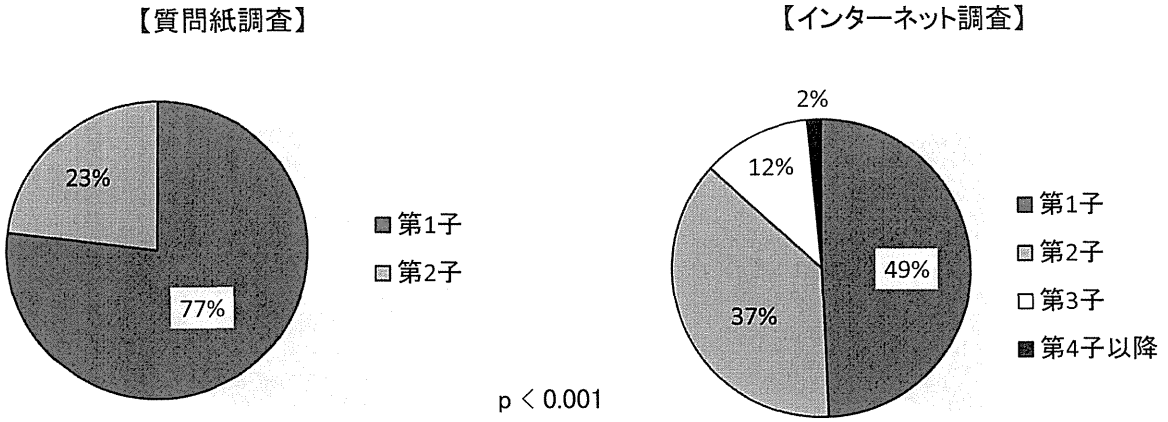


図 4. 妊娠中の子どもの出生順位
p 値 ；$\chi 2$ 検定

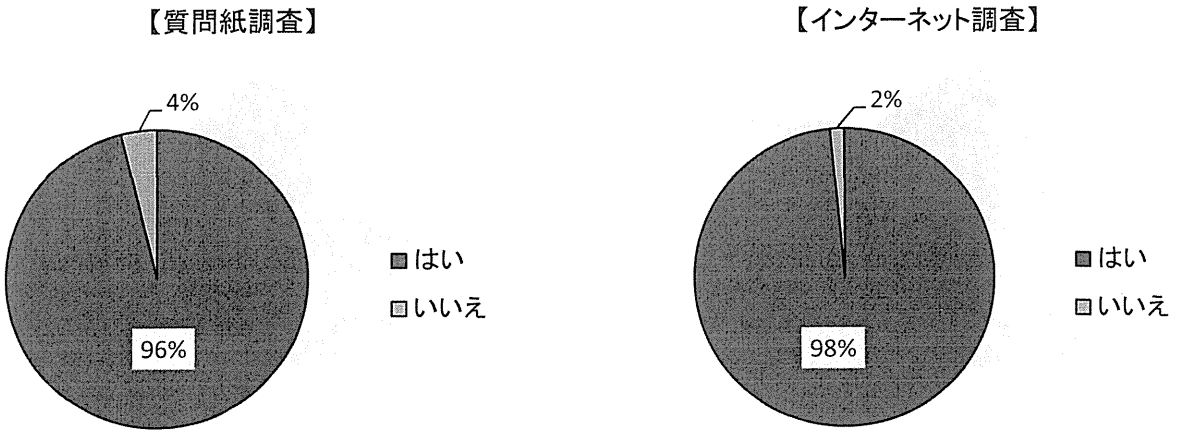


図 5. 葉酸の認識 「問. 葉酸という栄養素を知っていますか？」

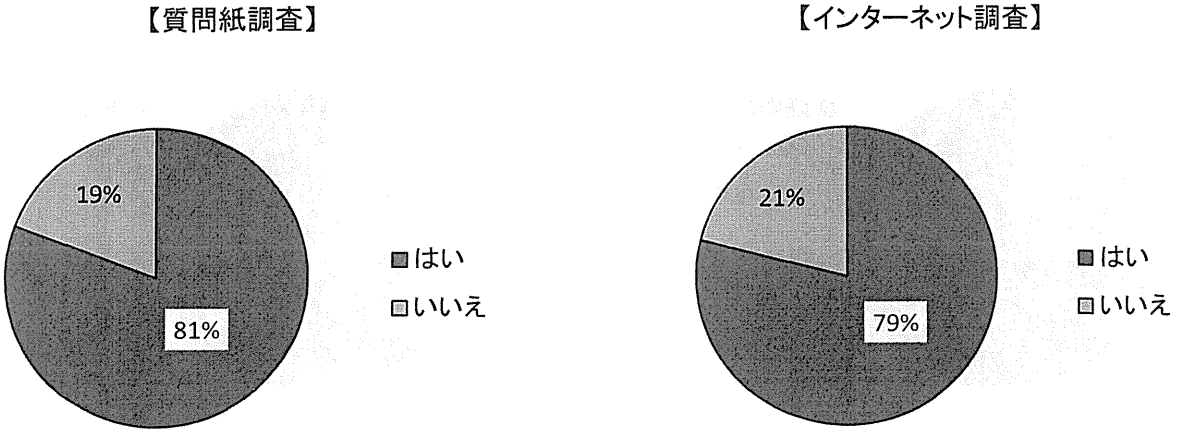
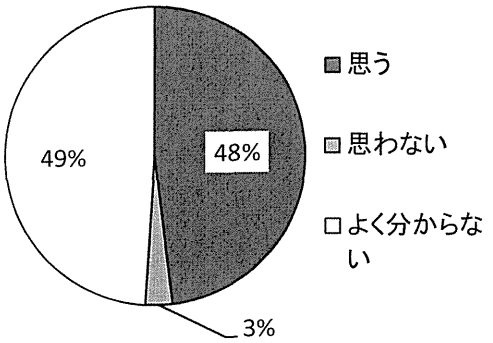


図 6. 葉酸の認識 「問. 葉酸はどのような食品に含まれているか知っていますか？」

【質問紙調査】



【インターネット調査】

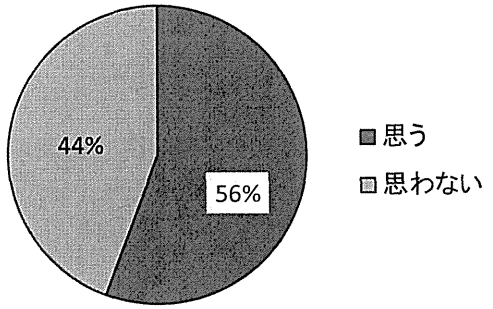
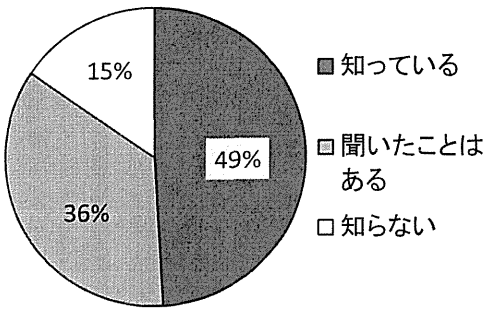


図 7. 葉酸の認識 「問. 食品にもともと含まれる葉酸と、サプリメントなどに添加されている葉酸では、吸収率が異なると思いますか？」

【質問紙調査】



【インターネット調査】

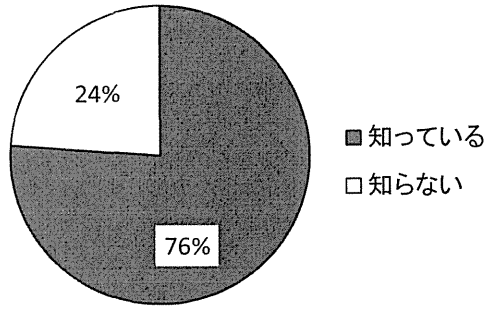
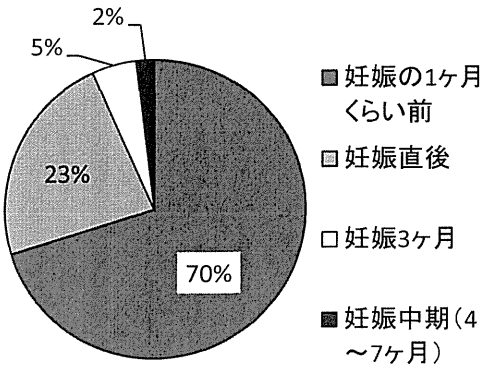


図 8. 葉酸と NTD の認識 「問. 葉酸の摂取が、胎児の神経管閉鎖障害リスク低減と関連することを知っていますか？」

【質問紙調査】



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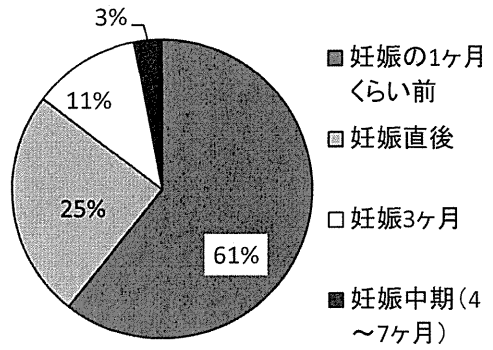
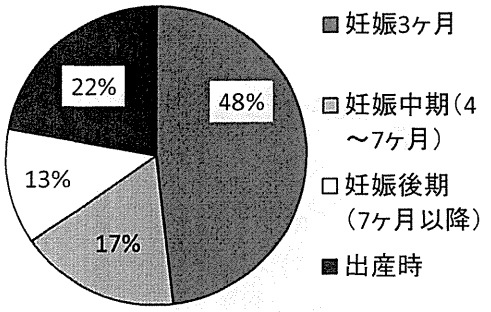


図 9. 葉酸と NTD の認識 「問. 神経管閉鎖障害リスク低減の為に葉酸を摂った方がよいと推奨されている時期は？」（開始時期）

【質問紙調査】



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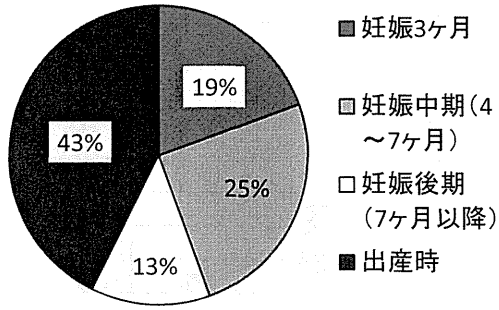
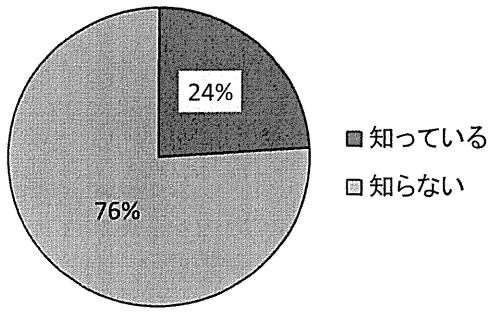


図 10. 葉酸と NTD の認識 「問. 神経管閉鎖障害リスク低減の為に葉酸を摂った方がよいと推奨されている時期は？」(終了時期)

【質問紙調査】



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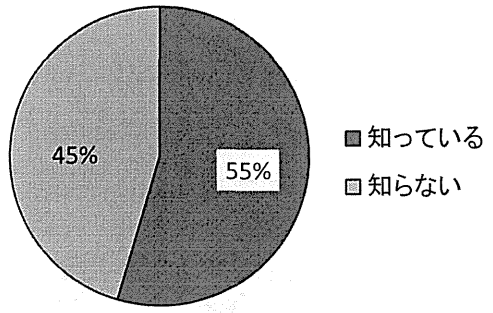


図 11. 葉酸と NTD の認識 「問. 神経管閉鎖障害リスク低減の為に勧められる葉酸の摂取量を知っていますか？」

【質問紙調査】

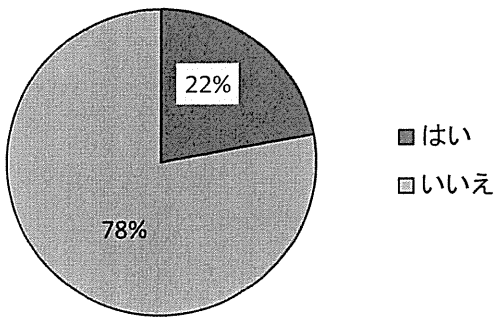


図 12. 葉酸摂取行動 「問. 妊娠前から妊娠に気づくまで、葉酸を意識的に摂っていましたか？」

【質問紙調査】

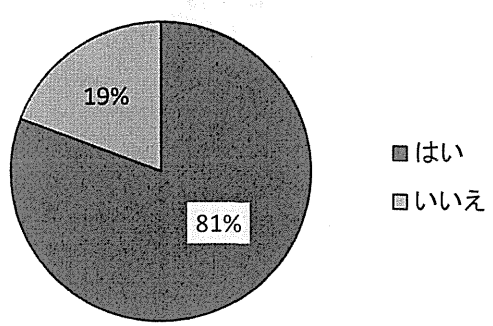


図 13. 葉酸摂取行動 「問. 妊娠に気づいてから、葉酸を意識的に摂っていましたか？」

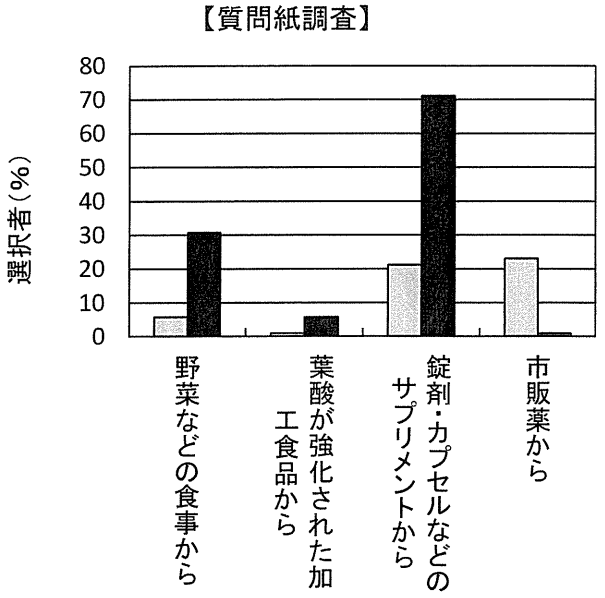


図 14. 葉酸摂取行動 「問. どのようにして摂っていましたか？」

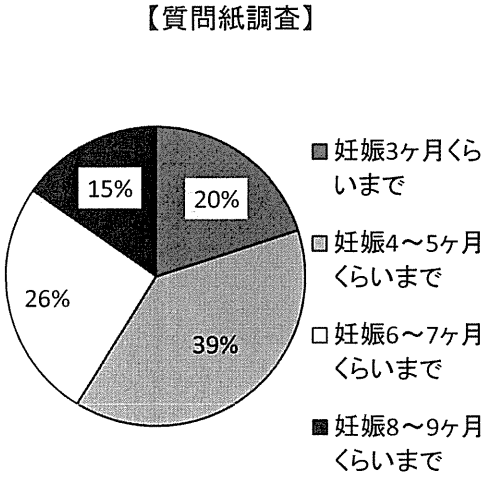


図 15. 葉酸摂取行動 「問. どのくらいの期間、葉酸を意識的に摂っていましたか？」

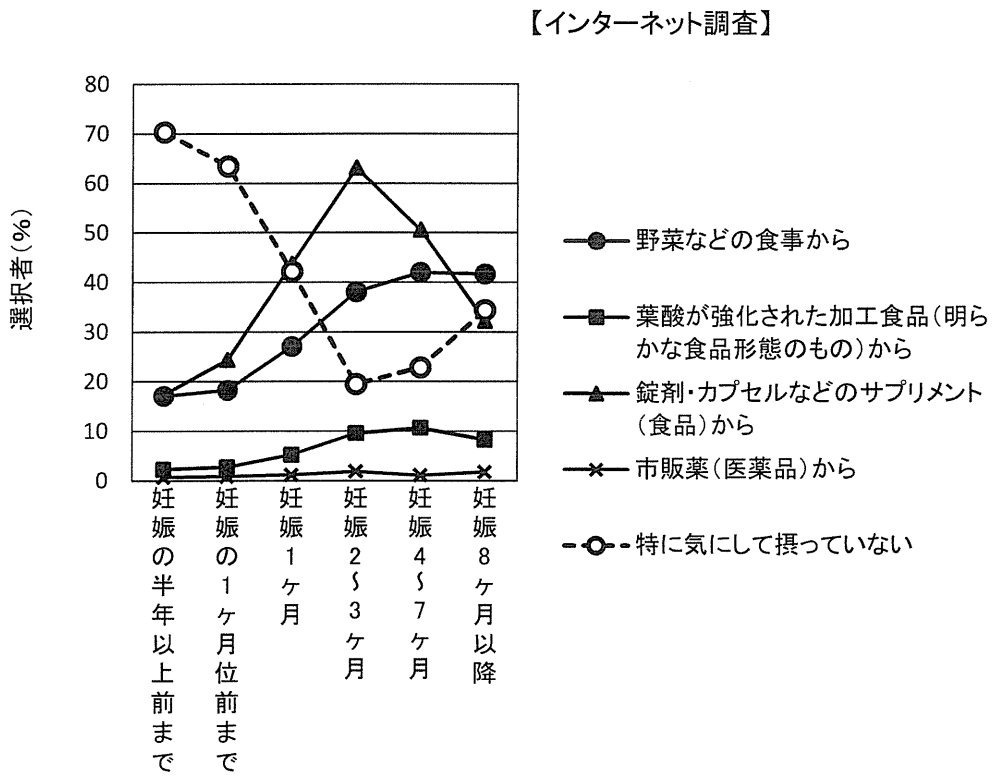
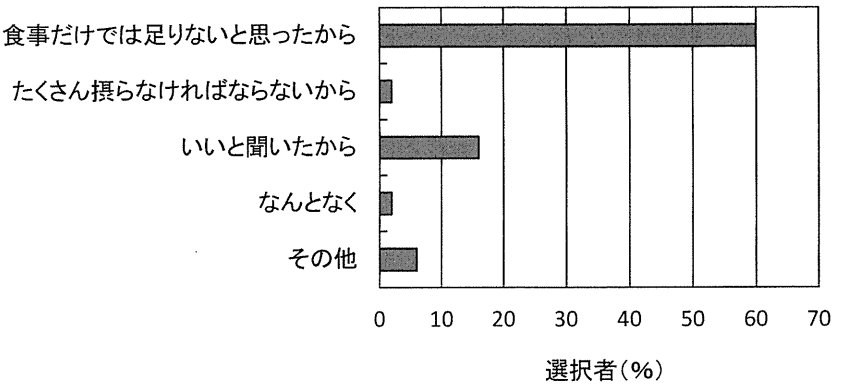


図 16. 葉酸摂取行動 「問. 各時期に、葉酸を意識的に摂っていましたか？」

【質問紙調査】



【インターネット調査】

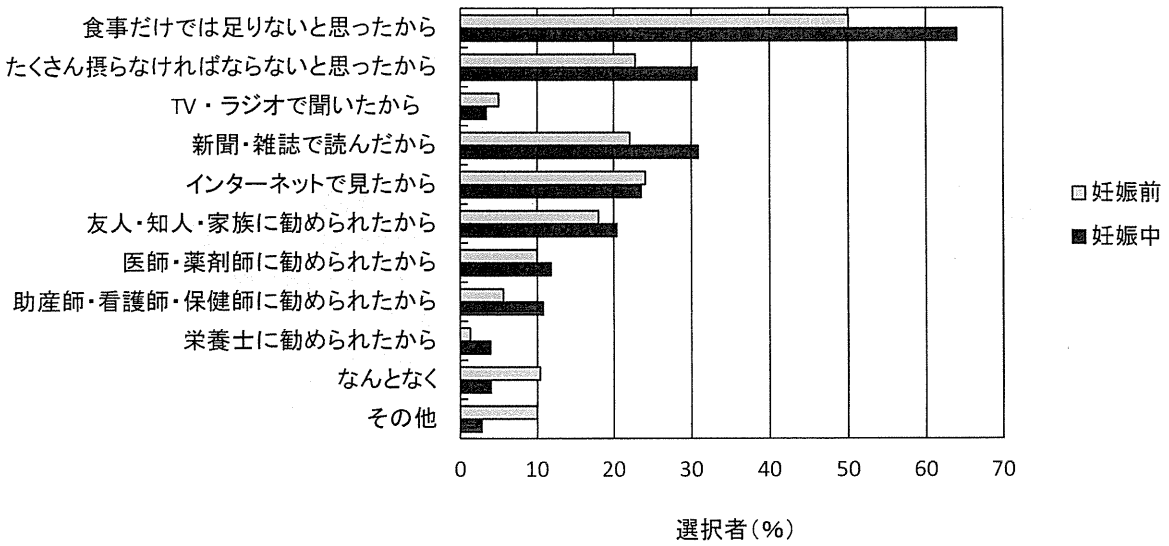
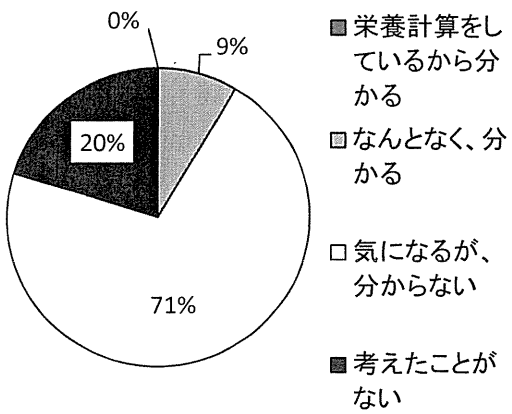


図 17. 葉酸摂取行動 「問. 葉酸の製品を利用しようと思った理由は何ですか？」

【質問紙調査】



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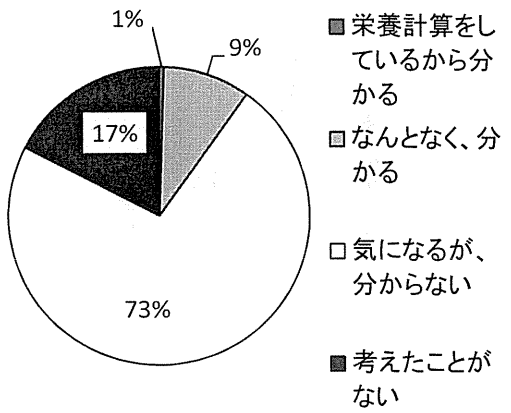
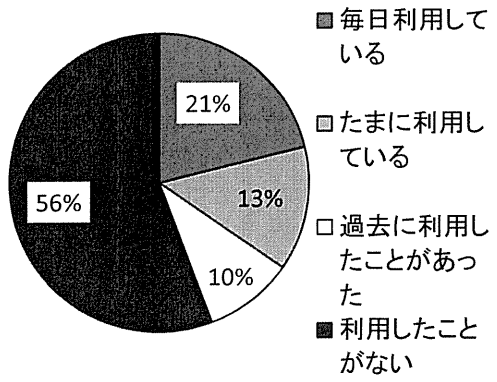


図 18. 葉酸と NTD の認識 「問. あなたは、ご自分が葉酸を食事からどれくらい摂っているか、分かりますか？」

【質問紙調査】



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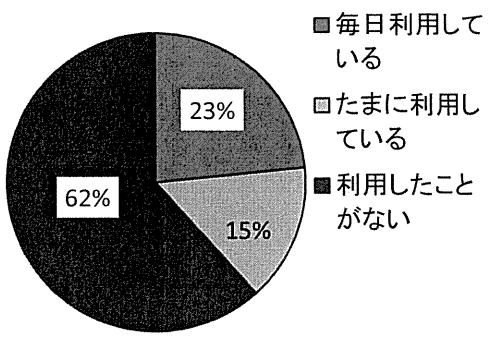
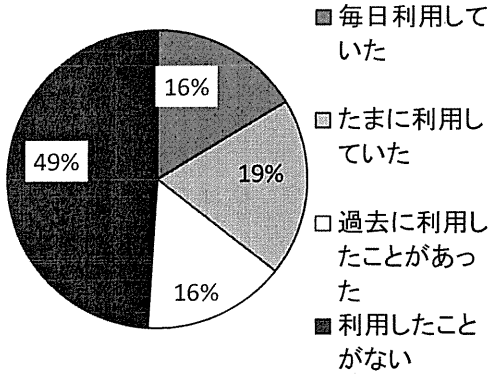


図 19. サプリメント摂取行動 「問. 妊娠中に葉酸以外のサプリメントを利用していますか？」

【質問紙調査】



【インターネット調査】

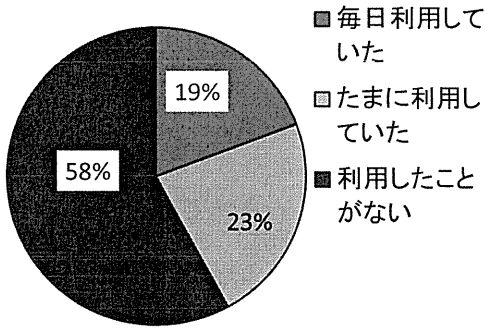


図 20. サプリメント摂取行動 「問. 妊娠前に葉酸以外のサプリメントを利用していますか？」

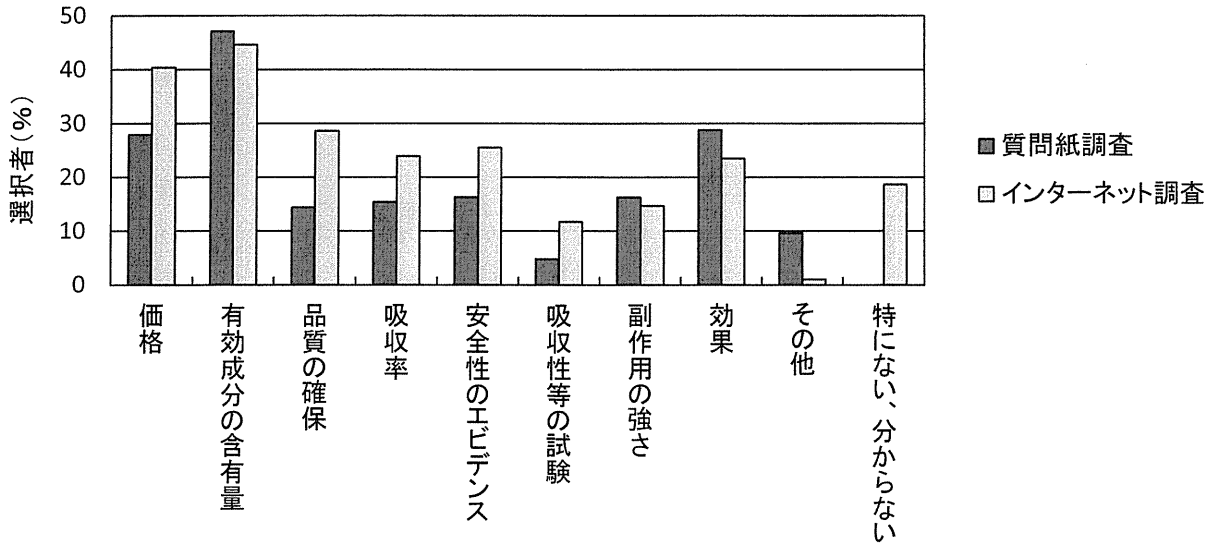


図 21. サプリメントの認識 「問. サプリメンと医薬品の違いは何だと思いますか？」

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
Toyoizumi K、 Yamada H、 Matsumoto K、 Sameshima Y.	Gargling with green tea for influenza prophylaxis: A clinical pilot study.	*Jpn J Clin Pharmacol Ther.*	44 (6)	459-461	2013
Unno K、 Tanida N、 Ishii N、 Yamamoto H、 Iguchi K、 Hoshino M、 Takeda A、 Ozawa H、 Ohkubo T、 Juneja LR、 Yamada H.	Anti-stress effect of theanine on students during pharmacy practice: Positive correlation among salivary α-amylase activity、 trait anxiety and subjective stress.	*Pharmacol Biochem Behav.*	111	128-135	2013
一丸佳代、井出和希、小野彩奈、北川護、成島大智、松本圭司、梅垣敬三、山田浩.	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改変.	臨床薬理	44 (5)	405-410	2013
梅垣敬三、山田浩、千葉剛、佐藤陽子、中西朋子、福山哲.	健康食品に関する健康被害事例の情報源およびその有用性評価.	食品衛生学雑誌	54 (4)	282-289	2013
梅垣敬三.	いわゆる健康食品の安全性確保.	食品衛生学雑誌	54 (6)	J-408-412	2013
梅垣敬三.	機能性成分の安全性と有効性　-最近の「健康食品」の安全性・有効性情報から-.	食品と容器	55 (1)	34-40	2014
佐藤陽子、中西朋子、横谷馨倫、千葉剛、梅垣敬三.	葉酸およびそのサプリメント摂取に対する妊婦、管理栄養士・栄養士、管理栄養士・看護師養成校の学生の認識.	栄養学雑誌	71 (4)	204-212	2013

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改変

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北川 護[*1] 成島大智[*1] 松本圭司[*1]
梅垣敬三[*2] 山田 浩[*1]

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

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[*a] These authors contributed equally to this study.

Information of adverse events associated with dietary supplements or health food, which is collected by manufacturers or healthcare facilities, is inconsistent. Therefore, a method to collect essential information from patients or consumers and evaluate the causal relationship of adverse events is necessary. We previously modified a dendritic algorithm for evaluating medication-related adverse events (Jones JK. Fam Community Health 1982; 5: 58-67) for use in dietary supplements or health food. In this study, we improved the dendritic algorithm, especially in the temporal relation between taking dietary supplements or health food and onset of adverse events, and compared to a different algorithm based on the scoring scale developed by Naranjo et al (Naranjo CA, et al. Clin Pharmacol Ther 1981; 30: 239-45). Using both algorithms, eight raters (pharmaceutical science students) assessed 200 cases of adverse events provided by the manufacturer's customer inquiry center. The κ coefficient of multi-rater reliability was 0.51 for the modified dendritic algorithm and 0.35 for the scoring scale. The time required to complete the evaluation tended to be shorter using the dendritic algorithm. In conclusion, the present results indicate that the improved dendritic algorithm may be reliable and suitable for universal usage. Pilot studies using the modified algorithm during history taking of consumers or patients to collect information on adverse events are needed to assess the utility of the algorithm in clinical practice.
(*Jpn J Clin Pharmacol Ther* 2013; 44(5): 405-410)

Key words: health food, adverse event, algorithm, causal relationship

緒 論

保健機能食品（特定保健用食品，栄養機能食品）およびそれ以外のいわゆる健康食品（以下，両者を総称して健康食品と略す）の摂取が原因と疑われる有害事象の発生が近年問題となっている[1~5]．この背景には，多種多様な製品が流通し消費される一方，製品に対する法的規制が十分になされていないということに加え，有害事象が発生した場合の評価法が確立されていないという問題がある[6]．健康食品の摂取による有害事象の報告は，販売店や製造販売元への問い合わせ，医療機関での診療記録などを基に保健所を介して厚生

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E-mail：hyamada@u-shizuoka-ken.ac.jp
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労働省に集約される．また，消費者庁を介した情報の集積も行われている．このように，多様な場で異なる専門性を持つ集団により収集された事例が集積されるという特性から，健康食品の摂取と有害事象の因果関係を正確かつ効率的に判断するための評価法が必要とされている．

健康食品の摂取に伴う有害事象の評価法が確立されていない反面，医薬品の投与に伴う有害事象の評価法として，Naranjo らの評価票[7]や Jones の樹枝状アルゴリズム[8]といった評価法が報告されている[9]．健康食品と医薬品は共に生体分子に働きかけることでその作用を発現するという共通点を持ち，薬物代謝酵素の誘導や阻害，受容体に対する結合などを介して作用を示す．我々はこれまでに，薬理学的・薬力学的アプローチを基盤とした Naranjo らの評価票や Jones の樹枝状アルゴリズムを改変し，最適化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確立を試みてきた[10~12]．その過程で，Jones の樹枝状アルゴリズムの内容を検討し，質問項目や分岐形式，カテゴリー分類を改変したアルゴリズムを構築した．構築したアルゴリズムを用いて事例を評価し，分析した結果，1）健康食品の摂取と有害事象の発生の時間的関連，2）パッチテストなどによる客観的根拠の取り扱いに関するカテゴリー分類上の課題が残った[12]．そこで，Jones の樹枝状アルゴリズムにこれらの課題を反映した質問項目，分岐形式，カテゴリー分類などの改変を加え，臨床応用可能な樹枝状アルゴリズムの最適化を試みた．さらに，これまでに Naranjo らの評価票を基に改変を重ねることで構築した評価票による評価を行い，両者を比較した．

方　法

先行研究で作成した Jones の樹枝状アルゴリズムの改変を健康食品の有害事象として報告される情報の特性とその重み付けを考慮して再検討し，カテゴリー分類，分岐形式を改変し，質問項目を追加した（Fig. 1）．改変は以下の 2 項目について行った（変更点 1 はカテゴリー分類における変更点，2 は分岐形式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，(a) 有害事象と健康食品の摂取との時間的関連が否定された事例と (b) 時間的関連がみられるが他の要因に起因する可能性が高い事例が同一のカテゴリー「関連なし（Remote, Doubtful）」に分類されていたため，(a) を「関連なし（Highly unlikely）」，(b) を「ほぼ関連なし（Unlikely）」として分類を細分化した．

2）有害事象の再現性が確認されていない事例である場合，客観的証拠（DLST，パッチテスト等）の有無が問われる質問が存在していなかった．客観的証拠は，因果関係を判断するうえで重要な要素であるため，再現性が確認されていない事例であっても，客観的証拠の有無が問われるように分岐形式を改変し，質問項目を追加した．

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）およびこれまでに Naranjo らの評価票を基に改変を重ね構築した評価票（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相談センターに寄せられた保健機能食品（特定保健用食品，栄養機能食品）および保健機能食品以外の健康食品の摂取に伴う有害事象相談事例 200 例に対して 8 名（薬学部 6 年生 2 名，5 年生 2 名，4 年生 3 名，大学院博士課程 1 名）が因果関係を評価した．8 名をランダムに 2 群に割り付け，一方の群（A 群）は 1. 改変評価票，2. 改変樹枝状アルゴリズムの順に，他方の群（B 群）は 1. 改変樹枝状アルゴリズム，2. 改変評価票の順に評価を行った．評価はそれぞれ独立して行い，1 と 2 の評価の間には 6 カ月の期間を設けた．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），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（Unlikely），関連なし（Highly unlikely），情報不足・評価不能（Lack of information）の 7 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，因果関係の強い順に，非常に確からしい（Highly probable），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），関連なし（Doubtful）の 5 段階にカテゴリー分類した．次いで，評価者間信頼性の指標として Fleiss の多評価者間 κ 係数を算出した．加えて，臨床における汎用性の観点から 200 事例の評価に要した時間を計測した．統計解析には R ver. 2.15.2（R Development Core Team, 2012）を用いた．

なお，本研究で利用した有害事象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

改変評価票，改変樹枝状アルゴリズムのいずれにおいても 200 事例の評価結果は，「可能性がある（Pos-

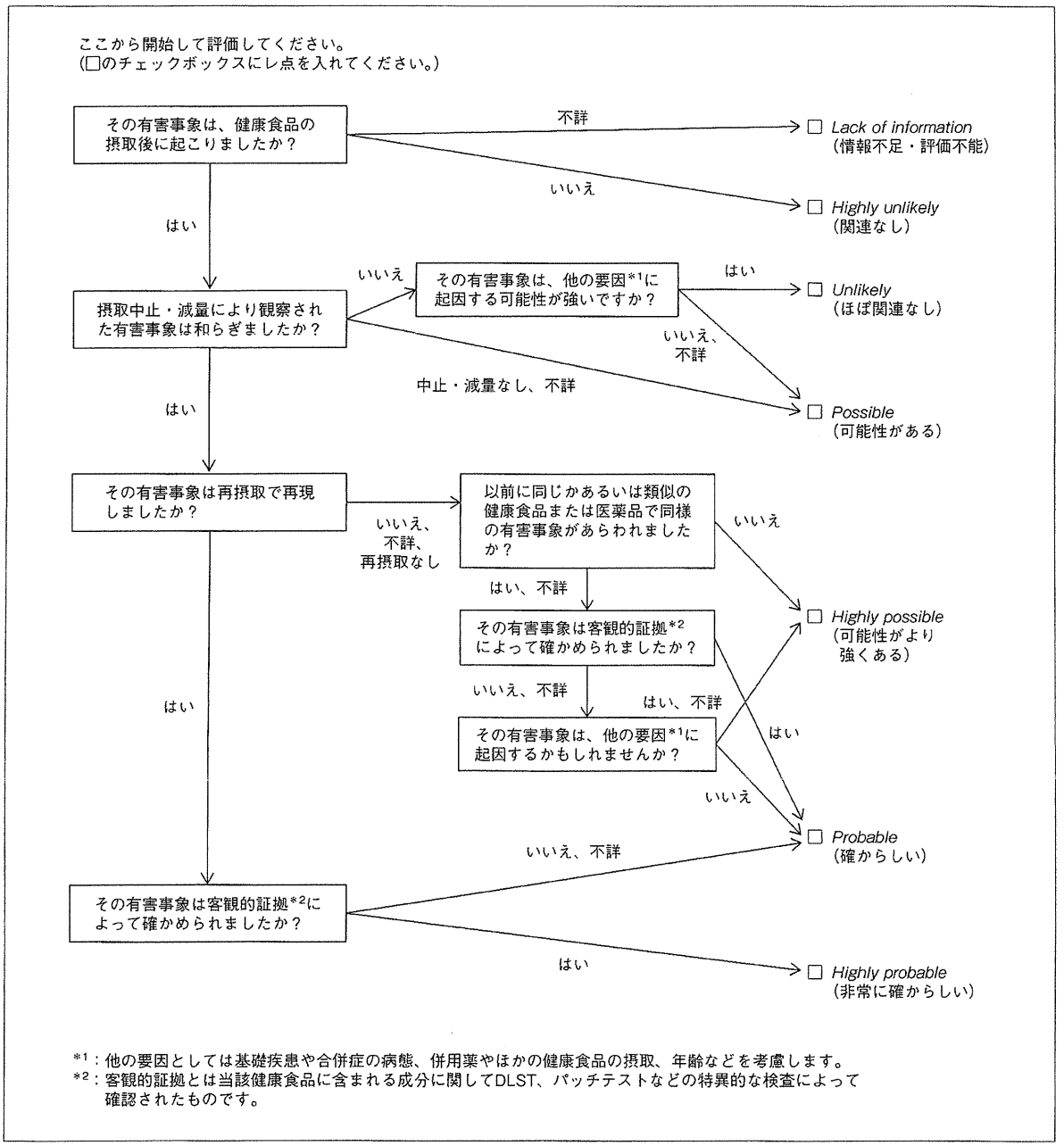

Fig. 1 改変樹枝状アルゴリズム
Jones の樹枝状アルゴリズムを基に，改変を重ねることで構築したアルゴリズム．

sible)」に集中し，「非常に確からしい（Highly probable)」に分類された事例はなかった（Fig. 2）．1. 改変評価票，2. 改変樹枝状アルゴリズムの順に評価を行った群（A 群）の κ 係数は，改変評価票 0.21，改変樹枝状アルゴリズム 0.53 であった．また，1. 改変樹枝状アルゴリズム，2. 改変評価票の順に評価を行った群（B 群）の κ 係数は，改変樹枝状アルゴリズム 0.50，改変評価票 0.54 であった．両群全体の κ 係数は，改変評価票 0.35，改変樹枝状アルゴリズム 0.51 であった．

評価時間は改変評価票 97±63 分，改変樹枝状アルゴリズム 79±27 分であった（Table）．

考　察

本研究では，Jones の樹枝状アルゴリズムに更なる改変を加え，健康食品の摂取に伴う有害事象の因果関係評価法の最適化を試みた．また，κ 係数，評価時間

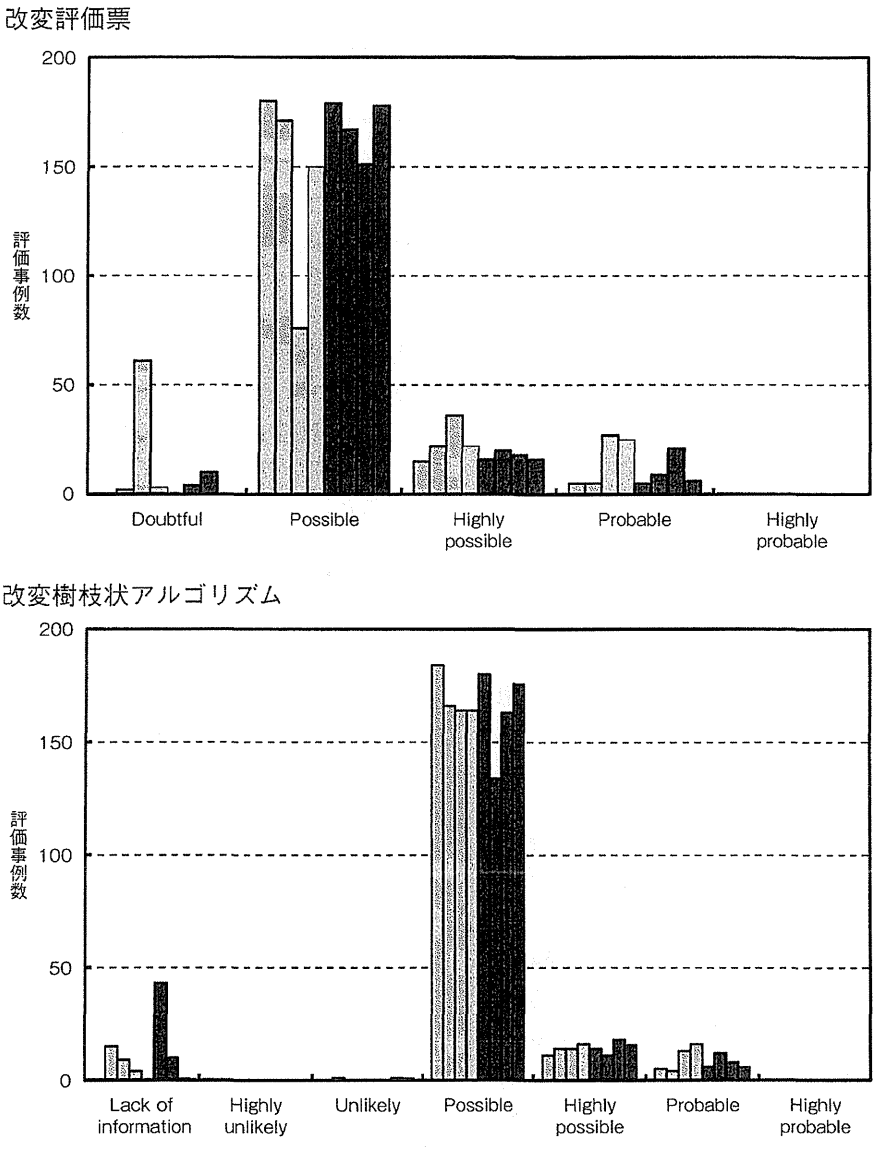

Fig. 2 改変評価票および改変樹枝状アルゴリズムによる有害事象評価結果の分布
改変評価票，改変樹枝状アルゴリズムの順に実施した群（A 群）：灰色，改変樹枝状アルゴリズム，改変評価票の順に実施した群（B 群）：黒色，評価した有害事象事例：200 例．

を指標として改変評価票との比較を行った．樹枝状アルゴリズムの改変前後での相違は，1）健康食品の摂取と有害事象の時間的関係から「関連なし（Remote, Doubtful）」を「関連なし（Highly unlikely）」，「ほぼ関連なし（Unlikely）」の 2 つのカテゴリーに細分化し，2）客観的証拠に対する重み付けを加えた点にある．このようにして構築した改変樹枝状アルゴリズムとこれまでに構築した改変評価票をランダムに 2 群に割り付けた 8 名の薬学生を評価者としてクロスオーバー方式で実施し，信頼性と臨床的な汎用性を評価した．

結果，両群全体における κ 係数は改変樹枝状アルゴリズムにおいて良好な値を示し，評価時間においても短い傾向がみられた．一方，改変評価票においては，先に改変評価票を実施した群で κ 係数が低く，改変樹枝状アルゴリズムでの評価後に改変評価票を実施した群では改変樹枝状アルゴリズムと同程度の κ 係数を示した．評価時間については個々のばらつきが大きく，今後より大規模な検討において比較する必要があると考えられた．また，改変評価票を先に実施した群において κ 係数が低い値を示した理由として，先行研究において示された改変評価票の使用におけるトレーニングの必要性が影響している可能性がある．評価者が健

Table 各評価法における多評価者間 κ 係数と評価時間の比較

		改変評価票	改変樹枝状アルゴリズム
多評価者間 κ 係数	A 群	0.21	0.53
	B 群	0.54	0.50
	両群	0.35	0.51
評価時間（mean±SD）（min）		97±63	79±27

康食品の摂取に伴う有害事象の因果関係評価に関与した経験のない学生であるという点からもその影響が顕著に表れたと推察される．したがって，改変評価票，改変樹枝状アルゴリズムのいずれも同程度の信頼性を有するものの，専門性の異なる集団から収集されるという有害事象報告の特性を考慮した場合，改変樹枝状アルゴリズムを活用することが適すると考えられた．しかしながら，評価者の資質による影響を十分に評価することは，類似の背景を持つ少数の評価者を対象とした本研究の限界を示しており，議論の余地が残された．

評価事例200例のカテゴリー分類は，これまでに改変評価票，樹枝状アルゴリズムで示した結果と同様，「可能性がある（Possible）」に集中した．これは，健康食品の摂取の中止や減量による情報が不足しているという情報の曖昧さが一因であると考えられた．また，「情報不足・評価不能（Lack of information）」に分類される事例も存在した．これは，消費者・患者から自発的に発信される情報のみでは，因果関係の判定に必要となる情報を十分に把握することが困難であり，お客様相談センターの担当者，販売店など情報の受け手の情報収集方式にも必ずしも一貫性がないことを示唆している．すなわち，一定の評価アルゴリズムを利用し，必要な情報を消費者・患者から聞き取り，詳細を把握することは因果関係評価を容易にし，結果として健康被害の拡大を阻止することに繋がると考えられる．また，漠然と有害事象の状況を問われる現状と比較して，順序立てて状況を問うことは消費者・患者にとっても情報提供のしやすさという点でメリットとなる．しかしながら，アルゴリズムに含まれる質問の回答様式の多くは「はい」，「いいえ」の2択ではなく，因果関係評価に対する一定の理解を要する．運用にあたっては，評価者に対する事例演習や説明が必要であると考えられた．

健康食品の利用目的として「病気の治療」を挙げる消費者も存在すること[13]から原疾患の有無や服用中の医薬品との相互作用も重要な問題となるが，改変評価票，改変樹枝状アルゴリズムのいずれにおいても疾患に関する情報は十分に得られず，因果関係評価における限界と改良の余地が残された．

また，有害事象には摂取によらず一定頻度で生じうるものも含まれる[14,15]．評価アルゴリズムを利用した大規模な情報の収集は，同一の症状を示す事象の集積にも繋がり，個別症例からは判定が困難な例を検出するうえでも活用が可能である．現在は，自発的に提供された情報に対してアルゴリズムを適用し，改変を重ねることでその質を高めることに留まっているが，消費者・患者からの聞き取り段階でアルゴリズムを活用することは，情報そのものの有用性を高め迅速に伝達することに繋がるだけでなく，シグナルマネジメントを基礎としたリスクマネジメントへの展開にも寄与するものと期待される．

結　論

今回最適化を図った改変樹枝状アルゴリズムは，改変評価票と比較して評価経験の少ない評価者においても高い信頼性を有し，評価時間の側面からも臨床現場における健康食品摂取に伴う有害事象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，臨床現場で使用する職種間の信頼性やアルゴリズムの適用による情報収集の効率化について有用性を検討する必要がある．

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Conflict of Interest
本研究論文の発表に関連して，開示すべき COI 関係にある企業等はない．

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Anti-stress effect of theanine on students during pharmacy practice: Positive correlation among salivary α-amylase activity, trait anxiety and subjective stress

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ABSTRACT

Purpose: Theanine, an amino acid in tea, has significant anti-stress effect on experimental animals under psychosocial stress. Anti-stress effect of theanine on humans was evaluated in 5th-year university students during pharmacy practice.

Method: The study design was a single-blind group comparison and participants (n = 20) were randomly assigned to theanine or placebo groups. Theanine or placebo (lactose) tablets (200 mg, twice a day, after breakfast and lunch) were taken from 1 week prior to the pharmacy practice and continued for 10 days in the practice period. To assess the anxiety of the participants, the state–trait anxiety inventory test was carried out before the pharmacy practice. Salivary α-amylase activity (sAA) was measured as a marker of sympathetic nervous system activity.

Results: In the placebo-group, sAA in the morning (pre-practice sAA) was higher than in theanine-group during the pharmacy practice ($p = 0.032$). Subjective stress was significantly lower in the theanine-group than in the placebo-group ($p = 0.020$). These results suggest that theanine intake had anti-stress effect on students. Furthermore, students with higher pre-practice sAA showed significantly higher trait anxiety in both groups ($p = 0.015$). Similarly, higher pre-practice sAA was correlated to shorter sleeping time in both groups ($p = 0.41 \times 10^{-3}$).

Conclusion: Stressful condition increased the level of sAA that was essentially affected by individual trait anxiety. The low levels of pre-practice sAA and subjective stress in the theanine-group suggest that theanine intake suppressed initial stress response of students assigned for a long-term commitment of pharmacy practice.

1. Introduction

Chronic psychosocial stress is associated with the development of depression, mood disorders, as well as cardiovascular and other age-related diseases (McEwen and Magarinos, 1997; Pedersen et al., 2001; Gareri et al., 2002; Bellinger et al., 2008; Thayer et al., 2010). Intervention of stress-induced alterations with dietary supplements is a potential therapeutic strategy for a healthy life. We have previously reported that the intake of theanine (γ-glutamylethylamide) suppressed the stress-derived malfunctions in aged mice that were chronically stressed under the confrontational housing (Unno et al., 2011, 2013). Theanine (L-theanine) is the most abundant amino acid in tea. The sweet umami taste of green tea is due to amino acids, especially theanine. Several studies have reported that theanine exerts neuroprotective effects (Nagasawa et al., 2004; Egashira et al., 2004, 2007, 2008; Cho et al., 2008; Kim et al., 2009), modulates the activity of neurotransmitters (Yamada et al., 2007; Kakuda et al., 2008) and reduces psychological stress (Kimura et al., 2007). In this study, we aimed to investigate the effect of theanine supplementation on stress responses in 5th-year college students of the school of pharmaceutical sciences. They were assigned to practice outside the university such as in a hospital or a drug store, for 11 weeks. Such a long-term commitment in new environments provides a stressful condition for young students. Salivary α-amylase activity (sAA), an oral cavity enzyme, was measured as a stress marker (Nater and Rohleder, 2009). Two main body systems

Abbreviations: ANS, autonomic nervous system; HPA, hypothalamus–pituitary–adrenal; sAA, salivary α-amylase activity; pre-practice sAA, sAA in the morning; post-practice sAA, sAA in the evening; STAI, the state–trait anxiety inventory; VAS, visual analog scales.

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are involved in the stress response, the autonomic nervous system (ANS) and the hypothalamus–pituitary–adrenal (HPA) axis. Measurement of sAA has been demonstrated as a useful tool for monitoring ANS reactivity to stress (Nater and Rohleder, 2009). This enzyme is increased rapidly in response to physiological and psychosocial stress (Almela et al., 2011; Nater et al., 2005, 2006; Rohleder et al., 2004). The secretion of salivary amylase is directly stimulated by innervation followed by hormonal regulation in response to changes in serum noradrenalin levels. Therefore, the salivary gland acts more quickly and sensitively responds to the psychological stress than cortisol (Yamaguchi et al., 2004). The measurement of sAA is an efficient and non-invasive assessment to study the effect of psychosocial stress. In the present study, considering possible individual variability in responding to the same stressful condition, trait anxiety, physical condition, subjective stress, achievement emotion and sleeping time were scored and integrated with the changes in sAA in each participant during pharmacy practice. Our results suggest that the theanine supplementation is beneficial in suppressing psychosocial stress in humans.

2. Methods

2.1. Participants

Twenty healthy 5th-year students of the University of Shizuoka, who participated in the experiment, were randomly divided into two groups with matching sex: theanine (n = 10, 7 men and 3 women; average age 22.5 ± 0.2 yr) and placebo (n = 10, 7 men and 3 women; average age 22.2 ± 0.1 yr) via sealed envelopes to receive theanine or placebo tablets. The students were assigned to practice outside the university, in a hospital or a drug store for 11 weeks. The first 10 days of the practice program were analyzed, because these days were assumed to be the most stressful. None of the participants indicated acute or chronic disease, regular medication intake, or habitual smoking. They were instructed to drink mainly water, and not to take theanine- and caffeine-rich beverages such as green tea, coffee, and black tea throughout the experiment. The study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of the University of Shizuoka. All the participants received verbal and written information about the study and signed an informed consent form before entering the study. This study was registered at clinicaltrials.gov (registration ID no. NCT01361204). The study period was from March to September in 2011.

2.2. Procedure

This study was a group comparison design and participants were randomly assigned to theanine or placebo groups. The participants did not know whether they were consuming theanine or placebo. To assess the anxiety of the participants, the state–trait anxiety inventory (STAI) test (Japanese STAI Form X-1, Sankyobo, Kyoto, Japan) was carried out before the pharmacy practice.

Theanine or placebo (lactose) tablets (200 mg, twice a day, after breakfast and lunch; Lyon et al., 2011; Kimura et al., 2007; Lu et al., 2004) were taken from 1 week prior to the pharmacy practice and continued for 10 days in the practice period, in total for 17 days.

The placebo tablet of lactose was in a similar color to a theanine tablet. A questionnaire including physical condition, subjective stress and achievement emotion was assigned for 10 days after each day's practice. The physical condition of the participant was assigned an ordinal scale (5, very good; 4, good; 3, normal; 2, a little bad; 1, bad). Subjective stress was evaluated using visual analog scales (VAS: 0–10) from very relaxed to highly stressed. Achievement emotion was assigned an ordinal scale (5, completely; 4, better; 3, a little better; 2, a little worse; 1, much worse). Sleeping hours were also recorded.

2.3. Measurement of sAA

To assess the physiological stress response, sAA was measured using a colorimetric system (Nipro Co., Osaka, Japan; Yamaguchi et al., 2004). Briefly, a substrate 2-chloro-4-nitrophenyl-4-O-β-D-galactopyranosylmaltoside is hydrolyzed by salivary amylase in the presence of maltose, a competitive inhibitor. This reaction turns a color of a reagent strip from yellow to white, which change is quantified using a salivary amylase monitor. One unit activity (U) per mass of enzyme is defined as the production of 1 μmol of the reduction sugar, maltose, in 1 min (NC-IUBNB, 1992).

Saliva was collected twice a day, in the morning after waking up and in the evening after practice, for 10 days during the practice. Prior to sampling, participants washed their mouths with water. After saliva was collected for 30 s using a sampling tip, each participant measured own sAA immediately every morning and evening for 10 days (including unassigned days, (i.e., a weekend), which measurement was excluded in the analyses).

To establish a no-stress and no-medication baseline of sAA, the participants measured sAA every morning and evening for 10 days during routine daily life at the university. The measurement was carried out before the pharmacy practice.

2.4. Statistical analysis

All results are expressed as mean ± SEM. The influence of stress on sAA was evaluated by two-way ANOVA and the Bonferroni test for differences between means. Correlation coefficients were obtained using a statistical analysis program (StatPlus, AnalystSoft Inc., online version). The comparison of correlation coefficients between placebo- and theanine-groups was carried out using Fisher's z-test. In each analysis, a p value < 0.05 was considered to be statistically significant.

3. Results

3.1. Changes of sAA

There was no significant difference in sAA levels between in the morning and in the evening during routine daily life at the university

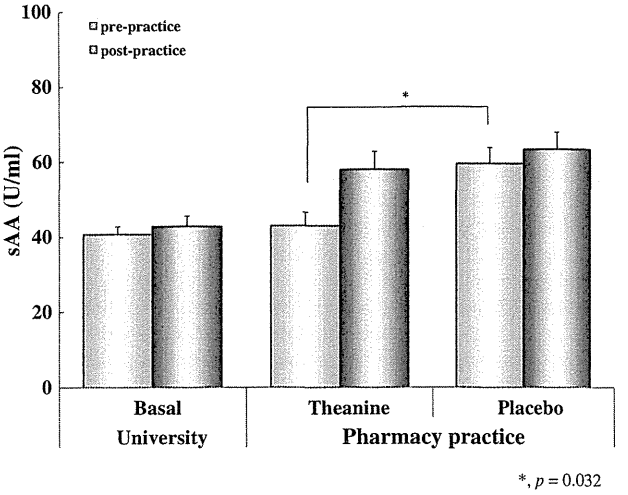

Fig. 1. Salivary α-amylase activity (sAA) of the participants during pharmacy practice was measured in the morning after waking up (pre-practice, gray bar) and in the evening after practice (post-practice, black bar). Theanine or placebo (lactose) tablets (200 mg, twice a day, after breakfast and lunch) were taken from 1 week prior to the pharmacy practice and continued for 10 days in the practice period. To assess a basal level, the participants measured sAA during daily life at the university with no-medication. The levels of sAA in unassigned days were not included in the analysis. Data are expressed as mean + SEM (*, $p = 0.032$).

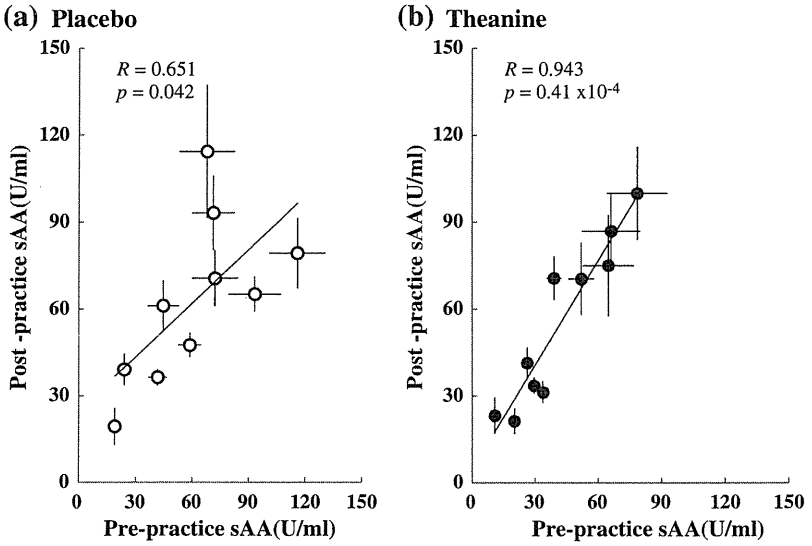

Fig. 2. Correlation between pre- and post-practice sAAs. (a), Placebo-group; (b), theanine-group. Each point of sAA represents the mean value of each participant that was calculated from sAA during pharmacy practice. Data are expressed as mean ± SEM.

(Fig. 1). During the practice, the level of pre-practice sAA (i.e., in the morning) (40 U/ml) was considered to be a baseline of sAA in the participants. The pre-practice sAA was significantly higher in the placebo-group than in the theanine-group ($p = 0.032$; two-way ANOVA) (Fig. 1). The theanine-group maintained the baseline observed during routine activity at the university. There was no significant difference in post-practice sAA (i.e., in the evening), however, the placebo-group showed a tendency of higher levels compared to the theanine-group ($p = 0.491$). In the theanine-group, the post-practice sAA tended to be higher than the pre-practice sAA ($p = 0.056$).

Considering individual variability, the mean values of pre- and post-practice sAA of each participant were analyzed. Participants of higher pre-practice sAA exhibited higher post-practice sAA (placebo, $R = 0.651$ and $p = 0.042$; theanine, $R = 0.943$ and $p = 0.41 \times 10^{-4}$; Fig. 2). The correlation between pre- and post-practice sAAs tended to be higher in the theanine-group than in the placebo-group ($p = 0.069$, Fisher-z test).

3.2. STAI value

The average STAI values were examined to assess anxiety based on the appraisal standard and there was no difference between two groups (Table 1). However, a positive correlation was observed between the values of STAI and pre-practice sAA in the placebo-group ($R = 0.742$, $p = 0.014$; Fig. 3a). Participants with high STAI value exhibited the high level of pre-practice sAA. In the theanine-group, a positive correlation between STAI and pre-practice sAA was similarly observed ($R = 0.560$, $p = 0.092$; Fig. 3b). The correlation coefficients between STAI value and post-practice sAA were low in both the placebo-group ($R = 0.505$, $p = 0.137$) and the theanine-group (R = 0.580, $p = 0.079$) (Fig. 3c and d).

Table 1
Characteristics of the placebo-group and the theanine-group.

	Placebo	Theanine
Age	22.5 ± 0.2	22.2 ± 0.1
Men/women	7/3	7/3
STAI value	41.0 ± 2.9	41.9 ± 2.4
Physical condition (score: 1–5)	3.65 ± 0.19	3.69 ± 0.23
Subjective stress (VAS: 0–10)	4.07 ± 0.33	3.10 ± 0.25*
Achievement emotion (score: 1–5)	3.26 ± 0.07	3.19 ± 0.06
Sleeping time (h)	6.20 ± 0.19	6.22 ± 0.25

* $p = 0.020$.

3.3. Subjective stress

Psychosocial stress was evaluated by each participant at the end of daily practice using VAS (0–10). The average score was significantly lower in the theanine-group than in the placebo-group ($p = 0.020$; one-way ANOVA; Table 1), which trait is notable from the first day of pharmacy practice (Fig. 4).

A positive correlation between subjective stress and post-practice sAA was observed both in the theanine-group ($R = 0.771$, $p = 0.0090$; Fig. 5b) and in the placebo-group ($R = 0.582$, $p = 0.077$: Fig. 5a). The correlation coefficients were not significantly different ($p > 0.05$, Fisher's z-test). Next, the participants were divided into three grades based on the subjective stress score (low: 0–3.4, middle: 3.5–6.4, and high: 6.5–10). The participants with low subjective stress exhibited significantly lower post-practice sAA in the theanine-group than in the placebo-group ($p = 0.0023$; one-way ANOVA; Table 2). No theanine-group participants showed high subjective stress. A close relationship between subjective stress and STAI was observed in theanine-group ($R = 0.866$ and $p = 0.0012$; Fig. 5d) but not in placebo-group (Fig. 5c).

3.4. Achievement emotion

Achievement emotion was evaluated by participants as an ordinal scale at the end of daily practice (Table 1). It was negatively correlated with post-practice sAA both in the placebo-group ($R = -0.440$, $p = 0.204$) and in the theanine-group ($R = -0.315$, $p = 0.376$) but not with STAI (Fig. 6).

3.5. Influence of physical condition and sleeping time

As pre-practice sAA was significantly lower in the theanine-group than in the placebo (Fig. 1), effects of physical condition and sleeping time were examined. No serious disturbance was reported in either theanine- or placebo-groups. The average values of physical condition and sleeping time were not different between the two groups (Table 1). No direct correlation between sAA and sleeping time was observed in each group (Fig. 7). However, when the sleeping time was compared between participants with low and high pre-practice sAA (with the cutoff

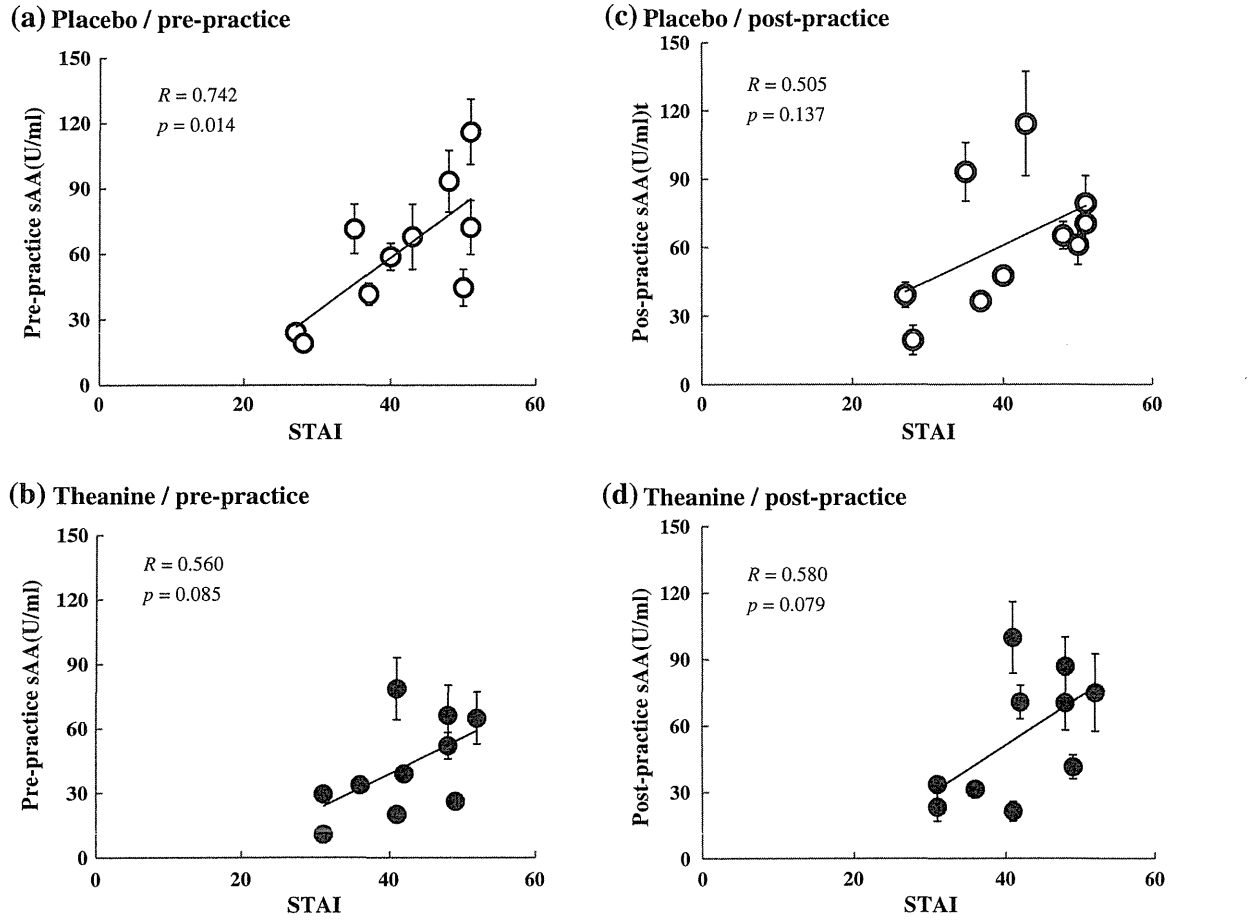

Fig. 3. Correlation between STAI and sAA. (a), STAI and pre-practice sAA of the placebo-group; (b), STAI and pre-practice sAA of the theanine-group; (c), STAI and post-practice sAA of the placebo-group; and (d), STAI and post-practice sAA of the theanine-group. Data are expressed as mean ± SEM.

value of 50 U/ml), it was significantly shorter in the participants with high pre-practice sAA ($p = 0.41 \times 10^{-3}$; one-way ANOVA). STAI value was also significantly higher in the participants with high pre-practice sAA ($p = 0.015$; one-way ANOVA; Table 3).

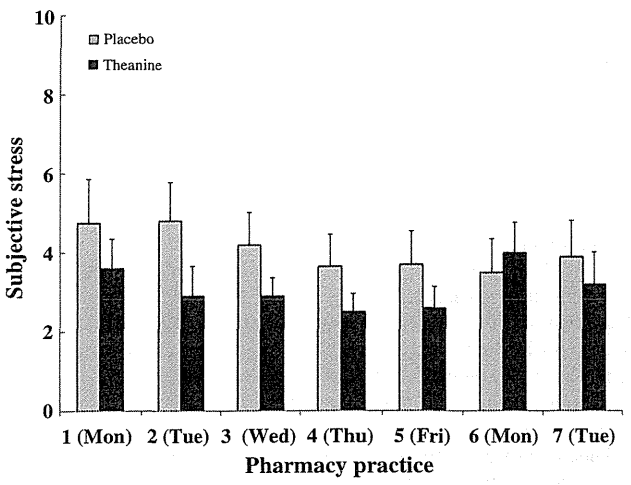

Fig. 4. Daily score of subjective stress for 7 days in placebo- (gray bar) and theanine-group (black bar). Data are expressed as mean ± SEM.

4. Discussion

4.1. Pharmacy practice during initial weeks is a stressful condition for students

It has been reported that sAA levels, a biomarker of ANS excitation, were low at the time of waking up (Adam et al., 2011) and the levels increased during the course of the day (Nater et al., 2007; Wingenfeld et al., 2010; Out et al., 2013). However, the circadian rhythm of sAA remains relatively constant throughout the day under a stress-free environment (Yamaguchi et al., 2006). Thus, in this study, the daily life at university was considered to be a stress-free state for the participants, which reflects the baseline of pre-practice sAA.

On the other hand, diurnal profile of sAA secretion has been reported to be altered by chronic stress or stress-related diseases. For example, levels of sAA after awakening increased rapidly in patients with posttraumatic stress disorder compared to healthy controls (Thoma et al., 2012). Diurnal sAA was associated with chronic stress and mood in healthy volunteers (Nater et al., 2007). In our study, increased levels of sAA in the morning and evening were observed in the participants of placebo-group during pharmacy practice. It suggests that pharmacy practice outside the university was a stressful condition for the participants, at least during the initial several weeks. While all the participants reported that they felt less stressed toward the end of the 11 week-practice (data not shown), theanine intake should be beneficial for reducing psychological burden during the initial weeks under stressful condition.

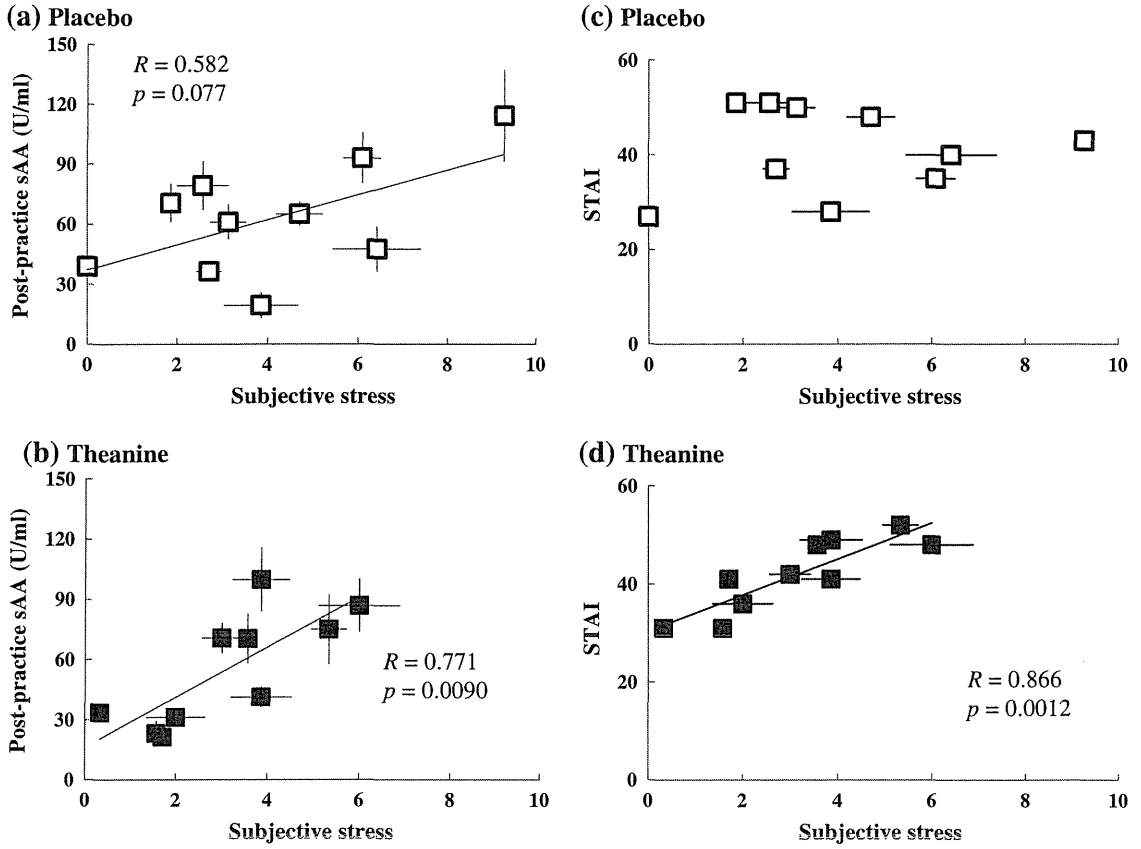

Fig. 5. Correlation between subjective stress and post-practice sAA (placebo, a; theanine, b), and between subjective stress and STAI (placebo, c; theanine, d). Data are expressed as mean ± SEM.

4.2. Interaction among sAA, trait anxiety and subjective stress

Although the levels of sAA considerably varied among the participants under the stressful condition, the pre-practice sAA was correlated with the STAI value, i.e., trait anxiety of each participant. The participants with low pre-practice sAA (<50 U/ml) exhibited significantly lower value of STAI than the participants of high pre-practice sAA (>50 U/ml) in both groups. These results indicate that trait anxiety of each participant essentially affects on the level of sAA. In addition, theanine intake had a role in suppressing the pre-practice sAA to the level of a non-stressful baseline under stressful condition. Although the post-practice sAA was increased, it was not statistically significant in the theanine-group. Moreover, the subjective stress was significantly lower in the theanine-group than in the placebo-group. The daily score of subjective stress was already low in the theanine-group from the first day of pharmacy practice, suggesting that the prior intake of theanine was effective for suppression of subjective stress.

In the participants with lower subjective stress (<3.4), post-practice sAA was significantly lower in the theanine-group than in the placebo-group. Some participants regarded the pharmacy practice as a relatively easy task, and reported low subjective stress. However, their nervous system could have been more excited than the self-reported stress level in the placebo-group. Thus, it is possible that they underestimated their true stress level. It has been reported that sAA was already high prior to the examination in dental school students with anticipation, while subjective distress was low (Robles et al., 2011). These results imply that the level of sAA is closely correlated with subjective stress, while excitation of the nervous system leads to higher sAA than the level of subjective stress.

As negative emotions such as anxiety, hopelessness and shame affect performance negatively (Pekrun et al., 2009), a participant with negative emotion could have evaluated his/her performance negatively. However, achievement emotion tended to correlate negatively to post-practice sAA but not to STAI.

Table 2
Relation between the level of subjective stress and post-practice sAA.

Group		Subjective stress		
		0–3.4	3.5–6.4	6.5–10
Placebo	n	5	4	1
	Post-practice sAA (U/ml)	57.3 ± 4.6	55.5 ± 6.4	114.3 ± 23.0
Theanine	n	5	5	0
	Post-practice sAA (U/ml)	37.7 ± 4.0*	68.1 ± 6.6	

* $p = 0.0023$.

4.3. Role of theanine in stress response

Orally consumed theanine is easily absorbed from the intestinal tract and partially transported into the brain competitively via the L-system at the blood–brain barrier (Yokogoshi et al., 1998; Terashima et al., 1999). The level of theanine intragastrically administered reached to the highest in the brain of rats after 5 h, and completely disappeared within 24 h (Terashima et al., 1999). In the theanine-group, the level of theanine in the brain might have been high from noon to evening and gradually decreased toward the next morning.

Theanine incorporation into the brain is reported to reduce the release of glutamate from presynapse to the synaptic cleft by strongly acting as a glutamine transporter (Kakuda et al., 2008; Kakuda, 2011). It then inhibits the incorporation of extracellular glutamine into neurons,

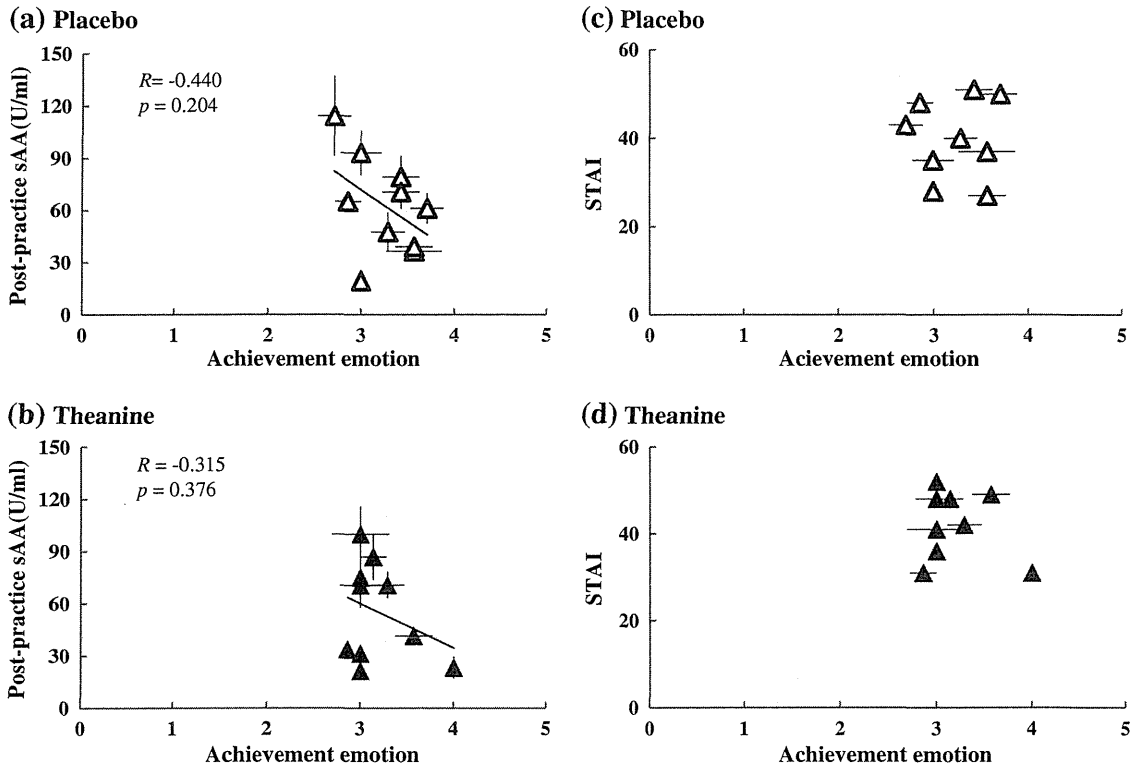

Fig. 6. Correlation between the achievement emotion and post-practice sAA (placebo, a; theanine, b), and between the achievement emotion and STAI value (placebo, c; theanine, d). Data are expressed as mean ± SEM.

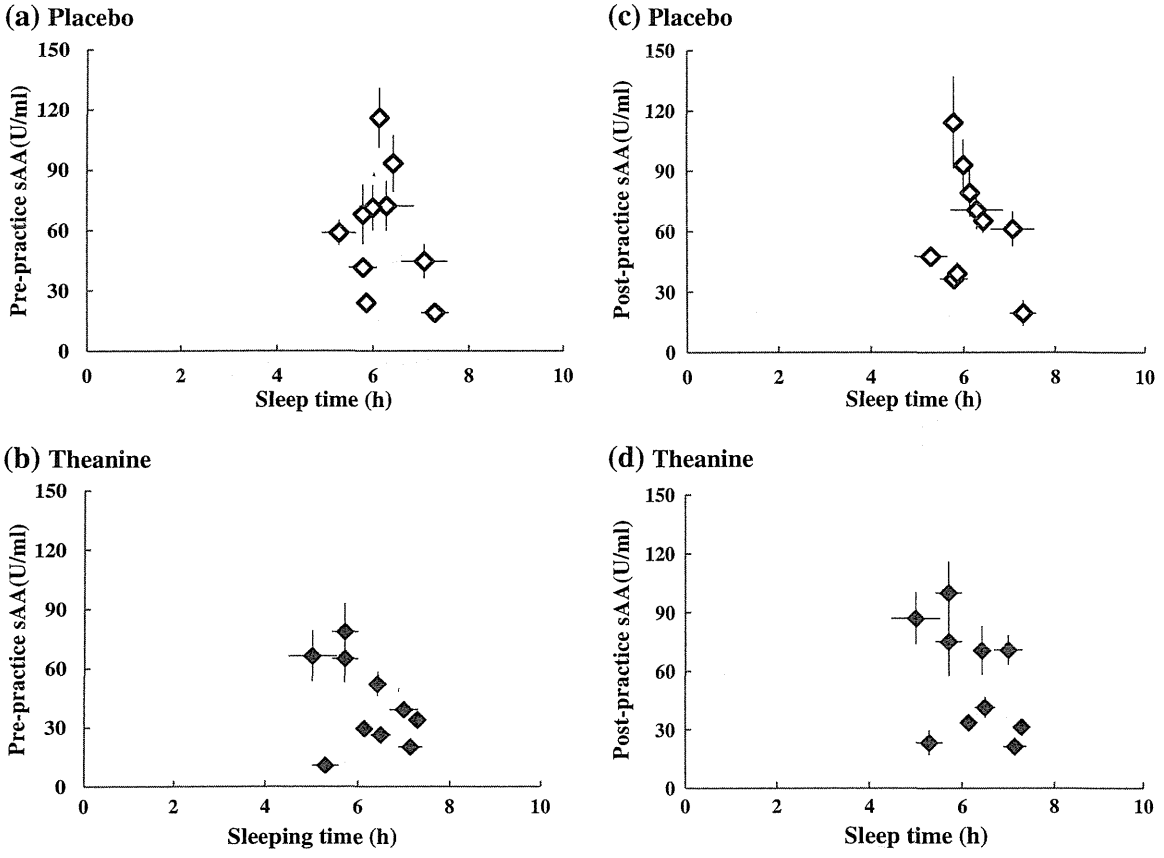

Fig. 7. Correlation between sleeping time and pre-practice sAA in the placebo-group (a and c) and in the theanine-group (b and d). Data are expressed as mean ± SEM.

Table 3
Relation between the level of pre-practice sAA and sleeping time.

Pre-practice sAA	Low		High	
	<50 U/ml		>50 U/ml	
Number of participants	Placebo	Theanine	Placebo	Theanine
	4	6	6	4
Sleeping time (h)	6.54 ± 0.10		5.94 ± 0.13* ($* p = 0.41 \times 10^{-3}$)	
STAI	37.2 ± 2.6		45.7 ± 1.79* ($* p = 0.015$)	

which suppresses the conversion of glutamine to glutamate, a potent excitatory amino acid, by glutaminase. We have shown that theanine intake completely suppressed HPA-axis alteration and behavioral depression in mice (Unno et al., 2013). These results suggest that theanine has an anti-stress effect through the suppression of adverse alteration of the HPA axis under stressful condition. Therefore, theanine intake may suppress the ANS and HPA axis excitability by reducing glutamate release, and lead to the low subjective stress.

Theanine intake has been reported to be effective in improving sleep quality (Lyon et al., 2011). The sleeping time of participants with high sAA (>50 U/ml) was significantly shorter than participants with low sAA (<50 U/ml) in both groups (Table 3). It has been reported that in children with short sleeping time, sAA of the baseline and peak levels were higher after a standardized psychosocial stress test than in those with average sleep duration (Räikkönen et al., 2010). Taken together, it implies that a short sleeping period does not allow the excited nervous system to recover, not only in children but also in adults. While some individuals who slept for a short period of time exhibited low sAA in this study, short sleep may have overactivated the ANS. Theanine incorporated into the brain may be effective for resetting stress response to basal level during sleep. In a modern stressful life, excitation of the ANS and HPA axis is rather anticipated as a daily stress response. Therefore, it should be beneficial when one can suppress excessive excitation and reset it to a basal level by the next morning. It is of interest to examine effects of sleeping time and quality on excitation of the nervous system.

5. Conclusions

Anti-stress effect of theanine on humans was evaluated in the students during pharmacy practice. The levels of sAA were measured as a marker for sympathetic nervous system activity. Pre-practice sAA was significantly lower in the theanine-group than in the placebo-group. The level of pre-practice sAA was predominantly affected by trait anxiety. Post-practice sAA was positively correlated to subjective stress. Theanine ingestion significantly decreased subjective stress. Sufficient sleep would also be helpful for suppressing excessive excitation. Taken together, sAA is a useful biomarker for evaluating physical and psychological conditions and theanine intake has a significant anti-stress effect on humans.

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