanine aminotransferase (ALT) (D), alkaline phospatase (ALP) (E). Values are given as the mean ± standard error (SE). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significantly different from the control group.*

fingerprint of pure forskolin (Fig. 3A) compared with that of the CFE used in the present study (Fig. 3B) indicated that forskolin was the major constituent amongst other components as detected by HPLC-ELSD. Thus, the effect of treatment with forskolin alone was then studied using 0.05% of the pure compound compared with the 0.5% CFE dose equivalent. Forskolin or CFE had a minimal effect on weight gain; bodyweights were similar amongst the control, forskolin and CFE groups with no statistical difference (Fig. 3C), although the CFE group was marginally lower over the whole duration of the experiment. There was no significant difference in average food intake between the control (5.14 ± 0.26 g day^{-1} mouse^{-1}), forskolin (4.89 ± 0.19 g day^{-1} mouse^{-1}) and CFE (4.94 ± 0.25 g day^{-1} mouse^{-1}) treated groups. Consistent with the dose and time response findings, CFE showed significant multiple-fold elevation of liver marker enzymes AST, ALT and ALP (increases of 130%, 415% and 258%, respectively; Fig. 3D). Also there was a significant increase (99%) in relative liver weight and a significant reduction (29%) in visceral fat in the CFE mice group (Fig. 3E, F). However, the forskolin diet elicited either little or no effect compared with the control; there were no treatment-related effects of forskolin alone on liver marker enzymes or the relative liver weight. In fact the relative visceral fat weight of forskolin-treated mice was moderately (but non-significantly) 20% lower compared with the control. Histological features of the representative views (H&E and oil red O) and grading of liver sections are shown in Figs 4, 5 and Table 2, respectively. The liver tissue of mice belonging to the control and forskolin groups showed a normal histological architecture (Fig. 4A, B). However, CFE-fed mice exhibited profound histological changes, predominantly microvesicular fatty change in hepatocytes (Fig. 4C) and midlobular hypertrophy including individual cell necrosis and cellular infiltration (Fig. 4D). The livers of CFE mice exhibited a significant fatty change, single cell necrosis of hepatocytes moderate with cellular infiltration and hepatocellular hypertrophy (Table 2). In addition, oil red O staining (Fig. 5C) indicated marked hepatocyte fat deposition (stained red) in the liver tissue from CFE-treated mice but was normal in control- and forskolin-treated mice (Fig. 5A, B).

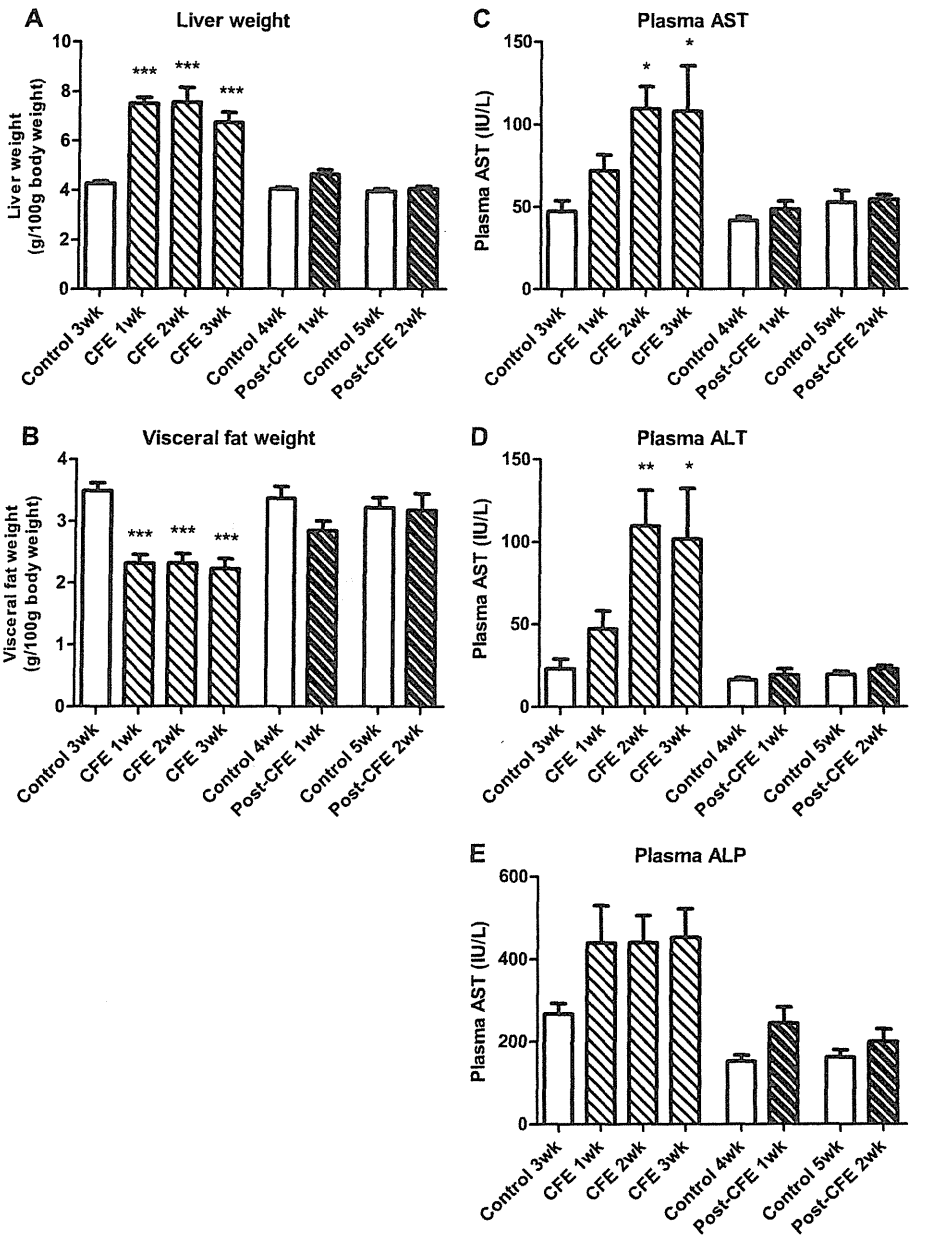

Figure 2. Time-dependent effects of *Coleus forskohlii* extract (CFE) on: relative liver weight (A); visceral fat weight (B); plasma markers of liver damage, aspartate aminotransferase (AST) (C), alanine aminotransferase (ALT) (D), alkaline phospatase (ALP) (E). Values are given as the mean ± standard error (SE). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ significantly different from the respective control group.

Discussion

Forskolin is one of the most extensively studied constituents of the *C. forskohlii* plant (Alasbahi and Melzig, 2012). Numerous positive bioactivity *in vitro* results have been reported with little *in vivo* results. Owing to the fact that the extract from the root of *C. forskohlii* showed CYP induction behaviour in mice (Virgona *et al.*, 2012), comparatively in the present study, CFE was evaluated for its hepatic effects of dose, duration of use and elucidation of the role of its major constituent 'forskolin' associated with these effects. We clearly demonstrated that CFE induced dose dependent hepatotoxicity; a significant effect was observed at a dietary CFE concentration greater than or equal to 0.5% and administration for longer than 1 week. Even although CFE produced decreased visceral fat tissue, it augmented not only liver mass but also hepatic lipid accumulation. Strong supporting evidence from the parameters measured indicated that forskolin, the main active compound in CFE, was not responsible for these events.

Anti-obesity effects by both decreased body weight gain and fat accumulation after CFE consumption has previously been demonstrated in ovariectomized rats (Han *et al.*, 2005). In the present study, a reduction of body weight gain was only observed in the highest dose (5%) although the degree of actual change was slight. This was considered at least partly to be related to the initial decreased food consumption perhaps

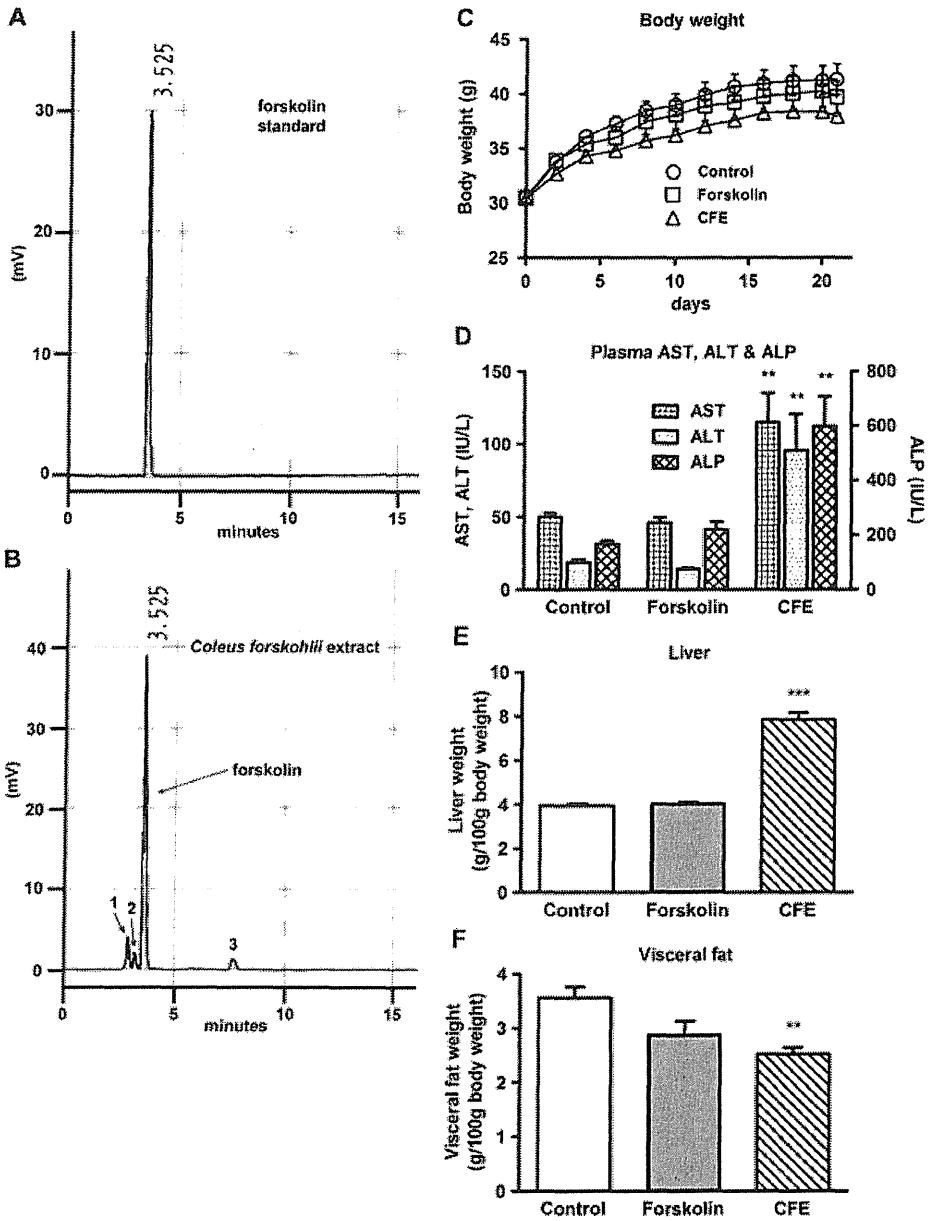

Figure 3. HPLC-ELSD analysis (A, B): chromatogram of pure forskolin at 3.525 min (A); chromatogram from the extract of *Coleus forskohlii* showing main forskolin peak at 3.525 min accompanied by 1, 2 and 3 unidentified components (B). Comparison of 0.05% pure forskolin diet with 0.5% *Coleus forskolli* extract (CFE) diet (C ~ F): body weight (C); plasma markers of liver damage, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phospatase (ALP) (D); relative visceral fat weight (E); relative liver weight (F). Values are given as the mean ± standard error (SE). $^{**}P < 0.01$, $^{***}P < 0.001$ significantly different from both the control and forskolin groups.

because of a repellent smell/taste of CFE at this concentration. Interestingly, extensive reduction in the visceral fat accumulation at the expense of an almost doubling of relative liver weight was seen in mice receiving 0.5% CFE but these phenomena did not occur in the pure forskolin-fed mice. In fact, a slight tendency towards decreased visceral fat mass, without any effect on liver weight, of mice receiving forskolin alone was noted. Thus, the effects of CFE on stored fat could be partially attributed to the lipolysis action of its forskolin content. Enhanced lipolysis leading to fat loss by forskolin has been reported both *in vitro* (Allen *et al.*, 1986; Okuda *et al.*, 1992) and *in vivo* (Han *et al.*, 2005). Obviously, impaired visceral adipose tissue development resulted in increased liver weight and thus the inability of CFE to suppress body weight gain cannot be ruled out. It appears that feeding CFE, especially at the highest dose, may lead to the induction of a whole or partial lack of adipose tissue, as in lipodystrophy or lipoatrophy. This quick collapse of visceral adipose tissue development caused by CFE appears to be reversible after only 1 week of post-CFE treatment.

The measurement of the activities of plasma biochemical parameters such as AST, ALT, and ALP in CFE mice dramatically showed several folds increase above the control. The transaminase enzymes such as AST and ALT and other hepatic markers including ALP are the most sensitive markers that play a major role in liver

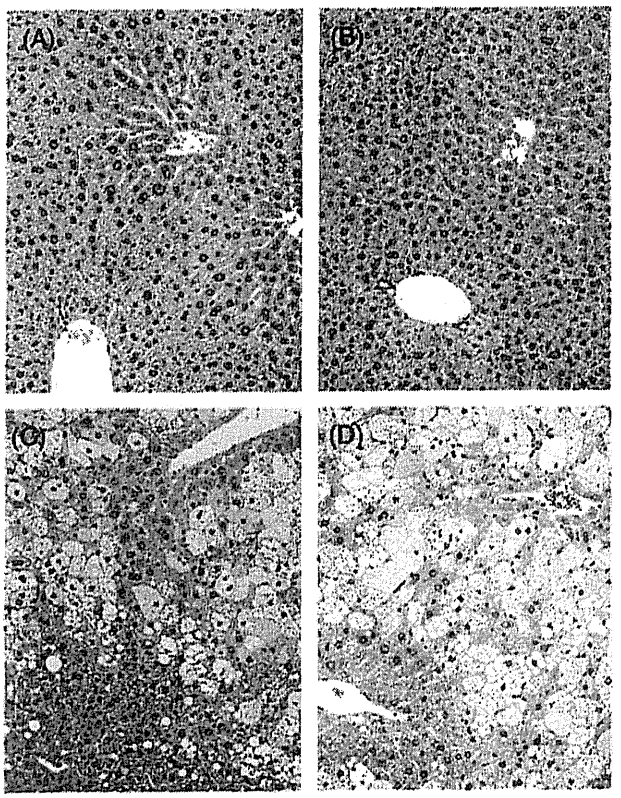

Figure 4. Representative histopathological changes of haematoxylin and eosin (H&E) stained liver sections of mice (original magnification 40×). Normal histological appearance of liver tissue of control mice (A); 0.05% forskolin diet group, liver section also show no abnormalities (B); liver sections of the 0.5% *Coleus forskolli* extract (CFE) diet group (C and D) – showed microvesicular fatty change in hepatocytes (c), apparent midlobular hypertrophy showing individual cell necrosis of hepatocytes, hepatocytes with cellular infiltration and clusters of foamy cells (D).

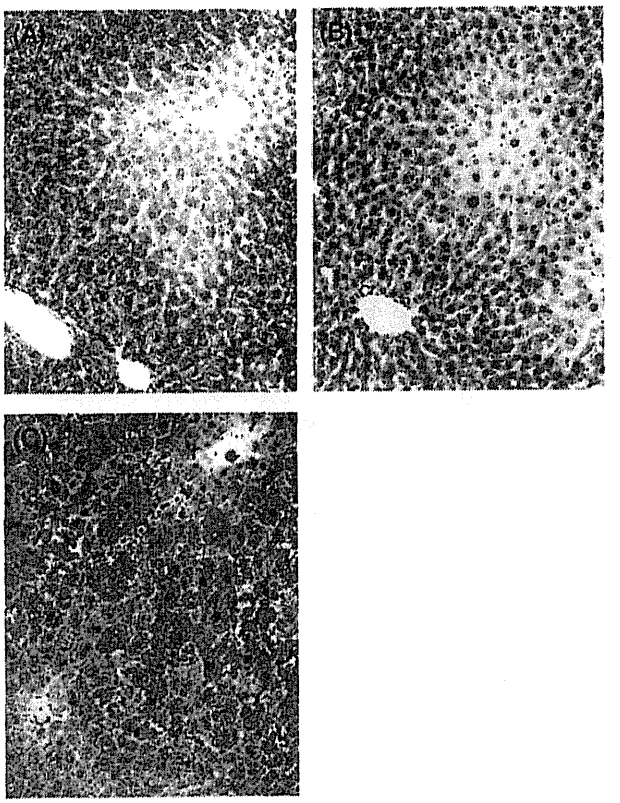

Figure 5. Representative histopathological changes of Oil red O-stained liver sections of mice (original magnification 40×). Control fed (a); 0.05% forskolin-fed mice (b); 0.5% *Coleus forskolli* extract (CFE) fed mice (c). Fat accumulation appears red in colour.

injury diagnosis (Sallie *et al.*, 1991). Elevation of AST, ALT and ALP activities in the plasma is the result of leakage from damaged cells and therefore reflects hepatocyte damage which is strongly associated with liver steatosis (Loria *et al.*, 2005).

In parallel with the alteration of liver function markers, these events were also confirmed by histological observation. In CFE mice, significant hepatic toxicity, including necrosis, hypertrophy and fatty change was observed. Hepatocyte fat accumulation was qualitatively characterized by the intensity of oil red O staining, which allows detection of lipid deposition. We found that CFE-treated mice had strong oil red O staining intensity indicating CFE caused predominantly microvesicular steatosis. Floettmann *et al.* (2010) found that once livers had exceeded a threshold weight of about 5.5% of total body weight that there is a correlation between increased liver weight and the presence of lipids analysed by oil red O staining intensities. In the present study, the relative liver weight of CFE mice at 7% is well above the threshold which further supports the fatty liver feature found from the oil red O staining result. The relative liver weight of forskolin-treated mice is about 4% and H&E and oil red O sections revealed no evidence of any lesion or fatty livers.

Several previous studies have established the association between impaired hepatic fat metabolism and the visceral fat depot (Unger *et al.*, 2010). Adipose tissue plays important roles in metabolic homeostasis which include an inert storage site for fat, and a major endocrine organ producing and releasing a variety of important bioactive substances into the bloodstream (Lara-Castro *et al.*, 2007). Deficiency of visceral adipose tissue has been associated with altered lipid metabolism most notably lipid accumulation in tissues such as the liver (Unger *et al.*, 2010). Adverse side effects as a result of a rapid and marked decrease in fat stored in adipose tissue, namely, severe liver steatosis have been reported in mice, and that the hepatic lipid accumulation is a result of uptake of mobilized fatty acids (FA) from adipose tissue and the liver's inability to sufficiently increase FA oxidation and export of synthesized triglycerides (Clement *et al.*, 2002; Wendel *et al.*, 2008). In the present studies, we demonstrated that a profoundly abnormal decrease in body adiposity observed in our CFE-fed mice drives fatty liver development clearly visible by the oil red O staining of lipid droplets. However, how the collapse of visceral fat mass by CFE triggers hepatocyte lipid deposition resulting in fatty liver remains to be defined.

An increased uptake of fatty acid in hepatocytes is associated with oxidative stress by overloaded mitochondrial beta-oxidation and is often found together with significant generation of reactive oxygen species, impaired exit of fatty acids and increased progression of steatosis (Gaemers and Groen, 2006). In addition, there is also a close relationship between steatosis and oxidative stress with reduced hepatic levels of glutathione (GSH) (Ibdah *et al.*, 2005). GST are a superfamily of multifunctional detoxification enzymes, which catalyze the conjugation of GSH to a wide variety of electrophilic compounds. GST isozymes also exhibit

Table 2. Grading levels of dietary treatment related histopathological liver changes of mice in the control and treatment groups

Histopathological feature	Control	+ 0.05% Forskolin	+ 0.5% CFE
Haemorrhage	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.58
Hepatocyte single cell necrosis	0.0 ± 0.0	0.0 ± 0.0	1.25 ± 0.25***
Hepatocyte hypertrophy	0.0 ± 0.0	0.0 ± 0.0	1.75 ± 0.48**
Fatty change	1.0 ± 0.0	1.0 ± 0.0	2.75 ± 0.25***

Values are expressed as means ± standard error (SE).
**,
*** Denote significant differences from the control, $P < 0.01$ and $P < 0.001$, respectively.

GSH peroxidase activity and catalyse the reduction of hydroperoxides of fatty acids, and phospholipids (Frova, 2006). Previous studies have demonstrated that induction of the GSTs protected against CCl4-induced hepatotoxicity in mice by catalysing the decomposition of lipid hydroperoxides generated from oxidative damage of cellular lipid molecules (Ohnuma *et al.*, 2011; Yang *et al.*, 2001). Also there is a clear manifestation of excessive formation of hepatic lipid peroxidation associated with the decline in the levels of antioxidant enzymes including GST (Fukao *et al.*, 2004). In our previous study (Virgona *et al.*, 2012), we found that both CFE and forskolin induced GST and CYP3A activities in the liver of mice. Thus it is therefore conceivable that the forskolin moiety of CFE enhanced GST to help mitigate the adverse effects that CFE had on lipid storage in the liver. Su *et al.* (1999) reported that CYP3A activity is highly responsive to relatively small changes in hepatic lipids produced by dietary manipulation in a rat model of microvesicular steatosis.

The importance of visceral adipose tissue should be considered as an integral component of the disorder, as evidenced in the 0.5% CFE-fed mice; a reduction in visceral adipose tissue probably influenced the regulation of hepatic lipid homeostasis in the liver and led to hepatotoxicity with fatty liver. In the present study, CFE extract promotes lipid accumulation in liver tissue at a dose 6 to 29 times higher than those generally reported for humans (Godard *et al.*, 2005; Henderson *et al.*, 2005) based on the body surface area normalization method (Reagan-Shaw *et al.*, 2008). Even if it is difficult to transfer the human doses to laboratory rodents, we can reasonably state that the doses used in the present study are high. In some human studies (Hori *et al.*, 2009) the risk of the development of non-alcoholic fatty liver disease (NAFLD) increased with the number of high-risk GSTs genotypes. Therefore, the increased possibility of NAFLD by the intake of CFE products in humans should not be overlooked.

As mentioned above, the aetiology of the hepatotoxicity is still unknown. However, one fact is certain that forskolin, the principle component of CFE, is not responsible for this hepatotoxicity. HPLC-ELSD analysis of the constituents of the CFE extract showed that additional peaks (marked as 1, 2 and 3, Fig. 3B), albeit small, were present at the retention time of approximately 3 and 8 min. Therefore, further studies on the isolation of these and possible other unknown constituents which may have a role in CFE-induced fatty liver are needed.

In summary, the present results indicate that unknown component(s) in CFE but not forskolin causes hepatotoxicity. The hepatotoxicity is dose dependent, and more importantly has quick onset which can be reversed within 1 week by withdrawal of CFE. The feature of hepatotoxicity consisting of hepatocellular damage and enzyme leakage resulted in elevated liver marker enzymes. Histologically, CFE induces fatty liver. The present study also demonstrates an important effect of dietary CFE on adiposity. Marked reductions in adipose mass probably contribute to the abrupt and massive increase in liver size, hepatocellular injury and liver steatosis. Further study on these observations should provide mechanistic insight into the metabolic and molecular mediators of adipose restriction and the occurrence of lipid accumulation in the liver.

Acknowledgments

The first author would like to dedicate this paper to the late Professor Takatoshi Esashi for his guidance in all aspects of this study.

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