Study on the infection-preventive characteristics of Japanese green tea

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Abstract

We investigated the infection-preventive characteristics of Japanese green tea(middle grade) in reference to its catechin(CAT) concentration. Colorimetric analysis showed that CAT was extracted most effectively at 80℃. When green tea extract(GTE) was prepared from the same green tea leaves (5g/80ml water) repeatedly six times at 80℃ for 1 min, almost the 2/3 of total CAT content was extracted during the first three extractions in which the second extraction gave the highest CAT concentration . Therefore, GTE prepared under this condition (5g/80ml water at 80℃ for 1 min) was referred to as standard GTE(SGTE) in this study. Bacteriological studies using *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* showed that the maximum inhibitory and bactericidal dilutions of the second SGET were ranged in 8- and 4-fold dilutions, corresponding to 0.33 mg/ml and 0.68 mg/ml CAT concentrations, respectively, against either bacterial species and the differences between these two values were within 2-fold dilutions, indicating that SGTE shows the bactericidal activity. However, SGTE required as long as 4 to 5 h for bactericidal time in sharp contrast to iodine with that of as short as 1 min. When SGTE with CAT concentration ranging 1.2 mg/ml to 2.7 mg/ml was incubated with influenza A/ Aichi virus at 37℃ for at least 30 min, SGTE also completely inhibited viral hemagglutination(HA) activity essential for the viral attachment to cell surface. The CAT-deficient SGTE, prepared by the treatment with $FeCl_3$, could not show both bactericidal and HA inhibitory activities, confirming that CAT in GTE is mainly responsible for these activities. In summary, GTE might be taken as a beneficial herbal medicine to promote the infection-safety conditions in the patients. However, on the application of GTE, its advantage/disadvantage and presence of sufficient CAT concentration should be tightly recognized.

Key words

Japanese green tea, catechin, bactericidal activity, influenza virus, delayed-type, infection control nursing.

Introduction

The green tea-drinking habit was established initially in China 3,000 to 4,000 yeas ago, and then introduced to many countries in the world. With the lapse of time, green tea extract (GTE) was gradually recognized as one of healthy drinks[1]. In the 19th century, chemical analyses of GTE identified several components such as caffeine, vitamins, amino acids and catechin molecules (CAT). Especially, CAT took the most attention, because Japanese GTE (moderate grade) included five different CAT, in which epigallocatechin (EpiC) is the main, at content of as much as 14% to 16%[1]. Recently, scientific evidences have been accumulated on the beneficial activities of GTE against various bacteria, fungi, toxins and influenza viruses[2-7].

Nowadays, various types of chemical disinfectants have become available and the use of these disinfectants seems to be the principle in the infection control nursing. Indeed, these disinfectants show powerful bactericidal and virocidal activities. However, it should be noteworthy that these disinfectants show adverse effects such as cytotoxic effect and hypersensitivity on the improper use and in the specific patients[8]. In contrast, GTE is routinely taken as one of favorite drinks for a long time and thereby free from these inevitable issues holding in these chemicals. In the light of these facts, GTE could be expected to contribute for promotion of the infection-safety condition in the patients, although its application might be limited in specific cares as one of traditional and complementary infection controls.

In order to expand the effective and adequate use of GTE in the infection control nursing, we investigated the infection-preventive characteristics of GTE in this study.

Materials and Methods

Preparation of GTE and CAT solution

Commercial Japanese green tea leaves (GTL) (moderate grade) in 80 ml water were extracted with occasionally stirring at various temperature (50℃ to 91℃) for 1 min followed by centrifugation (3,000 rpm for 10 min). The resultant supernatant was used as the first GTE. The remaining GTL were extracted repeatedly further five times by the same manner and referred to as the second to sixth GTE, respectively. It was shown that CAT reacts with metal ions such as $FeCl_3$, resulting in the formation of black precipitates easily removed by centrifugation[9]. Therefore, to prepare CAT-deficient GTE, GTE was treated with $FeCl_3$ at a final concentration of 10 mM followed by the centrifugation (10,000 rpm for 10 min). Pure (+)-catechin and EpiC were purchased
from Sigma. These GTE and pure CAT were sterilized by the filtration and stored at -80℃ until use.

Quantitative analysis of CAT in GTE

CAT concentration in GTE was quantified by the colorimetric analysis based on the ferrous tartrate method[1,10]. In brief, 5 ml of GTE at an appropriate dilution were reacted with 20 ml of ferrous tartrate reagents and then development of blue color was measured at optical density of 540 nm. The (+)-catechin was used for the standardization of CAT concentration.

Antibacterial assay

Staphylococcus (*S.*) *aureus* (ATCC25923), *Escherichia* (*E.*) *coli* (ATCC3630) and *Pseudomonas* (*Ps.*) *aeruginosa* (ATCC27853) were cultured in the nutrient broth at 37℃

overnight and then diluted with broth to yield 10^4 to 10^5 colony forming units (CFU)/ml as bacterial solutions used in the following assay. To examine the maximum inhibitory dilutions (MID) of GTE, 100 μl of two-fold dilution series of GTE were prepared with broth in a 96-well microplate and then cultured with the equal volume of bacterial solutions at 37℃ overnight. On the next day, turbidity of cultures was observed to determine MID which is the maximum dilution giving no turbidity. At the same time, small portions of cultures without turbidity were further inoculated into GTE-free broth, cultured and observed their turbidities as above. Finally, the maximum dilution of GTE giving no turbidity was taken as the maximum bactericidal dilutions (MBD).

Because the dilutions were used to express antibacterial activity, MID and MBD are corresponding to the minimum inhibitory and bactericidal concentrations, respectively, in the ordinary sensitivity test.

To examine the time-related bactericidal effect of GTE, the mixtures of GTE with bacterial solutions were incubated at 25℃ (0 h). At the indicated times after incubation, small portions of the mixtures were inoculated into the agar media to determine the remaining living bacteria expressed as CFU. In certain experiments, iodine solution (0.00063%) was used instead of GTE.

Assay of HA activity of influenza virus

The stock solution of influenza A/Aichi/2/68 (Hong Kong subtype) was diluted with phosphate buffered saline (PBS) and then incubated with an equal volume of GTE at 37℃. At the indicated times after incubation, two-fold dilution series of the mixtures were prepared with saline in a 96-well microplate followed by addition of an equal volume of chicken red cell solution (0.5%)[11]. As a control, PBS was used instead of GTE. After standing at 4℃ for 40 min, HA patterns were observed to determine HA titer expressed as the highest dilution with HA.

Results

CAT concentrations in GTE under different preparative conditions

When GTE was prepared from 5 g GTL in 80 ml water at various temperatures, CAT concentrations were 0.392 mg/ml and 0.875 mg/ml at 50℃ and 65℃, respectively, as shown in Fig.1. However, these relatively lower concentrations became abruptly higher with the increases of temperatures, attaining its peak at 80℃ (2.625 mg/ml), and then slightly decreased at 91℃ (2.458 mg/ml). Based on these findings, GTE was prepared at 80℃ throughout the following experiments.

As shown in Fig. 2, when GTE was prepared repeatedly six times from the same GTL as above, CAT concentrations were 2.1 mg/ml, 2.7 mg/ml, 1.65 mg/ml, 1.20 mg/ml, 0.9 mg/ml and 0.75 mg/ml for the first to sixth extractions, respectively, indicating that the second extraction gives the highest concentration and then these values gradually

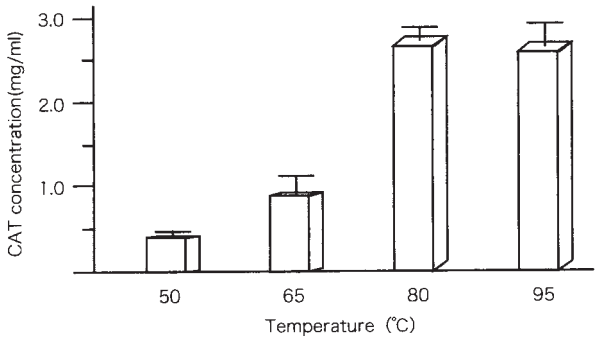

Fig.1. CAT concentration in GTE prepared by different temperature. GTE was prepared from 5g GTL in 80 ml water for 1 min at vatious temperatue and CAT concentration was quantified by the colorimetric method. Data are expressed as the mean±SD (thin bar) of three independent experiments.

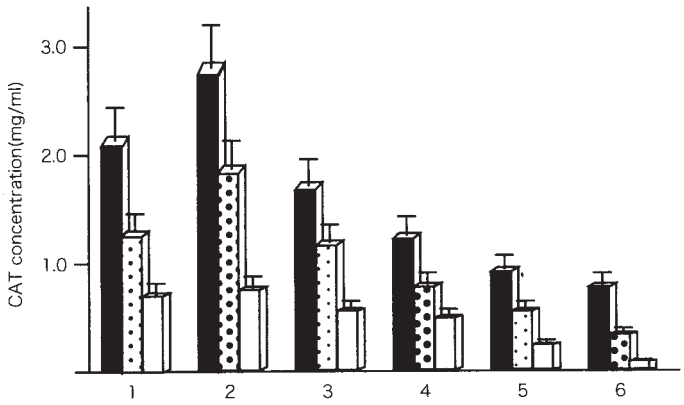

Fig. 2. CAT concentration in each GTE prepared by the repeated extraction from the same GTL. A total of 6 GTE preparations was obtained by the repeated extractions from 5g (solid bar), 3g (dotted bar) or 1g (open bar) of GTL in 80ml water for 1 min at 80℃. The same GTL was used throughout the extraction. CAT concentratons in each GTE were expressed as the mean ±SD (thin bar) of three independent expeiments.

decrease with the extraction numbers. This tendency, showing the highest CAT concentrations at the second extraction, was also observed in the cases of lower amounts of GTL (3 g to 1 g). In this connection, CAT concentrations of the second GTE were 1.18 mg/ml and 0.725 mg/ml for GTL of 3 g and 1 g, respectively. Taking together these findings, GTE prepared from 5 g GTL in 80 ml water at 80℃ for 1 min was referred to as standard GTE (SGTE) in this study.

Antibacterial activity of SGTE

Initially, MID and MBD of SGTE were examined against three bacterial species. As shown in Table 1, MID values of the second SGTE were ×8, ×4 and ×4 dilutions against *S. aureus, E. coli and Ps. aeruginosa,* respectively. On the other hand, MBD values of this SGTE were ×4, ×2 and ×4 dilutions against the corresponding bacteria species, respectively. Because the undiluted second SGTE containing 2.7 mg/ml of CAT, CAT concentrations were calculated as 0.338 mg/ml, 0.675 mg/ml and 1.350 mg/ml at the dilutions of ×8, ×4 and ×2, respectively. These MID and MBD values of the first SGTE were the same as those of the third ones against three bacterial species. With the repetitious extractions, these values became lower. In either SGTE, MID and MBD values were the same or closely resemble within two-fold against each bacterial species. These date indicate that SGTE exhibits its antibacterial activity by the bactericidal , but not by bacteriostatic, manner. Comparing with MIDs among the bacterial species, these values were also closely resemble, suggesting that antibacterial spectrum of GTE is broad. Conversely, CAT-deficient SGTE showed no antibacterial activity against either bacterial species, indicating that CAT in GTE is mainly responsible for this activity. Based on these findings, time-related bactericidal activity of SGTE and EpiC was examined comparing with that of iodine solution. As shown in Fig. 3A, the numbers of living cells were gradually increased with the lapse of time in the each control group of three bacterial species. By

Table 1. MID and MBD values of SGTE with or without CAT by the extraction times

Extraction times	CAT concentration in SGTE(×1) (mg/ml)	MID and MBD					
		S. aureus		*E. coli*		*Ps. aeruginosa*	
		MID	MBD	MID	MBD	MID	MBD
1	2.10	×4	×2	×1	×1	×2	×1
2	2.70	×8	×4	×4	×2	×4	×4
2*	<0.01	>×1	>×1	>×1	>×1	>×1	>×1
3	1.65	×4	×2	×1	×1	×2	×1
4	1.20	×4	×2	×1	×1	×1	×1
5	0.90	×2	×1	×1	×1	×1	×1
6	0.75	×2	×1	×1	×1	×1	×1

*CAT-deficient by the treatment with $FeCl_3$.

Table 2. Inhibitory effect of EpiC on the HA activity of influenza A/Aichi virus (Hong Kong subtype)

Concentration of EpiC (mg/ml)	Contact time (min)at 37℃	HA titer
10	60	< 2
5	60	< 2
2.5	60	< 2
1.25	60	< 2
0.62	60	32
0.31	60	64
5	10	64
5	20	32
5	30	< 2
5	45	< 2
Control	60	64

Table.3 Inhibitory effect of SGTE with or without CAT on the HA activity of influenza A/Aichi virus(Hong Kong subtype)

Extracion number	CAT concentration (mg/ml)	HA titer
1	2.10	< 2
2	2.80	< 2
2*	< 0.01	64
3	1.65	< 2
4	1.20	2
5	0.90	4
6	0.75	4
Control		64

*CAT-deficient SGTE by the treatment with $FeCl_3$.

contrast, in both SGTE- or EpiC-treated bacteria, these numbers decreased sharply during the first three hours after treatment, and finally became to the undetectable levels at 4 h or 5 h. On the other hand, each bacterial species was killed to the undetectable levels by iodine within as short as 1 min (Fig. 3B).

Inhibition of HA activity of influenza virus by EpiC and SGTE

Influenza viruses were treated with saline or various concentrations of EpiC for the indicated times at 37℃ and then the remaining HA tiers were examined. As shown in Table 2, HA titer of the control viruses treated with saline was 64. On the other hand, HA activity could not detected by the 1 h-treatment with more than 1.25 mg/ml of EpiC. The same treatment with 0.62 mg/ml EpiC induced the reduction in HA titer to 32 which is the half of the control, but the HA inhibitory activity was negligible at less than 0.31 mg/ml of EpiC. Time-related analysis of the inhibitory effect of EpiC (5 mg/ml) (the lower column in Table 2) showed that the 30 min-treatment is enough for the complete inhibition of HA activity. Therefore, the treatment time was fixed as 30 min in the following experiments using SGTE. As shown in Table 3, the first to fourth SGTEs could induce the complete reduction in HA titers, but the remaining SGTEs could not as well as CAT-deficient SGTE. These data indicate that SGTE exhibit inhibitory effect on the influenza viral HA activity by the 30 min-contact at 37℃, if SGTE contains CAT at concentrations of al least 1 mg/ml.

Discussion

It has been demonstrated that GTE and CAT exhibit bactericidal activity with a broad spectrum against various species including gram positive- and negative-bacteria and fungus[2-5], and also limit the growth of influenza virus through the inhibition of HA activity essential for the attachment to cells and uncoating process of influenza virus[6,7]. With the accumulation of these findings, beneficial roles of GTE have attracted attention in the health care facilities in Japan to promote the infection-safety condition in the patients. However, GTE should be supplied with the recognition of the infection-preventive characteristics, and thereby thoughtless and imprudent supply should be avoid. In this study, several findings useful on the application of GTE in the healthcare facilities were obtained through the examination using moderate grade GTL which is the most popular in Japan.

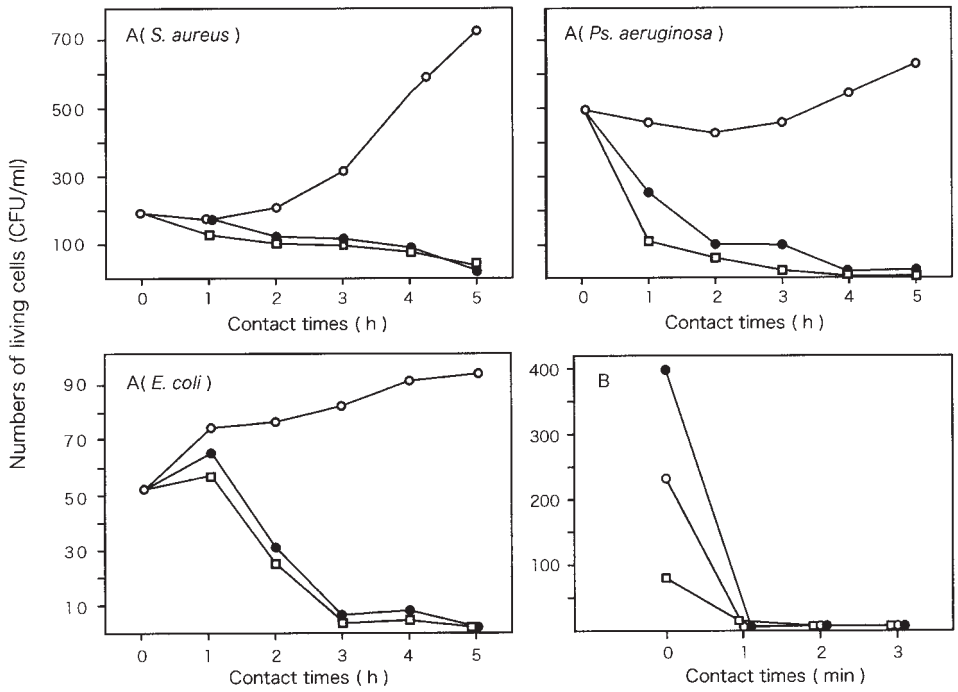

Fig. 3. Time-related bactericidal effect of EpiC, SGTE and iodine against three bacterial species.
The fresh cultures of bacteria at appropriate dilutions were incubated with 10 gm/ml Epic (open square), SGTE (2nd extraction) (closed circle) or without drugs (open circle) and then the numbers of living cells (CFU/ml) were counted at the indicaed times (A). Alternatively, *S. aureus* (open square), *E. coIi* (open circle) and *Ps. aeruginosa* (closed circle) were incubated with iodine solution and then the numbers of living cells were counted as above (B).

Colorimetric assay showed that CAT is extracted in a temperature-dependent manner with the peak at 80℃. Therefore, GTE prepared from GTL in 80 ml for 1 mi at 80℃ is referred to as SGTE. When the CAT-extractive efficacies were compared among the repetitiously extracted SGTE, the second extraction gave the highest CAT concentration regardless of the GTL amounts (1 g to 5 g). Based on CAT content as to 13.8% to 15.7% in dried GTL[1], almost two third of total CAT are estimated to be extracted during the first three extractions. This finding might be important for the GTE supply in the health care facilities, because that the repetitious preparation of GTE from the same GTL is popular in Japan.

The numbers of bacterial species used in this study were only three, but GTE showed a broad spectrum with bactericidal activity in accordance with previous papers[2-5]. However, time-related bactericidal assay showed that the complete reduction in the living cell numbers is observed only after the contact for as long as 4 to 5 h, in a sharp contrast to iodine with the complete reduction time of 1 min. Thus, the bactericidal manner of GTE could be said as the delayed type, whereas that of iodine is immediate-type. This finding seems to be also important for the health care personnel on the GTE supply. To overcome this disadvantage, it might be recommended to apply GTE repeatedly several times a day in cares. Indeed, successful GTE applications have been obtained empirically only by the repeated oral spraying, wiping and/or brushing with GTE (twice to five times a day) [12,13].

The first step of influenza virus growth is the attachment to cells which is detected as HA activity using red cells[11]. Therefore, inhibition of HA activity leads directly to that of

influenza virus growth. This study confirmed that GTE and EpiC could inhibit HA activity of influenza A/Aichi virus (Hong Kong subtype) as shown in the previous papers[6,7]. In addition to A/Hong Kong subtype, HA activities of all influenza virus types relating to the current influenza epidemic (A/USSR subtype and B type) are also inhibite by GTE (date not shown). However, in comparison with the previous papers[6,7], this study clarified in more details the HA inhibitory characteristics of GTE and EpiC, that is, the contact time and CAT concentration necessary for complete inhibition of HA activity are al least 30 min and 1.2 mg/ml, respectively.

Although GTE contains various biochemical components such as amino acids, vitamins[1], only CAT is taken as the major component responsible for the bacteria-killing and anti-influenza viral activities. When compared these activities between CAT-deficient and -containing CTE, the former GTE lost completely these activities. So far as we know, this study is the first report to rule out clearly the possible contribution of various components other than CAT in GTE on these activities. Conversely, at least 1 mg/ml of CAT should be contained in GTE both for HA inhibitory and bacterial killing effects.

In summary, GTE is a beneficial herbal medicine to promote the infection-safety conditions in the patients. However, on the application of GTE,its advantage/disadvantage (adverse effect-free but delayed effect) and the presence of a sufficient CAT concentration should be tightly recognized.

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感染予防からみた緑茶の特性に関する研究

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要　旨

緑茶（煎茶）の抗菌性とインフルエンザウイルス赤血球凝集（HA）活性阻害の特性を検証した．酒石酸鉄比色法による検討から，低温域（50℃と65℃）より高温域（80℃と91℃）でより多くカテキン（CAT）は抽出され，就中80℃が最大CAT量を示した．5g茶葉/80ml水，80℃，1分（標準緑茶液:SGTE）で6回抽出したところ，2回目で最高CAT量（2.7mg/ml）を示し，3回目までにほぼその2/3が抽出された．黄色ブドウ球菌，大腸菌と緑膿菌に対するSGTEの最大発育阻止と最大殺菌希釈度の差は，どの菌に対しても2倍以下であり，緑茶は，広範囲性かつ殺菌的作用を示すことが確認された．但し，ヨード液の1分に比べ，殺菌時間は4～5時間と極めて長いことが注目された．種々CAT量を有するSGTEを，種々時間37℃でインフルエンザウイルスと接触させたところ，CAT量1.2mg/ml～2.7mg/mlの範囲で，30分接触で完全にHA活性を阻害した．さらに，塩化第二鉄処理によるCAT除去SGTEには，抗菌作用も抗HA作用もないことから，種々ある緑茶成分のうち，CATが主な有効成分であることが示された．これらから，緑茶を感染予防として活用する際には，遅効性であること，また十分量なCAT含有（少なくとも1mg/ml）ことの認識は重要であると思われた．

キーワード

緑茶（煎茶），カテキン，殺菌作用，インフルエンザウイルス，遅効性，感染看護