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Review

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Increase in the hydroxyl radical-scavenging activity of *Panax ginseng* and ginsenosides by heat-processing

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Summary ***Panax ginseng*** **C.A. Meyer (Araliaceae), mainly cultivated in Korea and Northeast China, is processed before use based on its long history of ethnopharmacological evidence. Ginsenosides have been regarded as the main active components responsible for the pharmacological activities of ginseng. Although the Maillard reaction is known as a major source of compounds related to enhanced antioxidant activity by heat treatment in various crude drugs or foods, the chemical and free radical-scavenging activity changes of ginsenosides brought about by the Maillard reaction have not yet been elucidated. This paper gives a review of our recent findings, with emphasis on the hydroxyl radical (•OH)-scavenging activity changes of ginsengs and ginsenosides by heat-processing using an electron spin resonance spectrometer. 20(*S*)-Rg_3 showed the strongest activity, and the next was in the decreasing order of Rb_1, Rg_1, Rc, Rb_2, and Rd. The •OH-scavenging activities of ginsenosides were related to the ferrous metal ion-chelating activities of their aglycone, 20(*S*)-protopanaxadiol. In addition, the ferrous metal ion-chelating activities of ginsenosides were thought to be influenced by their types of hydrophilic sugar moieties. Moreover, Rb_1 was changed into 20(*S*)-Rg_3, 20(*R*)-Rg_3, Rk_1, and Rg_5 by heat-processing, and the sugar moieties at carbon-20 were separated. The generated amount of 20(*S*)-Rg_3 was higher than when Rb_1 was heat-processed without amino acids, and a significant increase in Maillard reaction products was noted. Based upon chemical and •OH-scavenging activity tests using Maillard reaction model experiments, the scientific evidence underlying the increase in free radical-scavenging activity of ginseng induced by heat-processing was elucidated.**

Keywords: *Panax ginseng*, heat-processing, Maillard reaction, ginsenoside, hydroxyl radical

1. Introduction

Ginseng (*Panax ginseng* C.A. Meyer, Araliaceae) is a medicinal herb that is mainly cultivated in Korea and Northeast China. Considered a valued medicine, it has been used in the Orient for more than 2,000 years. Ginseng and its components have been reported to exhibit a wide range of pharmacological and physiological actions, such as antiaging, antidiabetic, anticarcinogenic, analgesic, antipyretic, antistress, antifatigue, and tranquilizing properties, as well as the stimulation of DNA, RNA, and protein synthesis (*1-9*). These medicinal properties of ginseng have been suggested to be linked, although not totally, to ginseng's action to protect against free radical attack (*10-14*).

Traditionally, the root of ginseng has been processed to make white ginseng (roots air-dried after peeling) and red ginseng (roots steamed at 98-100°C without peeling) to enhance its preservation and efficacy. Red ginseng is more common as an herbal medicine than white ginseng in Asian countries, because steaming induces changes in the chemical constituents and enhances the biological activities of ginseng (*15-17*). A novel heat-processing method of steaming ginseng at a higher temperature than red ginseng was developed to achieve an even stronger efficacy than that of

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red ginseng, and this ginseng product was termed heat-processed ginseng (*18-20*) (Figure 1). Heat-processed ginseng has been reported to exhibit more potent pharmacological effects, such as antioxidant, vasorelaxation, anxiolytic-like, and antitumor activities, than those of conventional white or red ginseng by us and others (*19,21-24*).

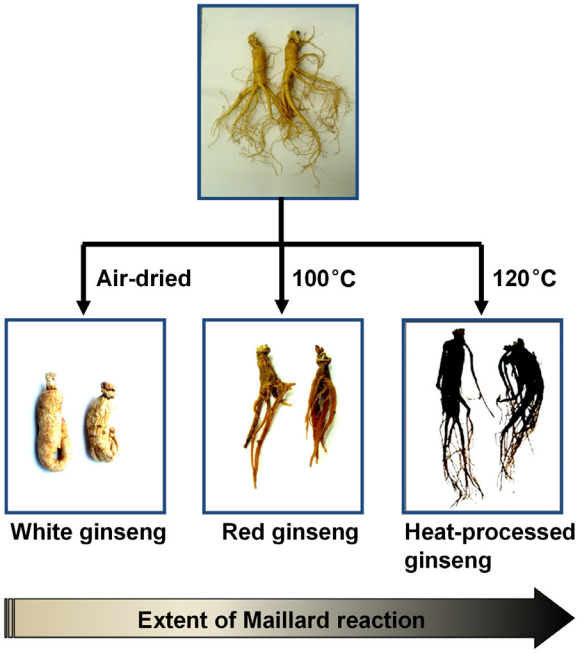

Figure 1. Classification of *Panax ginseng* by heat-processing methods.

The Maillard reaction of amino acids with sugar is a nonenzymatic browning reaction that takes place during the processing, cooking, and storage of foods. It is well-known that Maillard reaction products (MRPs) produced in both heat-treated food systems and in sugar-amino acid model systems exhibit antioxidant activity (*25-27*). The Maillard reaction occurs in the processing of red ginseng (*28*). MRPs in ginseng were reported to increase by heat-processing; these compounds are arginyl-fructosyl-glucose, arginyl-fructose, maltol, maltol-3-*O*-β-D-glucoside, *etc*. (*28,29*). To date, it is not clear what Maillard reaction compounds contribute to the antioxidant activity of MRPs, or how this activity develops over time (*27*). It is possible, therefore, that the formation of heat-processing-induced antioxidants is correlated with the extent of the Maillard reaction in ginseng, and this was experimentally studied by us.

2. Hydroxyl radical (•OH)-scavenging activities of ginseng

Figure 2 shows comparisons of the •OH-scavenging activities and browning levels of white ginseng, red ginseng, and heat-processed ginseng (*30*). White ginseng inhibited •OH production to about 45%, and it was further inhibited to about 40 and 34% by the addition of red ginseng and heat-processed ginseng, respectively, at a concentration of 0.5%. However, none of these effects were stronger than that of thiourea, the •OH-scavenging positive control. In addition, the absorbance value at 420 nm of white ginseng was 0.090 (A.U., arbitrary unit), and

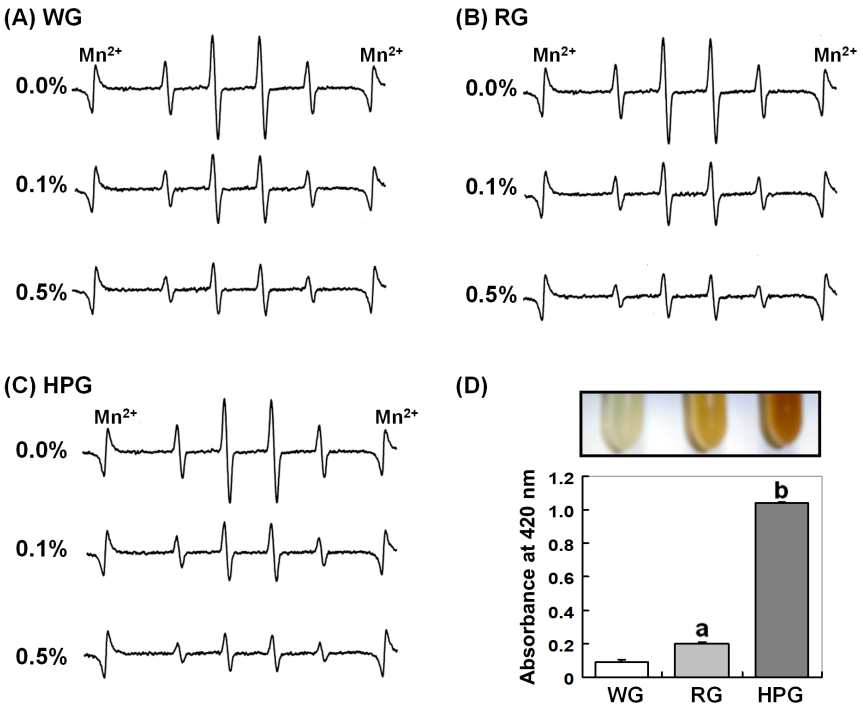

Figure 2. The •OH-scavenging activities of (A) white ginseng (WG), (B) red ginseng (RG), and (C) heat-processed ginseng (HPG). The changes in browning compound levels of *Panax ginseng* brought about by heat-processing **(D)**. $^{a}p < 0.05$, $^{b}p < 0.001$ *vs*. WG. (*30*)

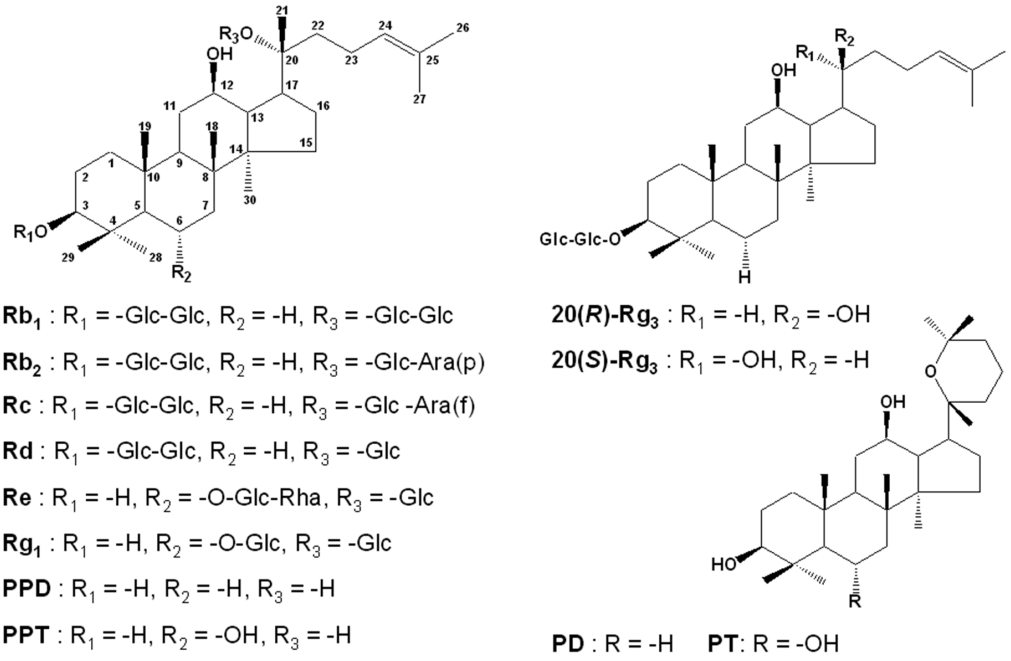

Figure 3. Structures of ginsenosides. -Glc: D-glucopyranosyl, -Rha: L-rhamnopyranosyl, -Ara(p): L-arabinopyranosyl, -Ara(f): L-arabinofuranosyl.

it was increased to 0.198 and 1.043 A.U. in red ginseng and heat-processed ginseng, respectively. Consequently, the •OH-scavenging activities of ginseng extracts were increased by heat-processing in a processing-temperature-dependent manner, but the browning level was more significantly increased by steaming. Therefore, the •OH-scavenging activities of ginsengs were not consistent with the levels of browning, and the effect of MRPs was thought to be minor.

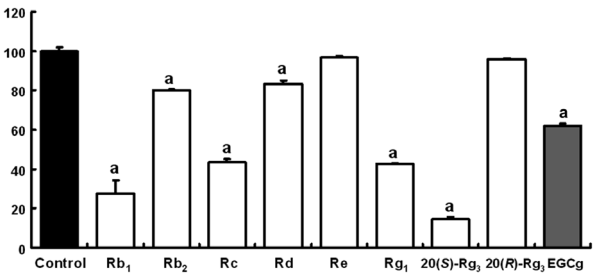

Figure 4. Comparison of the •OH-scavenging activities of ginsenosides at 2 mM when dissolved with distilled water. [a]$p < 0.001$ *vs*. control value. (*34*)

3. The structure and •OH-scavenging activity relationships of ginsenosides

Generally, ginseng root includes organic (80-90%) and inorganic (10-20%) substances. Organic substances contain a number of bio-active constituents, such as saponins (3-6%), carbohydrates (60-70%), nitrogenous substances (9-15%), fat-soluble components (2%), vitamins (0.5%), *etc*. (*31*). Ginsenosides have been regarded as the main active components responsible for the pharmacological activities of ginseng (*17,18,32*). Ginsenosides are glycosides of 30-carbon derivatives of the triterpenoid dammarane, as shown in Figure 3. They have a hydrophobic four-ring steroid-like structure with hydrophilic sugar moieties. About 30 different types of ginsenoside have been isolated and identified from the root of *Panax* species. Each also has at least two (carbon-3 and -20) or three (carbon-3, -6, and -20) hydroxyl groups (-OH), which are free or bound to monomeric, dimeric, or trimeric sugars (*17,33*). Therefore, we investigated the •OH-scavenging and ferrous metal ion-chelating activities of several ginsenosides using electron spin resonance (ESR) for the identification of active ginsenosides and their structure and activity relationships.

When the 8 ginsenosides were determined, 20(*S*)-Rg_3 showed the strongest •OH-scavenging activity, and the next were in the decreasing order of Rb_1, Rg_1, and Rc. These ginsenosides (2 mM) showed more than a 50% inhibitory activity against •OH generation than that of the control. The other ginsenosides such as Rb_2 and Rd showed a comparably lower activity, and Re and 20(*R*)-Rg_3 showed no significant inhibition. (–)-Epigallocatechin 3-*O*-gallate (EGCg) (2 mM), the •OH-scavenging positive control, inhibited •OH generation to about 62% (Figure 4) (*34*). From the comparisons of activities in diol-type ginsenosides, the additional -Glc and -Ara(f) connected to Glc at carbon-20 were thought to increase the •OH-scavenging activities of ginsenosides, as shown by the strong activities of Rb_1 and Rc. However, a low inhibitory activity was observed in diol-type ginsenosides containing additional -Ara(p) and no additional sugar moiety connected to

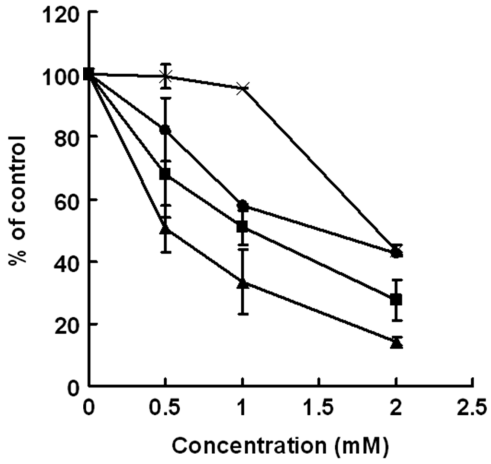

Figure 5. Comparison of the •OH-scavenging activities of Rb_1, Rc, Rg_1, and 20(*S*)-Rg_3. ■: Rb_1, ×: Rc, ●: Rg_1, ▲: 20(*S*)-Rg_3. (*34*)

Glc at carbon-20, as shown by Rb_2 and Rd, respectively (Figures 3 and 4). On the other hand, the effect of -Glc-Glc at carbon-3 was not certain because all diol-type ginsenosides contain this group. In the case of triol-type ginsenosides, the -Glc at carbon-6 was thought to increase the •OH-scavenging activity of ginsenoside, as shown by the strong activity of Rg_1, but an additional -Rha at the carbon-6 position had adverse effects, as shown by the nearly zero activity of Re (Figures 3 and 4).

On the other hand, 20(*S*)- and 20(*R*)-Rg_3 are epimers which increase during steaming by the deglycosylation of diol-type ginsenosides, but their •OH-scavenging activities show marked differences. 20(*S*)-Rg_3 showed the strongest activity compared to the other ginsenosides in this study (*34*), and many reports have provided supporting evidence that their antioxidant activity is closely related to the geometrical arrangement of the OH group, especially, at carbon-20. The alkene chain connected to carbon-20 in 20(*S*)-Rg_3 has a stable, fixed orientation and is packed tightly near the terpenoid, while that in 20(*R*)-Rg_3 protrudes further outside and has a flexible structure (*35*). This compact structure of 20(*S*)-Rg_3 is thought to influence the accessibility of water to the OH group of carbon-12 and -20. Therefore, 20(*S*)-Rg_3 is known to be more soluble in water than 20(*R*)-Rg_3. In addition, it was reported that the OH group of 20(*S*)-Rg_3 is better aligned with the OH acceptor group in the ion channels than that of 20(*R*)-Rg_3, and that it was important for Na+ channel regulation (*36*). Moreover, 20(*S*)-Rg_3 has been reported to provide neuroprotection against cerebral ischemia-induced injury in the rat brain through reducing lipid peroxides and scavenging free radicals (*37*). Therefore, it was thought that the strong •OH-scavenging activity of 20(*S*)-Rg_3 in ESR is closely related to the geometrical arrangement of the OH group, especially at cabon-12 and -20.

Figure 5 shows a comparison of the •OH-scavenging activities of ginsenoside Rb_1, Rc, Rg_1, and 20(*S*)-Rg_3,

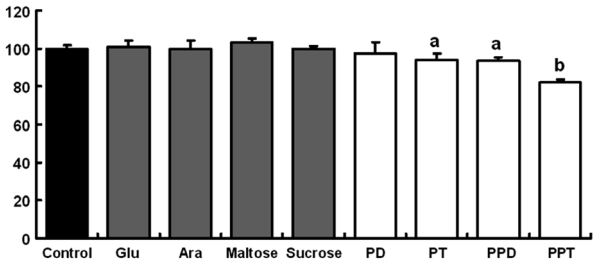

Figure 6. Comparison of the •OH-scavenging activities of sugar moieties and sapogenins at 2 mM when dissolved with distilled water. $^{a}p < 0.01$, $^{b}p < 0.001$ *vs*. control value. (*34*)

in concentrations ranging from 0.5 to 2 mM (*34*). The IC_{50} values of 20(*S*)-Rg_3, Rb_1, Rg_1, and Rc were 0.51, 1.04, 1.51, and 1.87 mM, respectively. EGCg, the •OH-scavenging positive control, showed an IC_{50} value of 3.76 mM (data not shown). From these results, the sugar moieties are thought to have pivotal roles in the •OH-scavenging activity of ginsenosides. The difference in the structures of ginsenosides is only due to the position and type of sugar moieties connected to the ring of the triterpenoid dammarane, and this mutual interaction was suggested to play an important role in the antioxidant effects of ginsenosides.

In the case of sugars and sapogenins, sugars showed no •OH-scavenging activities, and the activities of sapogenins were lower than predicted (Figure 6) (*34*). Although the activity of sapogenins was weak, there was evidence that the number of -OH groups is related to the •OH-scavenging activity of sapogenins. Panaxadiol (PD) and panaxatriol (PT), which have cycled side chains at carbon-20, showed no or weak •OH-scavenging activity, but it was improved in 20(*S*)-protopanaxadiol (PPD) and 20(*S*)-protopanaxatriol (PPT) containing one more -OH group at carbon-20. Moreover, the activity of PT and PPT was slightly higher than in PD and PPD, respectively (Figures 3 and 6). Therefore, it was interpreted that the aglycone of ginsenoside has some •OH-scavenging activity because of its -OH group at carbon-6 and -20. However, the •OH-scavenging mechanism can not be explained with only sapogenins because of their low activity. Consequently, the mechanism was considered to involve an associated function of sapogenins with their sugar moieties.

4. The chemical changes and Maillard reaction of Rb_1 brought about by heat-processing

The root of ginseng has been heat-processed to improve its medicinal efficacies in Korea based on the long history of ethnopharmacological evidence (*18,31,38*). Although an increasing body of evidence supports MRPs being involved in the increased activity by heat treatment in various crude drugs or foods (*39*), the effect of the Maillard reaction on the active components of ginseng and biological activities have not yet been fully elucidated. Ginsenosides have been regarded

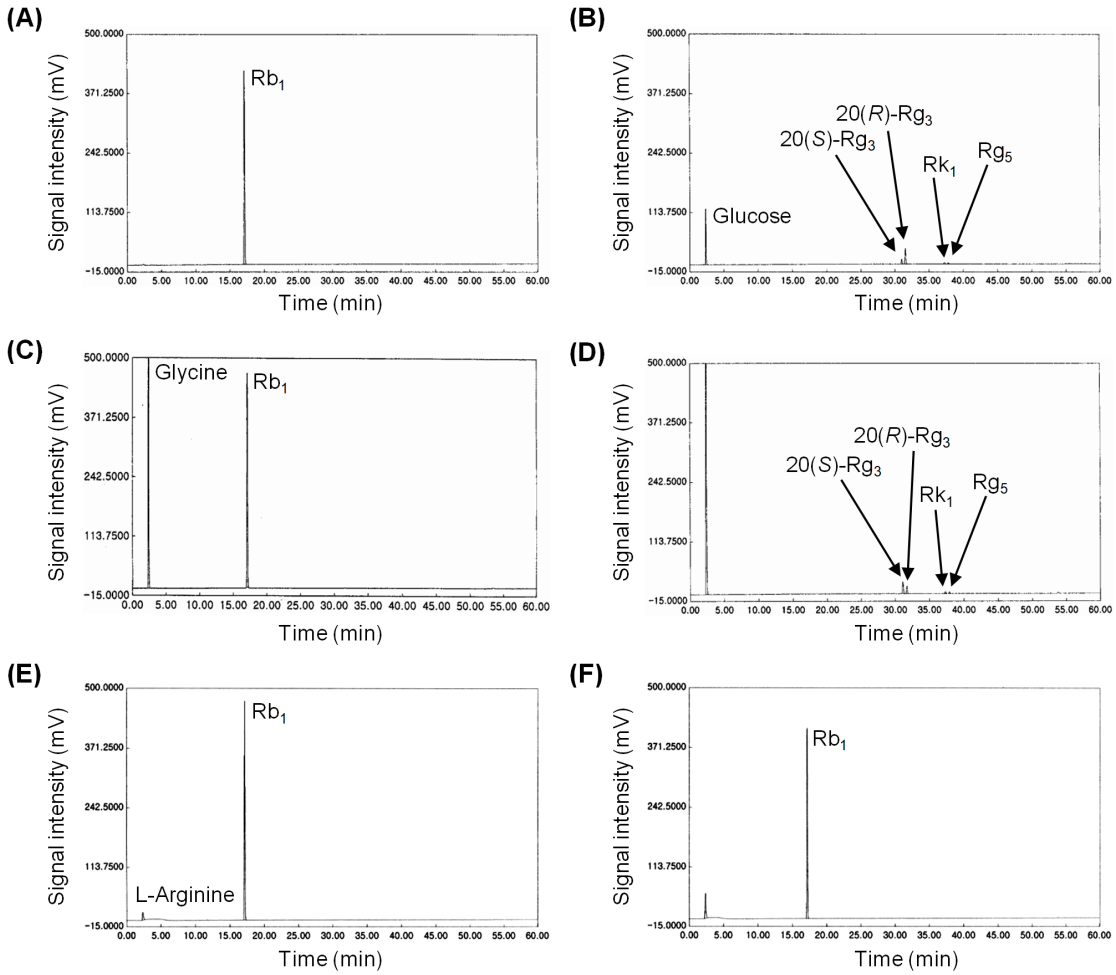

Figure 7. HPLC chromatograms of (A) Rb_1, (B) heat-processed Rb_1, (C) Rb_1-glycine mixture, (D) heat-processed Rb_1-glycine mixture, (E) Rb_1-arginine mixture, and (F) heat-processed Rb_1-arginine mixture. (*42*)

as the main active components responsible for the pharmacological activities of ginseng, and are well-known to be deglycosylated by heat-processing (*18*). The sugar moieties of ginsenosides can be a source of MRPs with amino acids contained in ginseng during heat-processing (*30*), and research on the Maillard reaction of ginsenosides is thought to be beneficial to understand the complex structural changes of ginsenosides brought about during the heat-processing of ginseng.

As one of the major ginsenosides contained in ginseng, Rb_1 is a diol-type triterpene glycoside, and the heat-processing-induced deglycosylation of two glucose molecules at carbon-20 of Rb_1 has been well-documented. Therefore, Rb_1 was used as a target ginsenoside to study a Maillard reaction model experiment in this study. To ascertain the generation of MRPs from ginsenosides and amino acids, we have analyzed Maillard reaction model experiments using Rb_1 and glycine or L-arginine. The sugar moieties of ginsenoside can be a source of MRPs with amino acids contained in ginseng during heat-processing. To identify the effects of amino acids on the heat stability or structural changes of Rb_1, Rb_1 was heat-processed with or without the same amount of glycine or L-arginine, because glycine is a frequently used amino acid in Maillard reaction model experiments (*40*) and L-arginine is the most abundant amino acid contained in *Panax ginseng* (*41*).

As shown in the HPLC chromatograms (Figures 7A and 7B), Rb_1 (1,000 μg) was changed into 20(*S*)-Rg_3 (146 μg), 20(*R*)-Rg_3 (201 μg), Rk_1 (102 μg), and Rg_5 (110 μg) by heat-processing, and the sugar moieties at carbon-20 of Rb_1 were deglycosylated. The separated sugar moiety was determined as glucose based on GC-MS analysis. Then, we added the same amount of glycine to Rb_1 to identify the effect of the Maillard reaction during heat-processing. Rb_1 (1,000 μg) was changed into 20(*S*)-Rg_3 (196 μg), 20(*R*)-Rg_3 (167 μg), Rk_1 (102 μg), and Rg_5 (108 μg) when heat-processed with glycine (Figures 7C and 7D), and the brown color level of heat-processed Rb_1-glycine mixture was significantly higher than that of Rb_1 or heat-processed Rb_1 (Figure 8) (*42*). The Maillard reaction is dependent on several factors such as the pH, time, temperature, concentration of reactants, and reactant type. The development of color is known as an important and clear feature of the Maillard reaction, and brown-

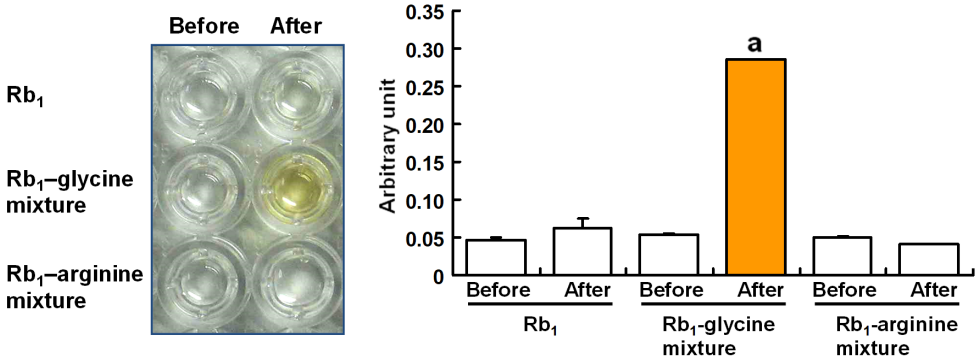

Figure 8. Comparison of MRP levels of samples. [a]$p < 0.001$ *vs*. Rb_1. (*42*)

colored nitrogenous polymers, called melanoidins, are known to be formed by this reaction (*43,44*). When changes in the contents of ginsenosides between heat-processed Rb_1 and heat-processed Rb_1-glycine mixture were compared, the generated amounts of 20(*S*)-Rg_3 and 20(*R*)-Rg_3 were inverse in these samples (Figures 7B and 7D). Therefore, the addition of glycine to Rb_1 for heat-processing was suggested to increase the generation of 20(*S*)-Rg_3, which has a strong •OH-scavenging activity, due to the Maillard reaction. However, the pH values of Rb_1 and Rb_1-glycine mixture were about 4.51 and 5.28, respectively. The pH value of the same amount of ginseng extract was about 5.35. The exact mechanism to explain the effect of glycine on the epimerization of ginsenoside is not certain at present, but the involvement of the Maillard reaction is certain. The addition of glycine to Rb_1 in heat-processing was thought to increase the generation of 20(*S*)-ginsenoside by the Maillard reaction.

At the same time, when Rb_1 was steamed with the same amount of L-arginine, about 0.5% of Rb_1 was lost during heat-processing, but the heat stability of Rb_1 was significantly improved (Figures 7E and 7F) compared to when Rb_1 was heat-processed with or without the same amount of glycine (*42*). In addition, there was no increase in the brown color by heat-processing of the Rb_1-arginine mixture (Figure 8) (*42*), and the pH value of the Rb_1-arginine mixture was about 10.37. High temperature and pH are known to promote the Maillard reaction, and L-arginine is the most abundant amino acid in *Panax ginseng* to generate MRPs such as arginyl-fructose and arginyl-fructosyl-glucose (*41,45,46*). However, the Maillard reaction did not occur when Rb_1 was steamed with L-arginine, and we paid attention to the structural characteristics of L-arginine. The substitution of L-arginine in protein is known to lead to significant heat stability enhancement in the presence of sugar substrates, most probably by interfering with nonenzymatic glycation (*47*). In addition, the guanidyl groups of L-arginine generally form long-range hydrogen bonds or electrostatic interactions with negatively charged groups, and this increased hydrogen bonding is one of the factors enhancing protein thermostability (*48,49*). Therefore, the improved heat stability of Rb_1 brought about by the addition of L-arginine was also thought to be closely related to its characteristics of interfering with nonenzymatic glycation and forming hydrogen bonds with Rb_1. However, we still have unanswered questions and need to conduct more precisely controlled examinations using other amino acids or using similar pH conditions with ginseng to elucidate the detailed mechanism behind the increase or decrease in the heat stability of Rb_1.

5. Conclusion

The recent introductions of various analytical methods with high sensitivity and specificity have been enriching our knowledge of ginseng, helping to identify new chemical entities from various ginseng species, and improving our understanding of this millennium herbal medicine (*17*). Based upon the chemical and •OH-scavenging activity tests using Maillard reaction model experiments, scientific evidence to explain the increase in the free radical-scavenging activity of ginseng induced by heat-processing was obtained. The •OH-scavenging active components such as 20(*S*)-Rg_3, Rg_5, and MRPs in *Panax ginseng* were significantly increased on heat-processing. The critical roles of the Maillard reaction were confirmed and supported by the following lines of observations: firstly, the generated amount of 20(*S*)-Rg_3 from Rb_1 was increased when heat-processed with glycine. Secondly, the generation of MRPs was positively correlated with the •OH-scavenging activity. Finally, certain amino acids such as L-arginine blocked the structural change of ginsenoside, leading to them having a stronger •OH-scavenging activity. Therefore, it is clear that the Maillard reaction is involved in the chemical and antioxidant activity changes of ginsenoside.

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Original Article

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Inhibitory effects of alpha-cyclodextrin and its derivative against sucrose-induced hyperglycemia in an *in vivo* evaluation system

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Summary **Cyclodextrins (CyDs) are cyclic oligosaccharides consisting of six to eight glucose residues. Administration of α-CyD (six glucose residues) inhibits sucrose-induced hyperglycemia in humans. Here we show that oral administration of α-CyD and dimethyl α-CyD suppresses sucrose-induced hyperglycemia in an *in vivo* evaluation system using silkworms. On the other hand, β-CyD (seven glucose residues), γ-CyD (eight glucose residues), and their derivatives did not show the suppressive effect. These findings suggest that dimethyl α-CyD is a new inhibitor against sucrose-induced hyperglycemia and the silkworm system is useful for evaluation of suppressive activities of α-CyD derivatives against postprandial hyperglycemia.**

Keywords: Cyclodextrin, hyperglycemic activity, silkworm, sucrose-induced hyperglycemia

1. Introduction

Elevated blood glucose levels due to excessive intake of sucrose, a major sweetener that is added to a variety of foods, lead to the development and worsening of lifestyle-related diseases, such as obesity and diabetes (*1*). Establishment of strategies for suppressing increases in blood glucose levels caused by excess sucrose intake is expected to prevent lifestyle-related diseases (*2*). Blood glucose levels after intake of sucrose are regulated by various different kinds of steps (*3*). Therefore, evaluation of active substances that suppress increases in blood glucose levels caused by sucrose intake requires experiments using whole animals.

Silkworm is an invertebrate animal and has several advantages as an experimental animal compared to mammals, including lower breeding costs and fewer ethical problems with regard to animal welfare (*4-8*). We have established *in vivo* experimental systems using silkworm for evaluating therapeutic activities of anti-diabetic drugs (*8-10*). A hyperglycemic silkworm model is useful for evaluating therapeutic activity of human insulin (*8,9,11*). We also developed a diabetic silkworm model for evaluation of anti-diabetic agents such as pioglitazone and metformin (*8,10*). Hemolymph glucose levels of silkworms are increased by intake of sucrose, the increase is suppressed by administration of α-glucosidase inhibitors that are used for treatment of diabetes in clinical (*12*). This means that *in vivo* evaluation systems using silkworms are useful to search substances for blood glucose control of humans.

Cyclodextrins (CyDs) are cyclic oligosaccharides consisting of six to eight glucose residues linked with α-1,4-glycosidic bonds. α-cyclodextrin (α-CyD) is composed of six glucose residues, β-cyclodextrin (β-CyD) of seven glucose residues, and γ-cyclodextrin (γ-CyD) of eight glucose units. CyD derivatives are clinically used for improving drug solubility and drug delivery (*13*). Moreover, α-, β-, and γ-CyDs have a capacity to inhibit α-amylase or glucoamylase, which degrade starch to maltose (*14*). Suppressive effect of α-CyD against increase in blood glucose level caused by intake of white rice has been demonstrated in human clinical trials (*15*). Administration of α-CyD also inhibits sucrose-induced hyperglycemia in humans (*16*). However, there is no report regarding comparative study of CyDs and its derivatives on the elevated blood glucose level after ingestion of sucrose.

In this study, we compared the suppressive effects of CyDs and its derivatives against sucrose-induced

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hyperglycemia in silkworms. α-CyD and dimethyl α-CyD have an activity to suppress sucrose-induced hyperglycemia in silkworms. Our findings suggest that silkworm system is useful to search for α-CyD derivatives that inhibit postprandial hyperglycemia by intake of sucrose.

2. Materials and Methods

2.1. *Silkworm rearing conditions*

Silkworms were reared according to the previously reported method (*12*). The eggs of the silkworm (Hu Yo × Tukuba Ne) were purchased from Ehime sericulture incorporated company (Ehime, Japan). Larvae hatched from the eggs were fed an artificial diet (Silkmate 2S, Nihon Nosan Corporation, Tokyo, Japan) and reared to the fifth-instar stage at 25-27 °C. A 10% (w/w)-sucrose or glucose diet was prepared by mixing Silkmate 2S and sucrose. A chemical-containing diet was prepared by mixing with 10%-sucrose diet.

2.2. *Determination of glucose level in silkworm hemolymph*

Glucose levels in the hemolymph were determined by the method described previously (*12*). Hemolymph was collected from the silkworms through a cut on the first proleg. Glucose levels in the hemolymph were determined using a glucometer (Accu-Check, Roche).

2.3. *Chemicals*

Cyclodextrins (CyDs) used in this study was listed in Table 1. HP-β-CyD with an average degree of substitution of hydroxypropyl group of 4.4 and HP-β-CyD with an average degree of substitution of hydroxypropyl group of 4.6 were kindly provided by CyDing Co., Ltd. (Kumamoto, Japan). Other CyDs used in this study were purchased from Wako Pure Chemical Industries (Osaka, Japan).

3. Results

3.1. *Evaluation of inhibitory effects of α-, β-, and γ-CyDs against sucrose-induced hyperglycemia in silkworms*

We first tested whether α-, β-, and γ-CyDs and their derivatives have capacities to suppress sucrose-induced hyperglycemia in silkworms. CyDs used in this study was listed in Table 1. We previously reported that acarbose showed suppressive effect against sucrose-induced hyperglycemia in silkworms (*12*). Therefore, acarbose was taken as a positive control of the experiment. The glucose levels in hemolymph of silkworms, which ingested diet containing 10% sucrose and α-CyD, was much lower than that of silkworms, which fed 10% sucrose diet (control) (Figure 1). On the other hand, the glucose levels in hemolymph of silkworms, which ingested diet containing 10% sucrose and β-CyD, γ-CyD, HP-β-CyD, HP-γ-CyD, M-β-CyD, or DM-β-CyD, were indistinguishable from the control (Figure 1). These results suggest that α-CyD has higher inhibitory capacity against sucrose-induced hyperglycemia than β-CyD, γ-CyD, and their derivatives.

Table 1. Cyclodextrins and its derivatives used in this study

Cyclodextrins	Number of glucose	Hydroxyl residues
α-CyD	6	R = OH
β-CyD	7	
γ-CyD	8	
HP-β-CyD	7	$R_{2,3,6} = OCH_2CH(OH)CH_3$
HP-γ-CyD	8	
M-β-CyD	7	$R_{2,3,6} = OCH_3$
DM-α-CyD	6	$R_2 = OCH_3$, $R_6 = OCH_3$
DM-β-CyD	7	

HP: hydroxypropyl. M: Methyl. DM: Dimethyl. R: 2, 3, 6-hydroxyl residues of glucose. $R_{2,3,6}$: either 2, 3, 6-hydroxyl residues of glucose. R_2: 2-hydroxyl residues of glucose. R_6: 6-hydroxyl residues of glucose.

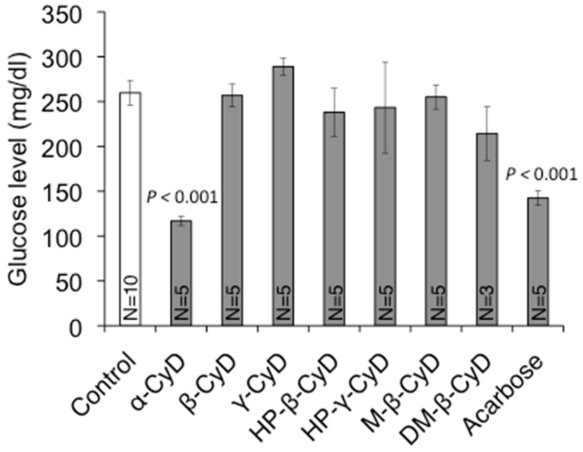

Figure 1. Effects of CyDs and their derivatives on dietary sucrose-induced increases of glucose levels in silkworm hemolymph. Silkworms were fed a diet containing 10% (w/w) sucrose with CyDs, their derivatives (200 mg/g diet), or acarbose (40 mg/g diet) for 1 h. Glucose levels in the silkworm hemolymph were measured (n = 3-10/group). Data represent mean ± SEM. Statistically significant differences between control and testing groups were evaluated using Student's t-test. Control, without chemical; α-CyD, α-cyclodextrin; β-CyD, β-cylrodextrin; γ-CyD, γ-cyclodextrin; HP-β-CyD, hydroxypropyl β-cylrodextrin; HP-γ-CyD, hydroxypropyl γ-cylrodextrin; M-β-CyD, methyl β-cylrodextrin; DM-β-CyD, dimethyl β-cylrodextrin.

3.2. *Inhibitory activity of an α-CyD derivative against sucrose-induced hyperglycemia in silkworms*

We next tested suppressive effects of DM-α-CyD,

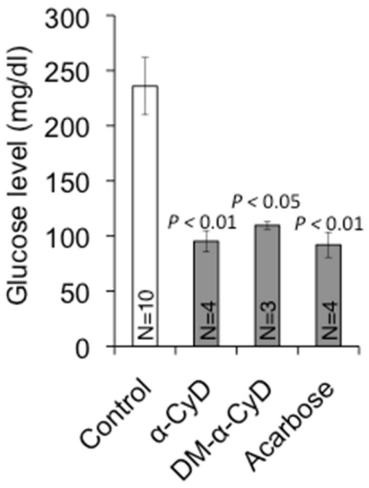

Figure 2. Inhibitory effect of dimethyl α-CyDs on dietary sucrose-induced increases of glucose levels in silkworm hemolymph. Silkworms were fed a diet containing 10% (w/w) sucrose with α-CyD, dimethyl α-CyDs (200 mg/g diet), or acarbose (40 mg/g diet) for 1 h. Glucose levels in the silkworm hemolymph were measured (n = 3-10/group). Data represent mean ± SEM. Statistically significant differences between control and testing groups were evaluated using Student's t-test. α-CyD, α-cyclodextrin; DM-α-CyD, dimethyl α-cyclodextrin.

an α-CyD derivative, against sucrose-induced hyperglycemia in silkworms. The glucose levels in hemolymph of silkworms feeding diet containing 10% sucrose and DM-α-CyD were much lower than that of silkworms feeding diet with a 10% sucrose (control) (Figure 2). The result suggest that DM-α-CyD also has inhibitory activity against sucrose-induced hyperglycemia in silkworms.

4. Discussion

In this study, we demonstrated that α-CyD suppressed sucrose-induced hyperglycemia using a silkworm system. β- and γ-CyDs did not show the suppressive effect in the system. Furthermore, we showed that dimethyl-α-CyD had an activity to suppress sucrose-induced hyperglycemia. CyDs are used for pharmacological applications, such as increase of solubility of drugs, therefore, their safety against humans are established (*13*). We propose here that identification of CyD derivatives by using the silkworm system will give fruitful results of finding new substances that suppress postprandial hyperglycemia in humans.

We also revealed that β- and γ-CyDs did not show suppressive capacity against sucrose-induced hyperglycemia in silkworms. The finding suggests that the structure of cyclic oligosaccharides composed six of glucose linked with α-1, 4-glycosidic bond are important for the suppressive functions. Moreover, dimethyl α-CyD, an α-CyD derivative, also showed the suppressive effect against sucrose-induced hyperglycemia in silkworms. This is the first report that dimethyl α-CyD has a capacity to suppress sucrose-induced hyperglycemia. To find a compound having higher capacities among derivatives of α-CyD is a subject in future.

Mammals such as mice and rats have been conventionally used for the evaluation of substances that exhibited suppressive action against increased blood glucose levels after ingestion of sucrose. Use of a large number of mammals for experiments is raising a problem of not only the cost but also animal welfare point of view. Silkworms have advantages as experimental animals being less expensive and having less ethical problems than mammals. Thus, we propose to use silkworms as a pre-step of using mammals for screening of effective substances for suppression of sucrose-induced hyperglycemia. Taken together, the *in vivo* evaluation system using silkworms may be useful to search α-CyD derivatives that have higher inhibitory activity against sucrose-induced hyperglycemia.

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Tooth whitening efficacy of pigmented rice gels containing carbamide peroxide

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Summary **Carbamide peroxide (CP) is commonly used as a tooth whitening agent. However, efficacy of CP can be enhanced if it is in the suitable delivery system. In the present study, CP loaded in pigmented rice gels were developed and investigated for their physicochemical properties and tooth whitening efficacy. The modified pigmented rice of two varieties, Homnil (HN) and Doisket (DS) were prepared and used as a gelling agent. The outer appearance of the obtained rice gels containing 10% CP (CP-HN and CP-DS, respectively) were transparent and homogeneous texture. The pH of both rice gel bases was neutral but became slightly acidic after incorporating with CP. The adhesive property of HN gel was significantly higher than DS gels. *In vitro* drug release profile exhibited that the release of CP from CP-DS was significantly higher than CP-HN and the commercial gel (CP-CG), respectively. *In vitro* tooth whitening efficacy in 45 normal teeth revealed that the tooth whitening efficacy of the gels was time dependent. At the end of the treatment, CP-HN showed significantly higher tooth whitening efficacy than CP-DS and CP-CG, respectively. It is concluded that the physicochemical properties, particularly the adhesive and dissolution properties, play an important role in the tooth whitening efficacy of the CP gels.**

Keywords: Homnil rice, Doisket rice, colored rice gels, tooth whitening, drug release

1. Introduction

Abnormal tooth's color or tooth discoloration is the stains on the teeth that are yellow, gray, chalky white, or brownish in color. It also refers to spots, blemishes, or lines on tooth surfaces (*1*). Stains on the teeth has many different causes; bacterial, food, aging, smoking, and some drugs such as fluoride, tetracycline, and doxycycline (*2*). Drinking colored beverage like coffee, tea, and red wine can also affect to the tooth color (*3*). All of these reasons cause a reduction in the brilliance of the enamel and dentin. Tooth discoloration affects physical appearance, beauty, and self-confidence (*4*). In adolescence, tooth discoloration can cause an effect on their psychosocial development (*5,6*).

Tooth whitening agents commonly used are hydrogen peroxide and carbamide peroxide (CP) (*7*). CP is a white crystal powder and a potent water-soluble agent. It acts as a strong oxidizing agent through the formation of free radicals, reactive oxygen molecules, and hydrogen peroxide anions. These reactive molecules attack the long-chained, dark-colored chromophore molecules and then split them into smaller, less colored and more diffusible molecules (*7,8*). CP is a popular tooth whitening ingredient because of its effectiveness and quite safe (*9,10*).

The most common formulation of tooth whitening products is gels, due to their preferable in terms of patient compliance, comfortable and easy to applyon the tray that used for tooth whitening (*11*). Gels have

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generally long chain molecular structure composed of many single molecules of monomers bonded to each other which called polymer. Gelling agents can be categorized into two groups: synthetic polymers and natural polymers. Nowadays the interesting in natural polymers produced from natural raw materials is increase because of environmental benefits (*12*). Rice grain is mainly composed of carbohydrate, which is also a natural polymer. The main components of rice carbohydrate are amylose and amylopectin. Amylose is a linear polymer chain and amylopectin is a branch polymer chain (*13,14*). The structure of starch in rice grain can be modified in order to create a preferable gelling agent (*15,16*). There has been reported the application of modified rice as a gelling agent in pharmaceutical fields especially using as the drug carrier gels in the buccal cavity (*17*) due to their biodegradability (*18,19*). Rice can be categorized into two groups based on the color of rice grains, pigmented and non-pigmented groups. The pigmented rice varieties have been received increasing interest currently due to their high bioactive compounds, such as phenolics, tocols, sterol derivatives which have high antioxidant, anti-inflammatory, and other health benefits (*20,21*). Therefore, the pigmented rice was selected to use in the present study. The physicochemical properties, such as pH, adhesive, and drug release properties of the pigmented rice gels obtained from two varieties were compared and the tooth whitening efficacy of the obtained CP loaded rice gels were investigated.

2. Materials and Methods

2.1. *Materials*

Thai pigmented rice grain varieties; Homnil (HN) and Doisket (DS), were obtained from local market in Chiang Mai province, Thailand. CP, silver nitrate, triphenylphosphine (TPP), and monochloroacetic acid were from Sigma chemical Co. (St. Louis, MO, USA). Dichloromethane, methanol, and glacial acetic acid were from RCI Lab-scan Co., Ltd. (Bangkok, Thailand). A commercial gel (CP-CG) containing the same concentration of CP was obtained from Ultradent Product Inc. (Salt Lake City, UT, USA). All other chemicals and solvents were of analytical grade or the highest grade available.

2.2. *Preparation of CP rice gels*

2.2.1. *Preparation of modified rice powder*

Raw pigmented rice powder samples of HN and DS prepared by wet milling method (*22*) were modified using etherification method previously described (*17*) with some modification. Briefly, 50% (w/w) sodium hydroxide aqueous solution was mixed with methanol at a ratio of 1:4 (w/w) in a 100-mL three necked round-bottom flask. The raw rice powder was added and stirred at room temperature until homogenous slurry was obtained. After that proper amount of monochloroacetic acid was added. The temperature of the mixture was controlled at 50 ± 5°C for 3 h with continuous stirring. At the end of the reaction, the mixture was neutralized to pH 7.0. The solid phase was collected and washed by 80% (w/w) methanol until the filtrate testing for chloride by using silver nitrate test was negative. The obtained slurry was dried at 50°C for 48 h. The obtained dried solid was pulverized and passed through the 80-mesh sieve. The modified rice powder after passing the 80-mesh sieve was kept in a desiccator for further use.

2.2.2. *Preparation of rice gel bases and CP rice gels*

Modified HN and DS powder samples were dispersed in DI water and heated to 90°C for 1 h with gentle stirring to obtain homogenous gels without air bubble. CP was gradually incorporated into the prepared gel bases to obtain a final concentration of 10% HN gels containing 10% CP (CP-HN) and DS gels containing 10% CP (CP-DS) were stored at 4°C until further use.

2.3. *Determination of pH*

The pH of the gel bases and the CP rice gels, CP-HN and CP-DS, as well as the commercial gel, CP-CG was determined at room temperature using a pH meter (Compact pH meter pH 22 Laqua twin, Horiba, Kyoto, Japan). The pH meter was standardized using pH 4 and pH 7 buffers before use.

2.4. *Adhesive property study*

The adhesive property of the rice gel bases, CP rice gels, and CP-CG was investigated using the method previously described (*23*) with some modification. The exact amount of 1 g of gels was applied on a 2 cm × 2 cm area of a porcine intestinal mucosal membrane. The membrane was freshly cut and fixed on the internal side of the beaker. The beaker was filled with 800 mL artificial saliva at 37 ± 1°C. The water was constantly stirred at 150 rpm. The time of the gels detachment from the membrane was recorded.

2.5. *In vitro drug release property*

In vitro drug release study was performed using dialysis bag with a molecular weight cut-off at 12,000 daltons (Cellu Sep® T4 regenerated cellulose tubular membrane, Membrane filtration products Inc., Seguin, TX, USA). The dialysis bag was activated by using the company method before starting the experiment. Briefly, the dialysis bag was soaked in 100 mL of distilled water and heated at 100°C for 30 min, then kept overnight at 4°C. The amount of 1 g of gel sample was placed in the

activated dialysis bag without air bubbles. The bag was tightly closed and immersed into the 50-mL medium (artificial saliva). The medium was maintained at 37 ± 1°C under constant stirring of 100 rpm. The samples were collected after 5, 10, 15, 20, 30, 40, 50, and 60 min. The volume of the collected sample was replaced with the fresh receptor medium. The amount of CP released was determined using high performance liquid chromatography (HPLC) (Hewlett Packard series 1100, Agilent technologies, Santa Clara, CA, USA) according to the previous method (*24*) with some modification. Briefly, 1,000 mL of 0.1 M TPP was added to 1,000 mL of the samples and constantly stirred for 2 h with light protection. The condition of HPLC was as follows; a reversed phase column Hypersil ODS (4.6 × 250 mm, Agilent technologies) was used as a stationary phase, UV detection was at 225 nm, a mobile phase with flow rate of 1.0 mL/min consisted of acetonitrile and water gradient. At starting time, the mobile phase was 50% acetonitrile and 50% water, then at 6.5 min of running time it was changed to 100% acetonitrile. At 10 min of running time the mobile phase was changed to 50% acetonitrile with 50% water, and this mixture was continued running until the retention time was completed at 25 min. The injection volume was 10 µL and the running temperature was 25 ± 0.2°C. A calibration curve was prepared using CP solution at concentration range of 50-200 µg/mL. The standard curve gave a linear response with correlation coefficient (r^2) of 0.9997.

2.6. *In vitro tooth whitening efficacy study*

2.6.1. *Tooth preparation*

Forty-five normal teeth were collected by dentists from normal volunteers of Chiang Mai University (CMU). This study was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University (No. 21/255). Normal teeth without hypoplastic defects were cleaned. The imperfections of the teeth were removed then stored at 4ºC in saturated 0.1% thymol solution until use.

2.6.2. *Evaluation of tooth whitening efficacy*

Color of the teeth was measured by using colorimeter (Fru WR10 portable precision colorimeter, Shenzhen wave optoelectronics technology Co., Ltd, Shenzhen, China) with color values recorded from 3 mm circular centers of tooth surface. The color values were calculated using the Commission international de l'Eclairage (International commission on illumination: CIE) b* (yellow-blue) scales (*25,26*).

The teeth were randomly allocated into 5 groups, including the test groups of CP-HN and CP-DS, the positive control group of CP-CG, and the gel bases groups of HN and DS as the negative control groups. Regimen of the treatment was 1 h/day, 4h/day and 8 h/day, duration of the study was 7 days. Each day, the exact amount of 0.1 mL samples were placed on enamel surface surrounded with 0.5 mL artificial saliva. The samples were kept in a close container with the controlled relative humidity of 100% and temperature of 25 ± 0.2ºC. After testing time was completed, the teeth samples were removed and washed by deionized water. Subsequently, the color was measured and calculated for the whitening efficacy of the gels.

2.7. *Statistical analysis*

Descriptive statistics for continuous variables were calculated and reported as a mean ± standard deviation (SD). Data were analyzed using a one-way analysis of variance (ANOVA) and Duncan's multiple range test ($p < 0.05$) using SPSS statistic software version 22.

3. Results

3.1. *Outer appearance and pH of the gels*

The obtained CP pigmented rice gels showed homogenous textures with moderate viscosity. Phase separation and drug precipitation were not found in all gels. The pH values of the gel bases was slightly different from the CP loading gels as shown in Table 1. The pH of rice gels bases was nearly neutral. After CP was incorporated, the pH of the rice gels was decreased.

3.2. *Adhesive property of the gels*

The adhesive property of the gels was different as presented in Table 1. Comparison between two pigmented rice gel bases, HN gel base showed longer detachment time than DS gel base indicating that HN has higher adhesive property than DS gel base. After CP was loaded, the adhesive property of the gels was slightly decreased. Considering the detachment time of gels containing CP, CP-HN had the highest detachment time followed by CP-CG and CP-DS.

3.3. *In vitro drug release property*

The determination of CP released from the gels was performed using HPLC method based on an oxidation

Table 1. pH values and adhesive property of the gels

Gels	pH	Adhesion, Detachment time (min)
HN	7.83 ± 0.03	64 ± 1
DS	7.64 ± 0.02	47 ± 3
CP-HN	6.62 ± 0.02	56 ± 3
CP-DS	6.16 ± 0.01	42 ± 2
CP-CG	6.51 ± 0.02	49 ± 2

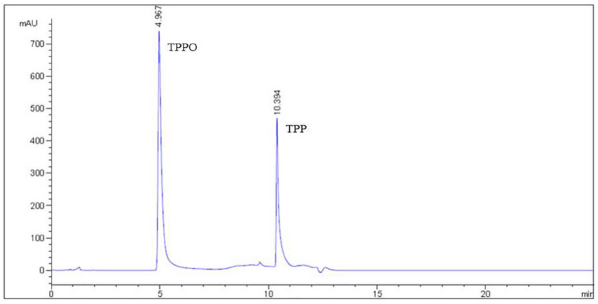

Figure 1. HPLC chromatograms of TPPO and TPP.

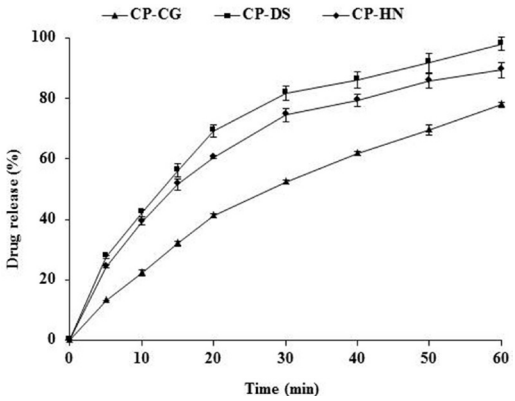

Figure 2. Release profiles of CP from CP-HN, CP-DS, and CP-CG.

of TPP into triphenylphosphine oxide (TPPO) (*27*). TPP and TPPO presented the HPLC chromatogram peaks at different retention times as presented in Figure 1. Determination of CP was made by external quantification using TPPO peaks area.

In vitro drug release of the two pigmented rice gels containing CP and CP-CG was presented in Figure 2. The results showed that the fastest drug release was obtained from CP-DS followed by CP-HN and CP-CG, respectively. During the release time, both CP rice gels demonstrated faster drug release than CP-CG. The amounts of the drug release from CP-DS, CP-HN and CP-CG after 60 min were 98.71 ± 2.11%, 89.48 ± 2.52%, and 77.90 ± 0.84% respectively.

3.4. *In vitro whitening efficacy*

Color of the teeth was measured by light reflection method using a colorimeter. This measurement reflects three color parameters; L*, a*, and b*. The parameter L* refers to the lightness (L), and ranges from black (L = 0) to white (L = 100). The parameter a* refers to red-green color, a positive a* refers to red and a negative a* refers to green. The parameter b* refers to yellow-blue color, a positive b* indicates yellow and a negative b* indicates blue. The L*a*b* values were calculated for color changing by using CIE L*a*b* system as the following equation; $\Delta E = [(\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2]^{1/2}$ (25,26). The ΔE values are often used in tooth whitening studies in order to indicate the perceptible tooth color changes after

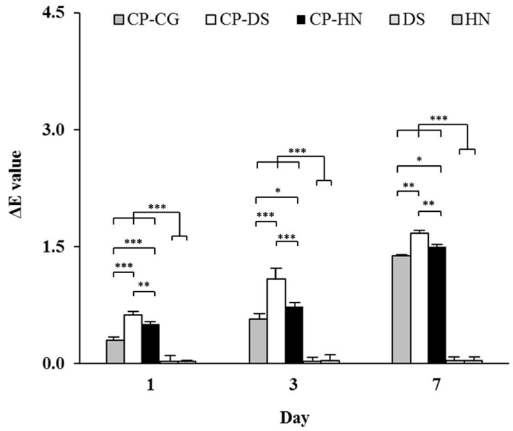

Figure 3. ΔE value progression of 1-h regimen, values are given as means ± SD. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

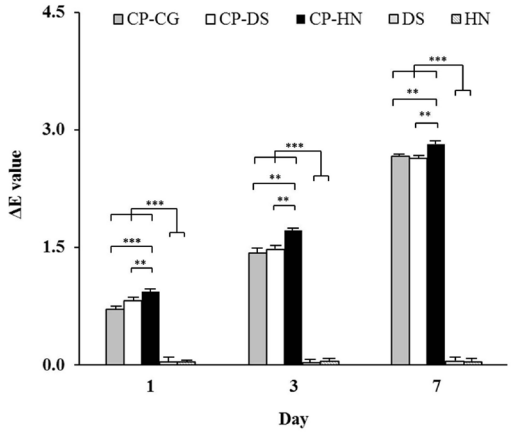

Figure 4. ΔE value progression of 4-h regimen, values are given as means ± SD. **$p < 0.01$, and ***$p < 0.001$.

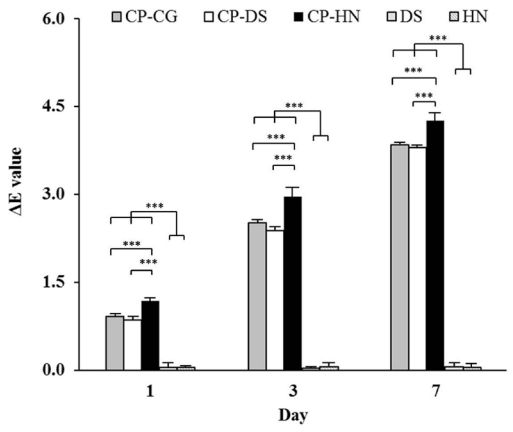

Figure 5. ΔE value progression of 8-h regimen, values are given as means ± SD. ***$p < 0.001$.

treatment.

The ΔE value progression at the different evaluation periods was presented in Figures 3-5. Comparing ΔE values of all groups, it was demonstrated that all treatment groups; CP-HN, CP-DS and CP-CG, showed significantly higher ΔE values than the negative control groups ($p < 0.05$). It was also visually observed that at day 7, the tooth color of the treatment groups was

whiter than that the tooth color at day 0. At the end of the treatment, the ΔE value of the treatment groups demonstrated significant tooth color improvement from day 0, day 1 and day 3 ($p < 0.05$). It was also found that 8-h regimen significantly improved tooth color when compared to 1-h and 4-h regimens ($p < 0.05$).

The ΔE value of 1-h regimen as shown in Figure 3 demonstrated that CP-DS group had the highest ΔE value followed by CP-HN group and CP-CG group, the mean ΔE value of day 7 was 1.67 ± 0.04, 1.50 ± 0.03, and 1.38 ± 0.02 respectively. However the results of 4-h regimen as shown in Figure 4 demonstrated that ΔE value of CP-HN was the highest. At day 7, the mean ΔE value of CP-HN was 2.82 ± 0.06. CP-CG and CP-DS showed similar efficacy with the mean ΔE values of 2.66 ± 0.03 and 2.64 ± 0.04, respectively. Similar results were found at 8-h regimen as shown in Figure 5, CP-HN showed the highest mean ΔE value followed by CP-CG and CP-DS with the mean ΔE values of 4.26 ± 0.09, 3.84 ± 0.12, and 3.79 ± 0.07 respectively. The results indicated that at the 1-h regimen, CP-DS was the most effective gels whereas at the 4-h and 8-h regimen CP-HN was the most effective gels.

4. Discussion

According to the high hydrophilicity of the modified rice obtained from carboxymethylated etherification (*28*), preferable hydrophilic CP-HN gels and CP-DS gels can be successfully prepared. Both CP-HN and CP-DS gels showed desirable transparency. CP can dissolve easily in water therefore the obtained CP rice gels shown homogenous texture without phase separation or drug precipitation. The pH of both rice gel bases was neutral but slightly decreased after CP was incorporated. Our results are in agreement with the work previously reported that the gels contained CP was slightly acidic (*29*). Therefore CP plays a role in the pH of the gels.

It has been known that adhesion is one of the most important properties in the pharmaceutical formulation in order to enhance localized drug delivery (*30*). Especially for tooth whitening formulations, a very high adhesive gels is preferable. It was reported that the enhancement of drug delivery through tooth surface and enamel could be successfully achieved by sufficient adhesive strength of formulations and teeth (*31*). Tooth whitening gels require high adhesive property to stay on the tooth surface to deliver maximum quantity of CP. The gels should not flow out from the tray to gingiva while using. High adhesion also helps to reduce side effect caused from peroxide damaged surrounding tissue (*32,33*). The results from the present study showed that the adhesive property of HN gels was higher than that of DS gels. Our result is in correspondence with the results that have been previously reported (*15,19*). This confirms that HN gels has higher adhesive property than DS gels. CP-HN and CP-DS showed similar adhesive property related to their respective gel bases. Moreover it was visually observed that HN and CP-HN strongly attached to the membrane and slowly dissolved to the medium. DS and CP-DS showed easier dispatched from the membrane. This might be due to the different compositions of both rice. The positive control CP-CG was swelled and ruptured to small pieces then detached from the membrane. This result shows the low adhesive property of the commercial gels.

The results in tooth whitening efficacy of CP pigmented rice gels in human teeth indicate that the whitening efficacy of the gels is time dependent. The efficacy of 8-h regimen was significantly higher than that of 4-h and 1-h regimens, respectively and the results of 7-day treatment is obviously better than 3-day and 1-day treatments, respectively. This result is in correspondence with the previous (*34,35*). Moreover, the results in the present study show that the adhesive and drug release properties of the gels play an important role in the tooth whitening efficacy. CP-DS gels showed the fastest drug release property compared to the other gels. At the 1-h regimen, CP-DS showed the highest tooth whitening efficacy. This result was in correspondence with *in vitro* release results. However at 4-h and 8-h regimens, CP-HN shows significantly higher effective tooth whitening efficacy than CP-DS and CP-CG. This result indicates that the adhesive property of the gels influences the tooth whitening efficacy in these regimens. CP-HN gel has the highest adhesive property among the test gels and could retain at the tooth surface area for the desired duration time whereas CP-DS and CP-CG gels with low adhesive property were easily flow out from tooth surface area and washed away by the surrounding medium. CP-CG showed higher adhesive property than CP-DS. However, the drug released from CP-CG was lower than CP-DS. Therefore after the 4-h and 8-h regimens, the tooth whitening efficacy of CP-DS and CP-CG were quite similar and significantly lower than CP-HN.

In conclusion, the pigmented rice gels containing CP can be successively prepared using carboxymethyl modified rice as gelling agent. Different rice varieties yield the gels with different physicochemical properties leading to the difference tooth whitening efficacy of the gels. Moreover, the efficacy of CP rice gels is time dependent. For the 1-h regimen, CP-DS was the most effective tooth whitening gels whereas at the 4-h and 8-h regimen CP-HN was the most effective tooth whitening gels.

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Original Article

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Effects of *Piper betle* fractionated extracts on inhibition of *Streptococcus mutans* and *Streptococcus intermedius*

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Summary **The overgrowth of certain strains of normal flora in oral cavity can cause many kinds of oral infections or diseases such as carries, periodontitis, and gingivitis. Prevention and treatment of these diseases are usually achieved by chemical antiseptics. However, these chemicals are found as negative impacts of human health hazards and accession of microbial resistance. The present study explores the potential of *Piper betle* extracts on inhibition of two oral pathogenic bacteria; *Streptococcus mutans* DMST 41283 and *Streptococcus intermedius* DMST 42700. *P. betle* demonstrated significantly higher inhibitory activity against both pathogenic strains than *Acacia catechu*, *Camellia sinensis*, *Coccinia grandis*, *Solanum indicum*, and *Streblus asper*. Among fractionated extracts of *P. betle* from several solvents, the extract from ethyl acetate (Pb-EtOAc) possessed the widest inhibition zone of 11.0 ± 0.1 and 11.3 ± 0.4 mm against both bacterial strains, respectively. Pb-EtOAc showed the same minimum inhibitory concentration of 0.5 mg/mL against both strains, whereas its minimum bactericidal concentrations were 2.0 and 0.5 mg/mL against *S. mutans* and *S. intermedius*, respectively. HPLC analysis demonstrated that the major active compound of Pb-EtOAc was 4-allylpyrocatechol. It was found that the killing kinetics of Pb-EtOAc against both test strains were time and dose dependent. Scanning electron microscopy micrographs showed the morphological changes and depletion of the tested pathogens indicating cell destruction after exposure to Pb-EtOAc. It is confirmed that Pb-EtOAc is potentially effective against both oral pathogens and might be used as natural alternative agents in prevention and treatment of oral infections caused by oral pathogenic bacteria.**

Keywords: 4-allylpyrocatechol, killing kinetics, cell morphology, oral infection, plant extract

1. Introduction

Oral health is categorized as the important issue to overall health and wellness. World health organization (WHO) concerns this situation and promotes many policies to boost a good oral health and prevent oral diseases (*1*). Poor oral health is mainly due to diseases or infections in oral cavity caused by overgrowth of some oral bacteria. *Streptococcus* is the major group of bacteria causing oral infectious diseases. *Streptococcus mutans* causes pathogenesis of dental caries whereas *Streptococcus intermedius* locates in subgingival tissue and causes gingivitis. The violence of these oral diseases leads to loose of teeth and periodontitis which is a chronic severe oral infection (*2,3*). Prevention and treatment of oral infection can be achieved by oral hygiene practices and use of some antiseptics. Various chemical antimicrobial agents were used as effective medicine and contain in oral health products. However, prolong use of these chemicals presents negative impacts

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of human health hazards and acquisition of microbial resistance. The use of natural products such as plant extracts are of increased interest due to safer than those chemicals for human and more environmental friendly. Therefore, several medicinal plants have been investigated for antibacterial activities in order to be an alternative choice for prevention and treatment of infectious diseases including oral infections (*4*). Many plant extracts including plant essential oils have been reported to have potential inhibitory effects against many strains of Gram-negative and Gram-positive pathogenic bacteria (*5-7*). The *in vitro* inhibition of some oral bacteria by the extracts of *Antrodia camphorate* (*8*) and *Prunus mume* (*9*) has been reported. However, there are still few reports of plant extracts on the inhibitory effects against oral pathogens. Therefore, it is essential to search for potential plants that have high inhibitory activity against the oral pathogens.

Piper betle is an edible plant in Family Piperaceae. This plant has been used as a main active composition in many Asian folk medicinal remedies for a long time. The extracts of *P. betle* have been reported to have several pharmacological effects such as anti-inflammatory, anti-allergic, wound healing, antiplatelet, antioxidant including antibacterial and antifungal activities (*10*). The bioactive compounds found in *P. betle* are several types such as allylpyrocatechol, eugenol, chavibetol, caryophyllene, and hydroxychavicol. The concentration of these compounds distributed in the plant is different depended on the different parts of the plant as well as source and season of harvesting (*11,12*). Among extensive reports on its biological activities, the report on inhibitory effects against the pathogenic microorganisms causing oral infectious diseases is still less. We previously reported the antimicrobial activity of *P. betle* against *Candida albicans* DMST 8684, *C. albicans* DMST 5815, *Streptococcus gordonii* DMST 38731 and *S. mutans* DMST 18777 and demonstrated that *P. betle* had significantly higher activities against these oral pathogens than *Andrographis paniculata*, *Momordica charantia*, *Phyllanthus emblica*, *Sesbania grandiflora* and *Psidium guajava* (*13*). In the present study, we further investigated the inhibition potential of *P. betle* against another strains of oral pathogenic bacteria; *S. mutans* DMST 41283 and S. intermedius DMST 42700, and compared its activity with five different plants having antibacterial activity against other microorganisms. Moreover, this study explores the bioactive compound of the most effective fractionated extract of *P. betle*. The work was to explore the inhibition potential of *P. betle* on oral pathogenic bacteria and that it can be used as an active agent for prevention and treatment of the oral infections caused by those pathogens.

2. Materials and Methods

2.1. *Culture media and chemicals*

Brain heart infusion broth (BHI) and brain heart infusion agar (BHA) were purchased from Difco (Maryland, USA). Human blood was supported by Maharaj Nakorn Chiang Mai Hospital (Chiang Mai, Thailand). Ethanol, ethyl acetate, and hexane were from RCI Labscan (Bangkok, Thailand). Dimethyl sulphoxide (DMSO) and glutaraldehyde were from Merck (Darmstadt, Germany). 4-Allylpyrocatechol (APC) was purchased from Fluka (Missouri, USA). Other chemicals and solvents are of the highest grade available.

2.2. *Plant materials and extraction*

P. betle and other five Thai medicinal plants, including *Acacia catechu*, *Camellia sinensis*, *Coccinia grandis*, *Solanum indicum*, and *Streblus asper*, were collected from the northern part of Thailand. The plants were identified and their voucher specimens were kept at the Herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand. The details of these plants are shown in Table 1. Fresh medicinal plants were dried in hot air oven at 50°C for 24-48 h before pulverizing into fine powder. To prepare their crude ethanol extract (CE), the plant powders were macerated in ethanol for 24 h. Subsequently, the mixture was filtered through a Whatman No.1 filter paper. The filtrate was collected and the plant residues were re-macerated in ethanol and filtered again. The obtained filtrates were gathered and subjected to a rotary vacuum evaporator (EYELA N-100, Tokyo, Japan) for removing the solvent. The obtained CE of each plant was kept in 4°C for further experiment.

The fractionated extracts of *P. betle* were prepared using hexane, ethyl acetate, and ethanol. The extraction method was according to the previous reported (*13*). Briefly, hexane was used as the first macerating solvent. The plant residue from hexane extraction was further macerated with ethyl acetate, and ethanol, respectively. The filtrates from each solvent were subjected to the rotary evaporator. After the solvents were completely removed, the fractionated extracts from hexane (Pb-Hexane), ethyl acetate (Pb-EtOAc) and ethanol (Pb-EtOH) were obtained. All extracts were kept at 4°C until use.

2.3. *Pathogenic strains and inoculum preparation*

Two strains of oral pathogenic bacteria; *S. mutans* DMST 41283 and *S. intermedius* DMST 42700 were used as the test microorganisms. *S. mutans* is a facultative anaerobic bacteria, but the optimal atmospheric condition for cultures should be anaerobic or contain only a low percentage of oxygen with 5-10% carbon dioxide whereas *S. intermedius* is an aerotolerant anaerobic commensal bacteria. The inoculum of these strains was prepared by culturing the oral pathogens in

broth media. Both strains were cultured in BHI at 37°C under anaerobic condition (5% H_2, 5% CO_2 and 90% N_2) for 24-48 h. The stock inoculum was adjusted to the turbidity of 0.5 McFarland standard using a McFarland densitometer (DEN-1 Biosan, Riga, Latvia).

2.4. *Antibacterial activity investigation*

The antibacterial activities of the extracts were investigated by means of two standard methods, the disk diffusion and the broth dilution methods.

The disk diffusion method was used to investigate antimicrobial activity of the CE of *P. betle* (Pb-CE) and the CE of other five plants (*A. catechu*, *C. grandis*, *C. sinensis*, *S. indicum*, and *S. asper*) as well as the fractionated extracts of *P. betle* (Pb-Hexane, Pb-EtOAc, and Pb-EtOH). The stock solutions of the test extracts were prepared by dissolving the extracts in DMSO. An exact amount of 20 µL of the extract solution was added onto the 6-mm diameter disk papers. The entire surface of BHA containing 5% human blood (bBHA) was swabbed with the stock inoculum of the test bacteria after adjusting to 0.5 McFarland standard. The extract loaded disks were placed on the surface of this medium. The plates were then incubated in an anaerobic condition at 37°C for 24 h. The diameter of inhibition zone was measured. Chlorhexidine 1.2 mg/mL solution (CHX) and DMSO were used as a positive and negative control, respectively.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *P. betle* extracts were evaluated by a broth dilution method. Stock solutions of Pb-Hexane, Pb-EtOAc, and Pb-CE were prepared by dissolving the extracts in DMSO. These stock solutions were diluted with BHI media to obtain the two-fold serial dilutions in a 96-well plate. The stock inoculum of each pathogen was added to the plates to have a final concentration of 1×10^6 cfu/mL. The plates were incubated in anaerobic condition at 37°C for 24 h. The lowest concentration of the extract that inhibited microbial growth after 24 h incubation was recorded as MIC. All dilutions were subsequently streaked on the entire surface of bBHA and further incubated in the same conditions as in the determination of MIC. After 24-h incubation, a minimum concentration of the extracts that showed complete inhibition of the bacteria was observed and recorded as MBC.

2.5. *HPLC analysis*

Pb-EtOAc was subjected to HPLC using a Hypersil ODS column (4.6 i.d. × 250 mm) with an Agilent 1100 HPLC system and UV detector set at 280 nm. An isocratic mobile system composed of methanol and water at a volume ratio of 7:3 was used to elute the samples at a flow rate of 0.70 mL/min for 20 min. The extract was dissolved in ethanol and filtrated through a 0.22 mm Millipore filter, type GV (Millipore, Bedford, Massachusetts, USA) prior to HPLC injection. The sample volume of 20 µL was injected and the HPLC was run in an ambient temperature. The samples were analyzed in triplicate. Standard APC was used as a biomarker and run separately under the same HPLC condition. The HPLC peak of the extract which appeared at the same retention time of APC was detected and expectedly identified as APC, a bioactive compound of the extract.

2.6. *Killing kinetic study*

Pb-EtOAc was used in this experiment. The extract solution was prepared by dissolving Pb-EtOAc in DMSO. The obtained solution was further diluted with BHI until the extract concentrations were 1-fold, 2-fold and 4-fold MBC. The inoculum of the pathogenic strains having microorganism concentrations of 1×10^6 cfu/mL was added to the extract solution in the 96-well plates. The plates were incubated in an anaerobic condition at 37°C for 24 h. Viable bacteria were determined at the time intervals of 0, 1, 2, 4, 6, 12, and 24 h by plating 20 µL of the known dilutions of the culture samples on the entire surface of bBHA. The plates were further incubated for 24-48 h. The plates with 30 to 300 colonies were used for colony forming unit (cfu) counts. The killing kinetic curves showing relationship between log cfu/mL and time were plotted. CHX and DMSO were used as positive and negative controls, respectively.

2.7. *Microbial morphology study*

Assessment of a possible mechanism of the active compound against oral pathogens, a morphological analysis of the test bacteria was carried out by treating the pathogens with Pb-EtOAc. Morphology of the pathogens before and after treatment was observed using scanning electron microscopy (SEM) (JEOL JSM-6610LV, Tokyo, Japan). The pathogenic strains having the concentration of 1×10^6 cfu/mL were added to the 0.5 mg/mL Pb-EtOAc solution in the 96-well plates. It was noted that this concentration was the MIC of Pb-EtOAc against all test pathogens. The cultures were then incubated in the same conditions as in the determination of MIC for 24 h. In the preparation of sample for SEM analysis, the suspension in each well was filtered through nylon membrane. Bacterial cells were fixed using 2% glutaraldehyde in phosphate buffer solution (pH 7.4) for 2 h-1 week. The cells were washed to remove glutaraldehyde and other suspended materials in the same solution for 10 min, this step was repeated 3 times. Then, the cells were dehydrated in increasing concentration of ethanol as follows; 50% for 5 min, 70% for 15 min, 85% for 15 min, 95 % for 15 min, and 100% for 15 min. This step was repeated 2 times. The chips of nylon membranes that covered with

Table 1. Plants and the percentage yield of their CE

Plant samples	Family	Local name	Voucher specimen	Used part	Yield (%)
A. catechu	Fabaceae	Seesiad	009208	Wood	2.7
C. grandis	Cucurbitaceae	Tamleung	007620	Leaf	18.1
C. sinensis	Theaceae	Miang	005584	Leaf	16.3
P. betle	Piperaceae	Plu	008612	Leaf	19.2
S. indicum	Solanaceae	Mawangton	023226	Fruit	22.5
S. asper	Moraceae	Khoi	005531	Bark	6.6

bacteria cells after dehydration were dried by critical point drying technique. Afterwards, the chips were mounted on aluminum stub and coated with gold in a sputter coater (JEOL JFC-1100E, Tokyo, Japan). The cells on the chip were observed under the SEM at 15 kV accelerating voltage with magnification of 15,000X. CHX was used as a positive control.

2.8. *Statistical analysis*

All experiments were done in triplicate and the results are expressed as mean ± SD. Statistical analysis was done by using one-way ANOVA followed by Tukey's HSD test using SPSS software version 17.0 and *p*-value at a level of 95% confidence limit.

3. Results

3.1. *Preparation of CE*

The CE of the studied plants showed different appearances and yields. All plant extracts showed different specific intense odor. Pb-CE was dark green fluidized mass whereas that of *C. grandis*, *C. sinensis*, *S. indicum*, and *S. asper* were a viscous mass. The CE of *A. catechu* appeared as dried powder. It was noted that the color of the extracts from the wood, bark, and fruit was rusty brown to light yellow, while that from the leaf was dark green. The yield of CE obtained from each plant was different as presented in Table 1.

3.2. *Comparing the inhibitory efficiency of P. betle with other plants*

The results as shown in Table 2 demonstrated that Pb-CE had the highest antimicrobial activity against both pathogenic bacteria. The inhibition zones of *S. intermedius* was slightly wider than that of *S. mutans*, indicating that *S. intermedius* was slightly more sensitive to Pb-CE than *S. mutans*. The CE of *S. asper* showed inhibition against *S. intermedius*, almost same as *P. betle*, but showed no activity against *S. mutans*. The CE of *A. catechu* showed inhibition against *S. intermedius* but significantly less than that of *P. betle* and no activity against *S. mutans*. The inhibition zones of the positive control, CHX, against *S. mutans* and *S. intermedius* were 14.4 ± 0.6 and 14.6 ± 0.6 mm, respectively, while the negative control, DMSO, showed no inhibition zone for both strains.

Table 2. Inhibition zone of plant CE against the test oral pathogens

Plant samples	Inhibition zone (mm)	
	S. mutans	*S. intermedius*
A. catechu	6.2 ± 0.1c	6.7 ± 0.3b
C. grandis	6.1 ± 0.1c	6.2 ± 0.1c
C. sinensis	6.1 ± 0.2c	6.3 ± 0.1c
P. betle	8.5 ± 0.7a	8.9 ± 0.3a
S. indicum	6.0 ± 0.1c	6.1 ± 0.1d
S. asper	6.4 ± 0.1b	7.8 ± 0.3a

Values are mean ± SD followed by different lowercase letters imply the significant differences ($p < 0.05$) between values in the same column.

Table 3. Inhibition zone of *P. betle* extracts against the test oral pathogens

P. betle fractionated extracts	Inhibition zone (mm)	
	S. mutans	*S. intermedius*
Pb-Hexane	6.2 ± 0.1b	8.0 ± 0.0b
Pb-EtOAc	11.0 ± 0.0a	11.3 ± 0.4a
Pb-EtOH	6.1 ± 0.2b	6.1 ± 0.1c

Values are mean ± SD followed by different lowercase letters imply the significant differences ($p < 0.05$) between values in the same column.

Table 4. MIC and MBC (mg/mL) of *P. betle* extracts against the test oral pathogens

P. betle extracts	*S. mutans*		*S. intermedius*	
	MIC	MBC	MIC	MBC
Pb-Hexane	2.0	2.0	1.0	1.0
Pb-EtOAc	0.5	2.0	0.5	0.5
Pb-CE	2.0	8.0	1.0	2.0

3.3. *Comparative antimicrobial activity of P. betle fractionated extracts*

Among the fractionated extracts of *P. betle*, Pb-EtOAc possessed obviously the highest antimicrobial activity with the inhibition zones of 11.0 ± 0.1 and 11.3 ± 0.4 mm against *S. mutans* and *S. intermedius*, respectively as shown in Table 3. Pb-Hexane showed the activity against only *S. intermedius* but significantly less potent than Pb-EtOAc. Moreover, Pb-Hexane showed no inhibitory activity against *S. mutans*. Pb-EtOH showed no inhibitory effect to both test pathogens.

3.4. *Determination of MIC and MBC of P. betle extracts*

The results obtained from broth dilution method could obtain MIC and MBC of the extracts. The results as shown in Table 4 confirmed that among *P. betle* extracts, Pb-EtOAc was the most effective extract of *P. betle* against both oral pathogenic bacteria. It was found that the MIC of Pb-EtOAc against both pathogens was the same value of 0.5 mg/mL. However, the MBC values against both strains were significant different, 2.0 mg/mL for *S. mutans* and 0.5 mg/mL for *S. intermedius*. Pb-Hexane and Pb-CE demonstrated similar level of inhibitory activity. Both extracts showed antimicrobial activity with the same value of MIC against both pathogens. Their MIC values were higher concentration than that of Pb-EtOAc, indicating less potential on pathogenic inhibition than Pb-EtOAc. CHX, a positive control for antibacterial activity, showed an inhibitory activity against *S. mutans* and *S. intermedius* with the same MIC and MBC values of $< 3 \times 10^{-4}$ mg/mL.

3.5. *HPLC analysis*

HPLC chromatograms of Pb-EtOAc in comparison with APC standard compound are shown in Figure 1. The results showed that Pb-EtOAc contained three major compounds at the retention time of 4.11, 5.43 and 6.67 min. APC showed a peak at a retention time of 4.10 min which is resemblance with the HPLC peak that found at 4.11 min of Pb-EtOAc.

3.6. *Killing kinetic study*

The killing kinetics of Pb-EtOAc against the two pathogens were investigated and the results indicated that the killing activity of Pb-EtOAc was dose and time dependent. At 1-fold MBC, Pb-EtOAc completely killed *S. mutans* within 6 h as shown in Figure 2. Increasing concentration of Pb-EtOAc, higher killing efficiency was obviously seen. The bacteria were completely killed within 2 h and 1 h by the concentrations of 2-fold and 4-fold MBC, respectively. Whereas CHX (0.006 mg/mL) could completely kill this pathogenic strain within 4 h. The killing kinetic of Pb-EtOAc against *S. intermedius* was shown in Figure 3. At 1-fold MBC, Pb-EtOAc completely killed the bacteria within 6 h. However, increasing concentration of the extract to 2-fold and 4-fold MBC, higher killing efficiency was obtained. The extract at these concentrations

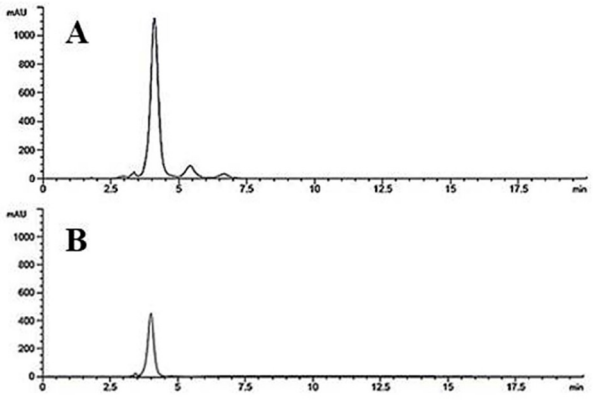

Figure 1. HPLC chromatograms of Pb-EtOAc (A) and 4-Allylpyrocatechol (B).

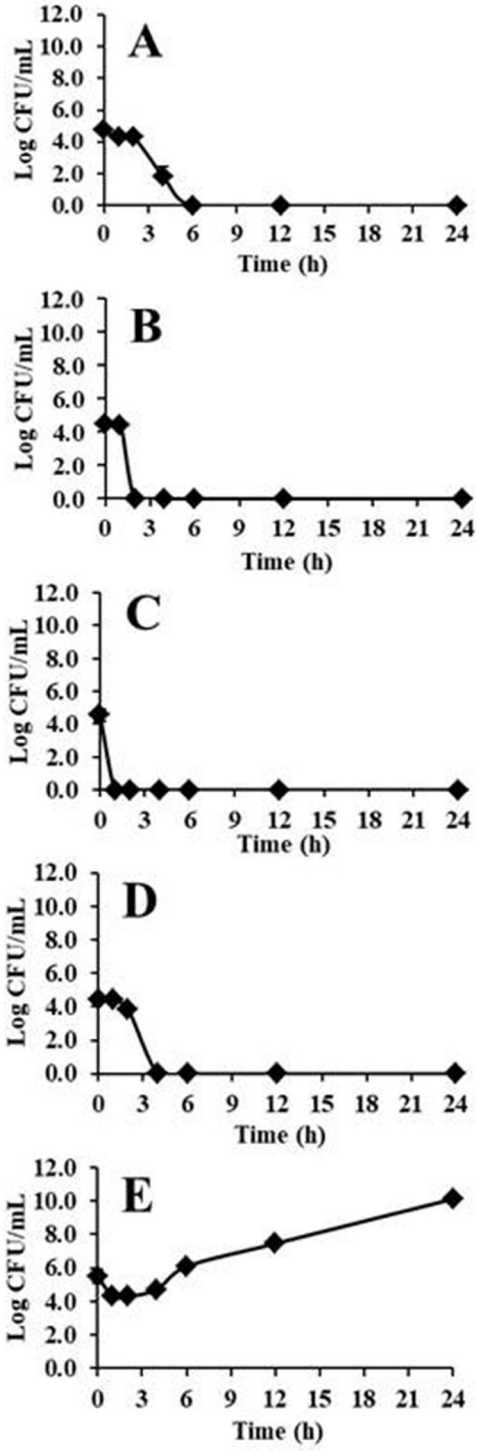

Figure 2. Killing kinetics of Pb-EtOAc at the concentrations of 1-fold MBC (A), 2-fold MBC (B), 4-fold MBC (C) in comparison with CHX (D) and DMSO (E) against *S. mutans* (*n* = 3).

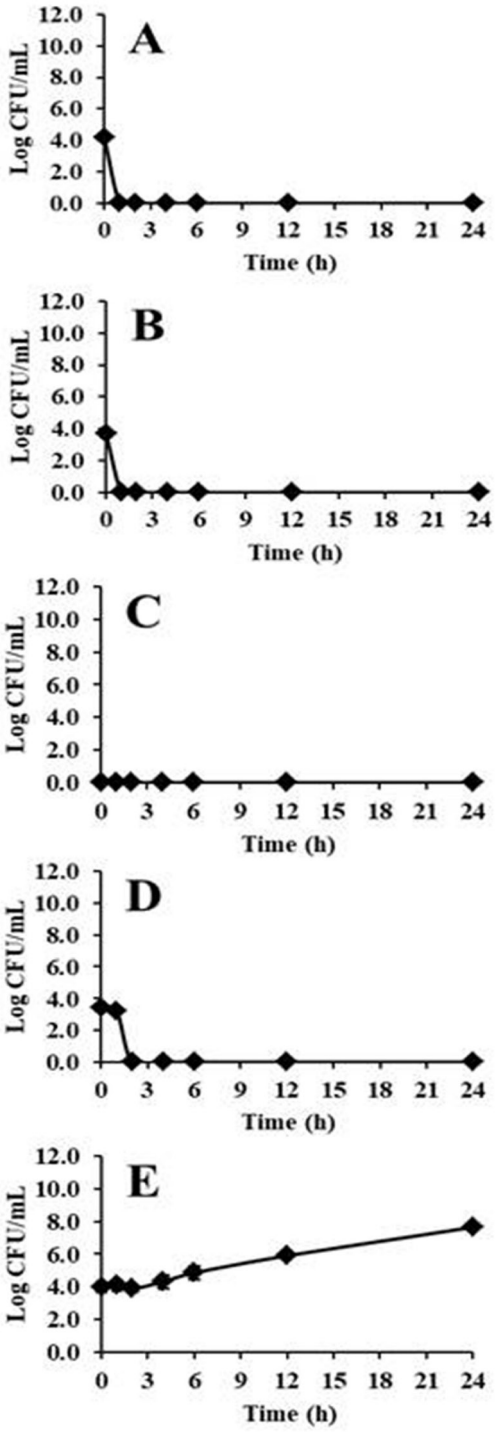

Figure 3. Killing kinetics of Pb-EtOAc at the concentrations of 1-fold MBC (A), 2-fold MBC (B), 4-fold MBC (C) in comparison with CHX (D) and DMSO (E) against *S. intermedius* (*n* = 3).

could completely kill the bacteria within 4 and 1 h, respectively. CHX at 0.006 mg/mL could completely kill this pathogen within 2 h.

3.7. *Microbial morphology study*

The morphology of the test oral pathogens were observed using SEM. The morphology of the untreated

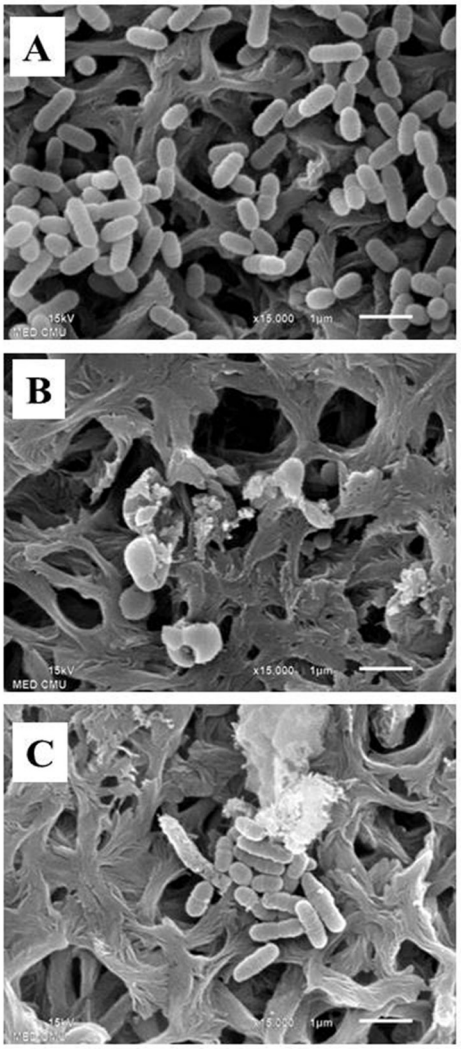

Figure 4. SEM micrographs of untreated and treated oral pathogens on nylon membrane; untreated *S. mutans* (A), treated *S. mutans* with Pb-EtOAc (B), and treated *S. mutans* with CHX (C).

S. mutans in BHI medium as control cells was elongated ellipse shape and smooth surface as shown in Figure 4A. After incubation with 0.5 mg/mL of Pb-EtOAc, the damage of *S. mutans* cells could be seen as presented in Figure 4B. Furthermore, morphology of the cells was changed. The size of the cells were swollen and disruption. The cell population was significantly reduced when compared to the control group, same as the positive control (Figure 4C). The control cells of *S. intermedius* in BHI media without being treated with the extract were complete round and smooth surface as shown in Figure 5A. After incubation with 0.5 mg/mL of Pb-EtOAc, the bacterial cells were damaged and the cell size was slightly enlarged with roughly surface as seen in Figure 5B. Moreover, the population of the cells was significantly decreased in comparison with a control sample. Treating with 0.006 mg/mL CHX, morphology and size of *S. intermedius* were slightly changed and the cell population was significantly decreased as presented in Figure 5C.

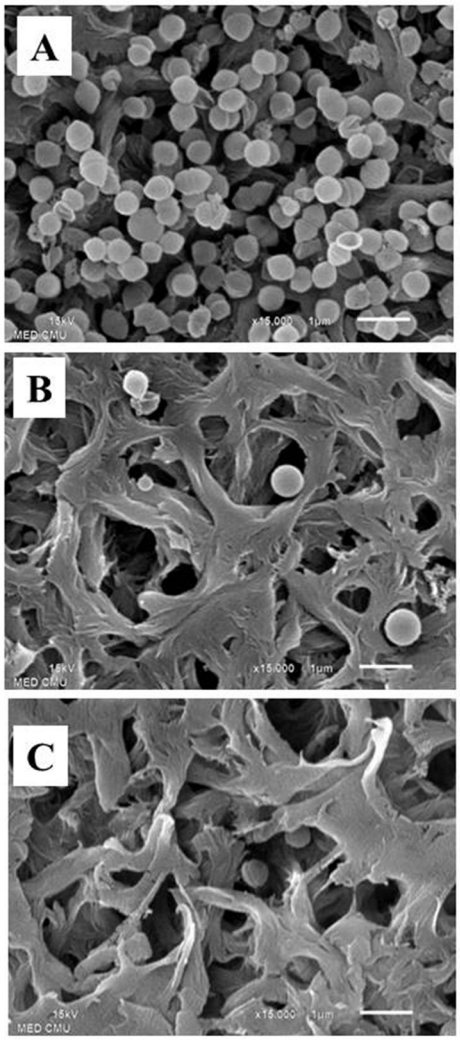

Figure 5. SEM micrographs of untreated and treated oral pathogens on nylon membrane; untreated *S. intermedius* (A), treated *S. intermedius* with Pb-EtOAc (B), and treated *S. intermedius* with CHX (C).

4. Discussion

Oral diseases infected by oral pathogenic bacteria do not affect only the oral cavity, but also influence the others parts in the body via blood circulation (*14,15*). For example, periodontitis caused by oral pathogenic bacteria has been reported to be a significant risk of developing cardiovascular diseases; atherosclerosis, myocardial infarction and stroke (*16*). Utilizing chemical aseptic or antimicrobial compounds to promote oral hygiene and inhibit oral pathogens causes several side effects (*17,18*). Moreover, microbial resistance has always been reported after long term using of these chemicals (*19*). The use of antimicrobial agents from plants instead of these chemicals, therefore, is our interest.

In our works on searching for potential plant extracts having high activity on inhibition of oral bacteria, *P. betle* is one of many interesting plants. In the current experiment, we compared the activity of *P. betle* with five different potential plants (*A. catechu*, *C. grandis*, *C. sinensis*, *S. indicum*, and *S. asper*) that have been previously reported to have antimicrobial activities (*20-24*). The yield of Pb-CE was 20.1%, slightly higher than that previously reported, which might be due to the different time and area of plant collection (*25*). Among the fractionated extracts of *P. betle*, the yield of Pb-EtOAc was the highest, followed by that of Pb-Hexane and Pb-EtOH. The difference of the yield among the different fractionated extracts is due to the different extracting solvents used and the solubility of the compounds existing in *P. betle* (*26*). These results confirm that not only the source of plants but also the extracting solvent and the method of extraction that affect the yield of the extracts. The antimicrobial activity of *P. betle* extract has been reported by other groups (*10*). However, they did not report its activity against the oral pathogens. We are the first group who demonstrate the activity of *P. betle* against oral pathogenic microorganisms. We previously reported its activity against the oral pathogenic fungi and certain bacterial strains (*13*). However, there are several bacteria existing in oral cavity that can be predominated and cause oral diseases, particular *S. mutans* and *S. intermedius*. The results of this study provide extensive data from the previous report and can be the scientific evidence to confirm the potential of *P. betle* on inhibition of various pathogenic bacterial strains in oral cavity as well as a supported scientific data of a historical use of *P. betle* by local people in the South and Southeast Asian countries as a mouth freshener (*27*).

In antibacterial activity investigation, the inhibition zone can approximately indicate the inhibitory effects of the test extracts, but MIC and MBC demonstrate deeper and more proper data for particularly comparative efficacy of the extracts. From the results of inhibition zone, MIC and MBC, it is confirmed that Pb-EtOAc was the highest potential extract on inhibitory activity against both oral pathogenic strains; *S. mutans* and *S. intermedius*.

Antimicrobial activity is usually regarded as bactericidal if the MBC/MIC ratio is ≤ 4 and bacteriostatic if > 4 (*28*). For Pb-EtOAc, the ratios of MBC/MIC calculated for *S. mutans* and *S. intermedius* were 4 and 1, respectively. From these results, the ratios of MBC/MIC are ≤ 4, indicating that Pb-EtOAc possesses bactericidal actions against both oral pathogens.

The pharmacological actions of some antimicrobial agents are dose dependent and some can be dose or time dependent (*29,30*). In our study on killing kinetics, the results indicate that the pharmacological action of antibacterial activity of Pb-EtOAc is dose and time dependent.

The morphological characteristic of bacterial cells is essential for understanding the possible mechanism

of action of antimicrobial agent against the pathogens. Generally, SEM is one of the potential tools used to analyze the morphology of the cells. It can provide the information on number, shape and size of cells (*31,32*). Using SEM, the morphology of *S. mutans* and *S. intermedius* was obviously observed in the current study. After treating with Pb-EtOAc, the morphology and size changes as well as population depletion of both strains were well seen. These effects caused by Pb-EtOAc are similar to the effects caused by CHX, the positive control. CHX was previously reported that it can destroy pathogen cells by disruption of cell membrane with its positive charge (*33*). The results from this study support that the antibacterial activity of Pb-EtOAc against *S. mutans* and *S. intermedius* is disruption of cell membrane of those pathogens leading to the leakage of essential cell compositions and cell dead.

The active compounds in plant extracts can be possibly identified by HPLC analysis using known active compound as a marker (*34*). Many compounds including APC have been reported from *P. betle*. APC has been reported to have many biological actions, for example induce apoptosis of cancer cells (*35*) and inhibit platelet aggregation (*36*). For antimicrobial activity, this compound has been reported to have an inhibitory activity against *Staphylococcus aureus* and *Escherichia coli* (*37*). In the present study, HPLC chromatogram of Pb-EtOAc demonstrates many peaks indicating many compounds existing in the extract. However, the major peak of Pb-EtOAc appears at the same retention time as the peak of APC. Therefore, the active compound of *P. bettle* for antibacterial activity against the oral pathogens is considered to be APC. The inhibitory activity against anaerobic bacteria of APC has never been reported elsewhere. This is the first report demonstrates this activity of the compound, particularly against anaerobic strains of oral pathogens.

In conclusion, the findings in the present study confirm that *P. betle* is a potential medicinal plant for inhibition of *S. mutans* and *S. intermedius*, the oral pathogenic bacteria and the major cause of dental caries and gingivitis. Pb-EtOAc is the most effective extract of *P. betle*. The major active compound of Pb-EtOAc is APC. The antibacterial activity of Pb-EtOAc bactericidal action and it is time and dose dependent. The mechanism of action of Pb-EtOAc is the ability to destroy the pathogen cells by causing disruption of cell membrane causing the leakage of essential components of the cells and cell dead.

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Original Article

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D-cycloserine nasal formulation development for anxiety disorders by using polymeric gels

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Summary **D-cycloserine (DCS), a partial agonist at N-methyl-D-aspartate (NMDA) receptors, is used as an enhancer of exposure therapy for anxiety disorders. The purpose of the present study was to investigate the feasibility of using polymeric gels to increase the viscosity of the formulation and thereby increase the nasal residence time and sustained release of DCS *in vitro*. Hydroxypropyl methylcellulose (HPMC), hydroxypropyl cellulose (HPC), and methyl cellulose (MC) were prepared at concentrations of 0.5 to 5% w/v. Pluronic F-127 (PF-127) was prepared at concentrations of 15 to 35% w/v. pH, viscosity and *in vitro* DCS release behavior of the formulated gels were analyzed. All four gels that were tested, demonstrated sustained DCS release behavior over a 24-hour period, but with different rates. Based on the results of this study, HPMC, HPC, MC, and PF-127 are capable of increasing the viscosity of nasal gel formulations and of releasing DCS in sustained manner. Therefore, these polymeric gels can be suitable carriers for DCS nasal gel formulation.**

Keywords: D-cycloserine, polymeric gels, hydroxypropyl methyl cellulose, hydroxypropyl cellulose, methylcellulose, Pluronic F-127

1. Introduction

Anxiety disorders are the most common mental disorders in America, affecting 15.7 million people each year and more than 30 million people at some point in the course of their lifetimes (*1,2*). Anxiety disorders have a serious impact on the society and health care system. According to data from the National Comorbidity Study (*3*), approximately $42.3 billion per year were spent on anxiety disorders in 1990 in the United States. Additionally, anxiety disorders may cause reduced productivity at work, which can be a burden on the society (*3*).

D-cycloserine (DCS), an antibiotic for tuberculosis, has been tried as an enhancer of exposure therapy for anxiety disorders (*2*) as it was discovered to act as a partial agonist at the glycine modulatory site of the N-methyl-D-aspartate (NMDA) receptor with high affinity for this receptor (*4,5*). DCS has also been used to improve the negative symptoms in schizophrenia (*6,7*), and to facilitate improvements in functional impairments among children with autism (*8*). Moreover, DCS has been used in the treatment of other psychiatric disorders such as acrophobia (*9*), social-phobia (*10*) and obsessive-compulsive disorder (*11,12*).

The availability of the DCS at the NMDA receptor site depends on its dosage and on the levels of the blood/cerebrospinal fluid (CSF). DCS has excellent central bioavailability (*13,14*) and is excreted primarily by the kidneys with a half-life of 9 hours (*15*). For treatment of tuberculosis, 250 mg tablets of DCS are typically used at 500-750 mg daily in chronic dosing (*16*). In contrast, utilization of DCS to enhance exposure treatment in humans has required only 50-500 mg in isolated dosing rather than in chronic dosing (*10*). While the pharmacokinetics of DCS used for treatment of tuberculosis is known, the relationship between pharmacokinetics of DCS and the effect on behavior is not firmly established.

The optimal delivery of drugs to the brain in conditions such as Alzheimer's disease and anxiety disorders is crucial. A drug can be more effective for the treatment of anxiety disorders if it is given direct access

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to the brain through the nasal passage than if it is given in the current capsule-based oral formulation. Nasal delivery could significantly improve onset of action for DCS and reduce the required dose (*17*). For these reasons, the nasal administration method has received lot of attention lately (*18-20*), and it has become clear that a new formulation and delivery system for DCS will have important treatment implications. Because the olfactory receptor cells are in direct contact with both the environment and the central nervous system (CNS), delivering the drug *via* nasal passage allows drugs to bypass the blood brain barrier (BBB) and to be delivered to the CNS directly (*21,22*).

Many researchers have tried to use a nasal delivery system to bypass the BBB since many drugs are not able to cross the BBB (*18-20*). Particularly, interest is shown in nasal delivery of drugs for neurodegenerative diseases such as Alzheimer's disease (*23*) and Parkinson's disease (*24*). Nasal delivery has many advantages besides circumventing the BBB. The nasal epithelium is composed of monolayer of cells with a relatively high permeability (*25*). The nose has a large surface area with numerous microvilli that enhance drug absorption rate, which may provide a rapid onset of action. Additionally, the sub-epithelial layer is highly vascularized and venous blood from the nose does not pass the liver, therefore, it circumvents first-pass metabolism in the liver. Nasal delivery also avoids acid and gastrointestinal enzyme degradation. Consequently, it will require lower doses, have more rapid onset of pharmacological actions, and have fewer side effects (*26,27*). By eliminating the need for systemic delivery, unwanted systemic side effects are reduced (*21,22*). Nasal delivery could be used for targeted drug delivery that releases the drug at or near the intended physiologic site of action. Nasal delivery also could be a strategy for an extended-release or sustained-release drug delivery if the dosage frequency allows at least a two-fold reduction as compared to conventional dosage form (*28*).

One of the limitations of nasal drug delivery is its inadequate nasal drug absorption due to nasal mucociliary clearance. The clearance function of the nose is a protective system against foreign materials such as bacteria and viruses from reaching the lungs and is very important in order to prevent respiratory tract infections (*29*). The mucocilliary clearance function moves noxious substances towards the nasopharynx and the substances are eventually transported into the gastrointestinal tract (*30*). Increasing formulation viscosity with polymeric gels may provide a means of increasing the residence time in the nasal cavity by decreasing the mucociliary clearance. Increasing the residence time in the nasal cavity may prolong the absorption and facilitate the uptake of the drug, therefore providing longer therapeutic effects of nasal preparations (*31*).

In the present study, different concentrations of four polymeric gels; Hydroxypropyl methylcellulose (HPMC), methylcellulose (MC), hydroxypropyl cellulose (HPC), and Pluronic® F-127 (PF-127); that are known to increase viscosities of solutions in concentration dependent manner were prepared and analyzed. We hypothesized that if a polymer (HPMC, HPC, MC, or PF-127) is used as a drug carrier in DCS nasal gel formulation, then the polymer will increase the viscosity of the formulation and facilitate sustained DCS release. The aim of this preliminary study was to screen DCS gel formulations prepared using different polymers for optimal pH, viscosity and *in vitro* release behavior. Overall, the long-term goal of this study was to help evaluate the feasibility of using these polymers for the development of DCS nasal gel formulations in the future.

2. Materials and Methods

2.1. *Materials*

DCS was obtained from Acros Organics™ (NJ, USA). HPMC, MC, and HPC were also purchased from Acros Organics™ (NJ, USA). Pluronic F-127 gel was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). A ServoDyne™ Digital Mixer (Cole-Parmer®, Vernon Hills, IL, USA) was used to mix the powdered polymers into distilled water. Distek dissolution apparatus (Distek, North Brunswick, NJ, USA) and high-performance liquid chromatography (HPLC) (Waters, Milford, MA, USA) were used for the *in vitro* DCS release study. The solvents used were HPLC grade distilled water and phosphate buffer (Fisher Scientific, Hampton, NH, USA). RPMI 2650 cells (ATCC® CCL-30™), Eagle's minimal essential medium (EMEM), fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin, and trypsin EDTA solution (1X ATCC® 30-2101™) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Microplate reader used was Epoch 2 Microplate Spectrophotometer, BioTek® (Winooski, VT, USA).

2.2. *Preparation of gels with HPMC, MC, and HPC*

Different concentrations of HPMC, MC, and HPC were prepared. "Hot/Cold technique" was used as these gels are non-Newtonian pseudo-plastics that must be heated first for an even suspension in distilled water (*32*). Then, the gels were dissolved in the distilled water by cooling in an ice bath. Briefly, 400 mL of distilled water was heated on a hot plate until it reached approximately 100°C. The beaker was removed from the hot plate and placed in a rubber ice bucket under a mixing apparatus so that the propeller was submerged but not touching the bottom of the beaker. When the stirring process began, weighed amounts (for 0.5, 1, 2, 3, 4, 5%, w/v) of HPMC, MC, and HPC powders were slowly added and mixed into the distilled water. After 2.5 minutes, ice

was added around the sides of the beaker in the rubber ice bucket. Stirring was continued at 190 rpm until the 20-minute interval had come to completion.

2.3. *Preparation of gels with PF-127*

Pluronic gels were prepared by cold method (*33*) in different concentrations (15, 20, 25, 30, 35%, w/v) and screened to compare effect of different concentration. Briefly, a weighed amount of Pluronic F-127 was slowly added to around 60-70 mL of water (at 10°C) in a beaker with continuous magnetic stirring. Aqueous PF-127 mixture was kept overnight at 4°C, and the final volume was adjusted to 100 mL with deionized water.

2.4. *pH and viscosity*

The pH of polymeric gels was analyzed for 3 weeks using Mettler Toledo pH meter (Columbus, OH, USA). The viscosities of prepared solutions were analyzed with a Brookfield DV-III Ultra Rheometer (Brookfield Engineering Laboratories, Middleboro, MA, USA) using a cone and cup attachment system. The prepared gels were placed in the cup and the spindle used was lowered perpendicular into the sample. The spindle was rotated at constant rpm. The temperature was set at 32°C as the physiological temperature of the nasal mucosa ranges between 32-34°C (*34*). Each concentration (0.5 mL) of HPMC, MC and HPC was analyzed in Brookfield's RheoCalc computer program using the Bingham equation. The unit commonly used is centipoise (cP), where 1 cP = 10^{-2} P = 10^{-3} Pa·s = 1 mPa·s. The viscosity of PF-127 was not measured because it was beyond the limit of the instrument and the spindle available. Few literatures (*35,36*) were selected for the relationship between the concentration of PF-127 and the viscosity.

2.5. *In vitro DCS release behavior study*

In vitro release of DCS from the gels was performed using a Distek dissolution apparatus (Distek, North Brunswick, NJ, USA). The procedure was modified so that it is suitable for the analysis of the gels. Briefly, 15 mL of each gel and 300 mg DCS was added to a 50 mL centrifuge tube with plug seal cap (Corning®, Corning, NY, USA). The tube was wrapped in aluminum foil and left on a Wrist Action Shaker (Burrell, Pittsburgh, PA, USA) overnight in order to ensure uniform mixing. A blue membrane clip shut (Spectrum Laboratories, Rancho Dominguez, CA, USA) was used to clip one side of an 8 cm piece of molecular-porous membrane tubing (Spectra/Por®, Rancho Dominguez, CA, USA) that was soaked in a beaker of phosphate buffered saline (PBS) buffer for 20 minutes. DCS gel (2 mL) was added using a positive displacement pipet (Eppendorf, Westbury, NY, USA) into the membrane and the top of the bag was closed with a piece of string. Then, the attached string was used to tie the bag onto the bottom of the drive assembly of the Distek system. Once the vessels filled with 500 mL of PBS each had equilibrated to water temperature, the assembly drives were lowered into the vessels and locked into place to submerge the membranous sack in 500 mL PBS buffer. The Distek system was turned on and rotated at 50 rpm to mimic flow of body fluids. Samples (2 mL) were collected at predetermined time intervals for up to 24 hours (every 10 minutes for the first half hour, then every 15 minutes for the following half hour, and then every hour after that for a total of 6 hours as well as one 24-hour sample the next day) and analyzed by HPLC (Waters, Milford, MA, USA). Whenever each sample was removed, an equivalent amount of buffer was added. The samples were run on HPLC for 3.5 minutes at 220 nm wavelength, 30°C, 10 µL injection volume and flow rate of 0.5 mL/min. The mobile phase was a 90:10 mixture of a 10 mM sodium phosphate buffer (pH 7.4): acetonitrile. The peak areas were converted to the amount of DCS in mg using the previously calibrated standard curve of DCS. The amount of DCS release from each gel in phosphate buffer was plotted against time to know the release pattern. Based on the data, drug release behavior of polymeric gels was observed.

2.6. *Drug transport assay in Calu-3 cells*

For the drug transport assay Transwells were obtained from Corning Incorporated (Corning, NY). T-75 flasks were obtained from Thermo scientific (Rochester, NY, USA). Dimethyl Sulfoxide (DMSO) was obtained from Sigma (St. Louis, MO, USA). PBS 1X, sterile was used and was obtained from Mediatech, Inc (Manassas, VA, USA). Trypan Blue was obtained from MP Biomedicals, LLC (Solon, OH, USA). Serum-free cell freezing media, Trypsin EDTA, Fetal Bovine Serum, EMEM cell culture medium, and Calu-3 cell line (HTB 55) were all obtained from ATCC (Manassas, VA, USA). DCS was obtained from Research Products International Corp. (Mt. Prospect, Illinois, USA) and was used without any further purification. All chemicals, buffer reagents, and solvents used were of analytical grade and were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). HPLC grade water and acetonitrile were also purchased from Fisher Chemicals (Fair Lawn, NJ, USA) and used throughout this study.

2.7. *In vitro cytotoxicity test in RPMI 2650 cells*

The cell line was grown in the T75 tissue culture flasks at 37°C in 95% air-5% CO_2. Culture medium used was EMEM with 10% FBS (v/v). When cell line was 70-80% confluent the cells were detached with Trypsin-EDTA solution and 100 µL of cells with media were

seeded onto 96-well plate at a final density of 10,000 cells/100 µL per well. After overnight incubation, various concentrations of DCS dissolved in autoclaved distilled water were added to each well to determine the cytotoxicity. The concentrations of DCS used were in the range of 1.25-100 µM. The cells were incubated for 72 hours at 37°C in 5% CO_2. After incubation, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well. The cells were incubated at 37°C for 4 hours. The formazan crystals formed were dissolved in a solubilizing buffer containing 20% SDS and 0.1 N HCl. The plate was left overnight, and absorbance values of the samples were read at wavelength of 570 nm with microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek®, VT, USA). The relative cell viability was calculated from the absorbance values as a percent of untreated cells (control).

2.8. *Cell culture for drug transport assay*

The Calu-3 cell line (HTB-55) purchased from the American Type Cell Culture Collection (ATCC, Rockville, IN) were grown in 75 cm^2 flasks in complete Eagles's minimal essential medium (EMEM) containing 10% (v/v) fetal bovine serum solution and maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Cells were propagated and subcultured according to ATCC recommendations. To establish the air-liquid interface model, cells were seeded onto Transwell polyester inserts at a density of 5×10^5 cells/cm^2 in 1.5 mL apical and 2.6 mL basolateral medium. The apical medium was removed 24 hours after seeding and cells were fed every alternate day with fresh basolateral medium only. The monolayers were allowed to differentiate under air interface feeding conditions over 10-15 days.

2.9. *Transepithelial electrical resistance (TEER) of cell layers*

The transepithelial electrical resistance (TEER) of Calu-3 monolayers was measured over time using a Millicell ERS-2 Epithelial Volt-ohm meter (EMD Millipore Corporation, Billerica, MA) with STX-01 chopstick electrodes. Pre-warmed sterile PBS 1× was added to the apical and basolateral sides of the Calu-3 monolayer. The monolayer was equilibrated for 30 minutes in a humidified atmosphere of 95% air/5% CO_2 at 37°C prior to resistance measurements. TEER was calculated by subtracting the resistance of a blank insert. The resistance of the cell monolayers in each well was measured 7 times between days 2 and 15 of culture.

2.10. *Transport experiments*

Transport experiments were conducted on days 10-15 in culture. Before each experiment, the cells were washed three times with sterile PBS 1×. The apical and basolateral layer were washed with pre-warmed sterile PBS and allowed to equilibrate for 30 minutes at 37°C. After the equilibration, the TEER of the monolayer was checked, and then the entire medium in both compartments was discarded. Fresh pre-warmed sterile PBS was acquired, and 2.6 mL of this were placed in the basolateral compartment and 1.5 mL of a solution of DCS was placed in the apical compartment. A sample of 1 mL was collected from each basolateral compartment at specific time intervals within a 3-hour period: 0.25, 0.5, 1, 2, and 3 hours. After each sampling period, the PBS in the basolateral compartment was discarded and replaced with a fresh 2.6 mL of pre-warmed sterile PBS to each basolateral compartment, and then placed in a humidified atmosphere of 95% air/5% CO_2 at 37°C. At the end of the entire sampling period, the remaining solution in the apical compartments was also collected for HPLC analysis. The samples were purified by filtration through 0.45 µm membrane filter and transferred to a HPLC vial for analysis. After the collection of the last samples, the TEER of the monolayers was again monitored.

2.11. *HPLC method for DCS analysis*

For the analysis of DCS in unknown samples, a previously published stability-indicating HPLC method developed in our lab for the separation and the detection of DCS was used (*17*). All the chromatographic studies were performed on a Waters Alliance e2696 separations module/2489 UV/Vis detector. The separations were performed on Atlantis T3 5 µm Column (250 × 4.6 mm, Waters, Milford MA, USA). Column effluents were monitored at the wavelength of 220 nm for a run time of 8 minutes at the temperature of 30°C. For the mobile phase, 90% of 10 mM sodium phosphate buffer (pH = 7.5) and 10% acetonitrile was used. The mobile phase was filtered and degassed before use. The flow rate was 0.5 mL/min with the injection volume of 10 µL.

2.12. *Statistical analysis*

Experiments were performed at three different times and means were compared by analysis of variance (ANOVA). Data analysis was performed with Microsoft Excel (Microsoft Corp, Redmond, WA, USA) and a p-value of < 0.05 was considered statistically significant.

3. Results

3.1. *pH*

The pH of gels was found to be in the range of 6.66 (5% HPC) to 7.57 (35% PF-127) (Figure 1). Table 1 shows the pH value of each gel for three weeks. All

polymeric gels showed general trend of stability (The pH difference of ≤ 0.1 from week 1 to week 3) except 5% MC (0.14), 2% HPMC (0.19) and 30% PF-127 (0.11).

3.2. *Viscosity*

Table 2 shows the viscosity profile of HPMC, MC, and HPC polymeric gels at different concentrations. The viscosity of the preparation used in this study ranged between 1.4 cP to 43,000 cP. 4% HPMC showed the highest viscosity (43,000 cP) and 0.5% HPC showed the lowest viscosity (1.4 cP). Viscosity of the 5% HPMC could not be measured because it was beyond the limit of the instrument and spindle available but the viscosity of 5% HPMC was higher than 4% HPMC by visual inspection. Likewise, the viscosity of PF-127 was not measured because of instrument limitations. For the relative comparison to cellulose derivative gels, the data from previously published literature (*35,36*) was used.

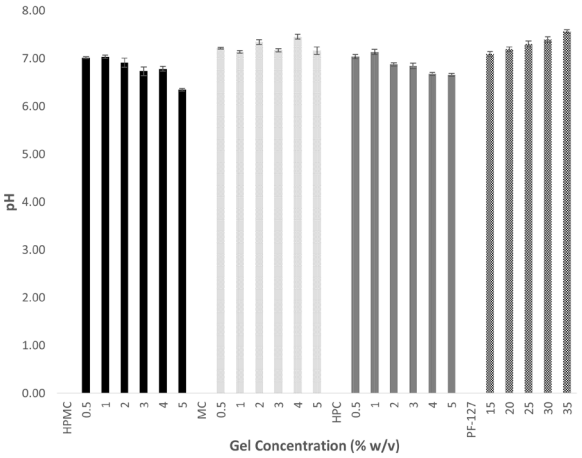

Figure 1. Average pH of Polymeric Gels. The pH of polymeric gels was analyzed using Mettler Toledo pH meter. Data are expressed as mean ± standard deviation (n = 3).

The data and visual inspection clearly indicate that as the concentration of polymer increased, there was an increased viscosity. HPMC, HPC, and MC showed trend of exponential increase in viscosities with an increase in concentration (analyzed by trend line equations). These polymeric gels showed strong correlation between the concentration of gel and the viscosity (R^2 > 0.97-0.99) (Figures 2A-2C). Compared to MC and HPC, the viscosities of HPMC and PF-127 increased relatively much higher as concentration increased. The viscosity of HPMC was in the range of 14.4 cP to 42,296.0 cP for 4% HPMC and it is expected that 5% HPMC would be much higher. The viscosity of MC was in the range of 1.69 cP to 137.5 and HPC was in the range of 1.4 cP to 54.3 cP.

3.3. *Comparison of the effect of different polymeric gels on DCS release*

The drug-release behavior from four different polymeric gels was performed *in vitro* using Distek dissolution apparatus discussed in methods section. All four gels

Table 1. Comparison of pH of gels over 3 weeks

HPMC concentration	Week1	Week2	Week3	AVG pH	ST DEV	CV
0.50%	7.04	7.01	7.00	7.02	0.02	0.00
1.00%	7.01	7.08	7.02	7.04	0.04	0.00
2.00%	7.01	6.91	6.82	6.91	0.10	0.01
3.00%	6.74	6.82	6.64	6.73	0.09	0.01
4.00%	6.83	6.79	6.73	6.78	0.05	0.01
5.00%	6.33	6.38	6.34	6.35	0.03	0.00
MC concentration	Week1	Week2	Week3	AVG pH	ST DEV	CV
0.50%	7.20	7.20	7.23	7.21	0.02	0.00
1.00%	7.11	7.14	7.16	7.14	0.03	0.00
2.00%	7.40	7.32	7.31	7.34	0.05	0.01
3.00%	7.17	7.20	7.14	7.17	0.03	0.00
4.00%	7.52	7.42	7.43	7.46	0.06	0.01
5.00%	7.25	7.12	7.11	7.16	0.08	0.01
HPC concentration	Week1	Week2	Week3	AVG pH	ST DEV	CV
0.50%	7.09	7.01	7.02	7.04	0.04	0.01
1.00%	7.20	7.11	7.10	7.14	0.06	0.01
2.00%	6.91	6.87	6.85	6.88	0.03	0.00
3.00%	6.91	6.82	6.81	6.85	0.06	0.01
4.00%	6.71	6.65	6.67	6.68	0.03	0.00
5.00%	6.69	6.64	6.64	6.66	0.03	0.00
PF - 127 concentration	Week1	Week2	Week3	AVG pH	ST DEV	CV
15.00%	7.15	7.06	7.07	7.09	0.05	0.01
20.00%	7.25	7.17	7.16	7.19	0.05	0.01
25.00%	7.35	7.23	7.33	7.30	0.06	0.01
30.00%	7.45	7.40	7.34	7.40	0.06	0.01
35.00%	7.60	7.56	7.54	7.57	0.03	0.00

Table 2. Comparison of viscosities of polymer gels

Polymeric Gel Type	Concentration (% W/V)	Viscosity (Cp)	Trend line equation	R-squared value
HPMC	0.5	14.4	$y = 8.7768e^{223.24x}$	0.97
	1	94.4		
	2	1,728.3		
	3	7,930.7		
	4	42,296.0		
	5	Not Done		
MC	0.5	1.69	$y = 1.4428e^{94.927x}$	0.98
	1	4.26		
	2	11.53		
	3	29.17		
	4	66.53		
	5	137.5		
HPC	0.5	1.4	$y = 1.108e^{81.172x}$	0.99
	1	2.5		
	2	6.6		
	3	14.8		
	4	29.4		
	5	54.3		

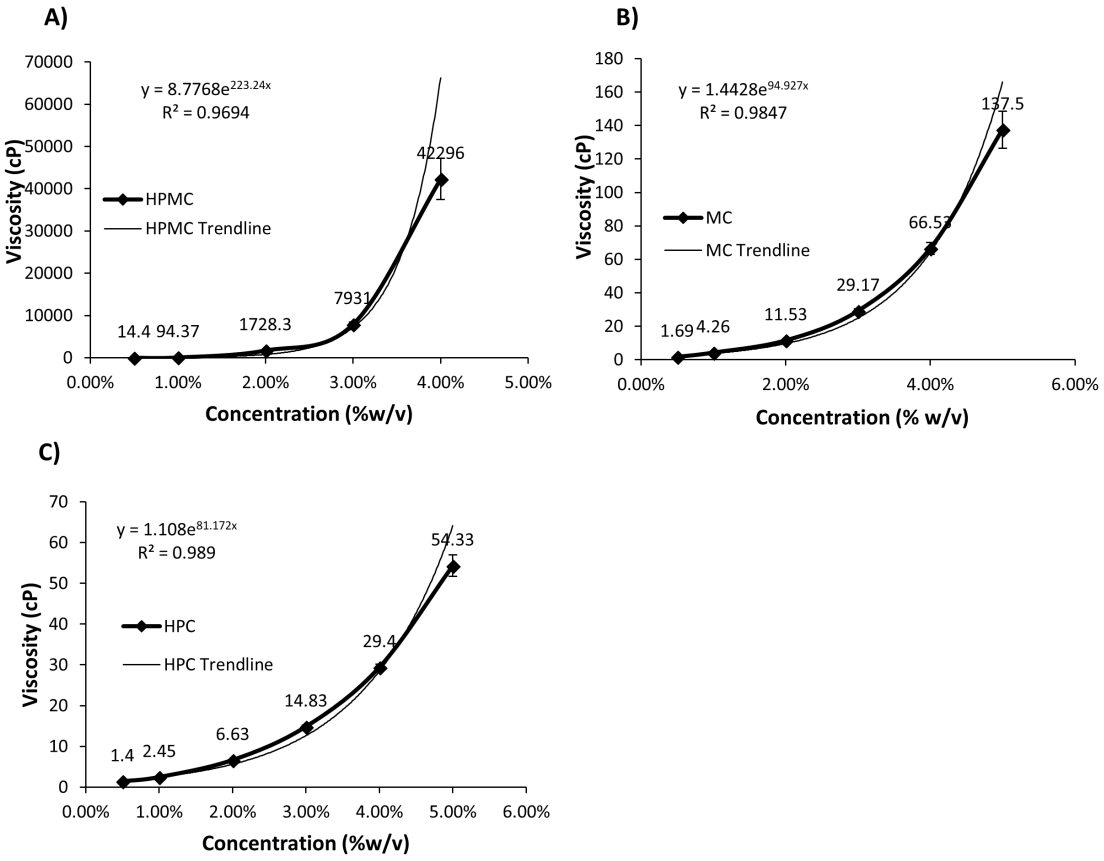

Figure 2. Relationship between the concentration of polymeric gels and the viscosities. The viscosity of polymeric gels was analyzed using the Brookfield DV-III Ultra Rheometer. The data is expressed as a function of concentration of polymeric gels (% w/v) versus the viscosity (cP). Data are expressed as mean ± standard deviation ($n = 3$).

tested showed sustained DCS release over a 24-hour period, but with different rates. All cellulose derivative polymers (HPMC, MC, and HPC) showed the general trend of burst release resulting in greater than 50% of DCS release in the first hour except 5% HPMC, 4% HPC, and 5% HPC. On the other hand, PF-127 could not release 50% of DCS in 24 hours (Figure 3). One percent and 0.5% of HPMC could release > 90% of DCS in two hours. In 24 hours, 99% and 96% of DCS were released from 0.5% and 1% HPMC. Two, 3, and

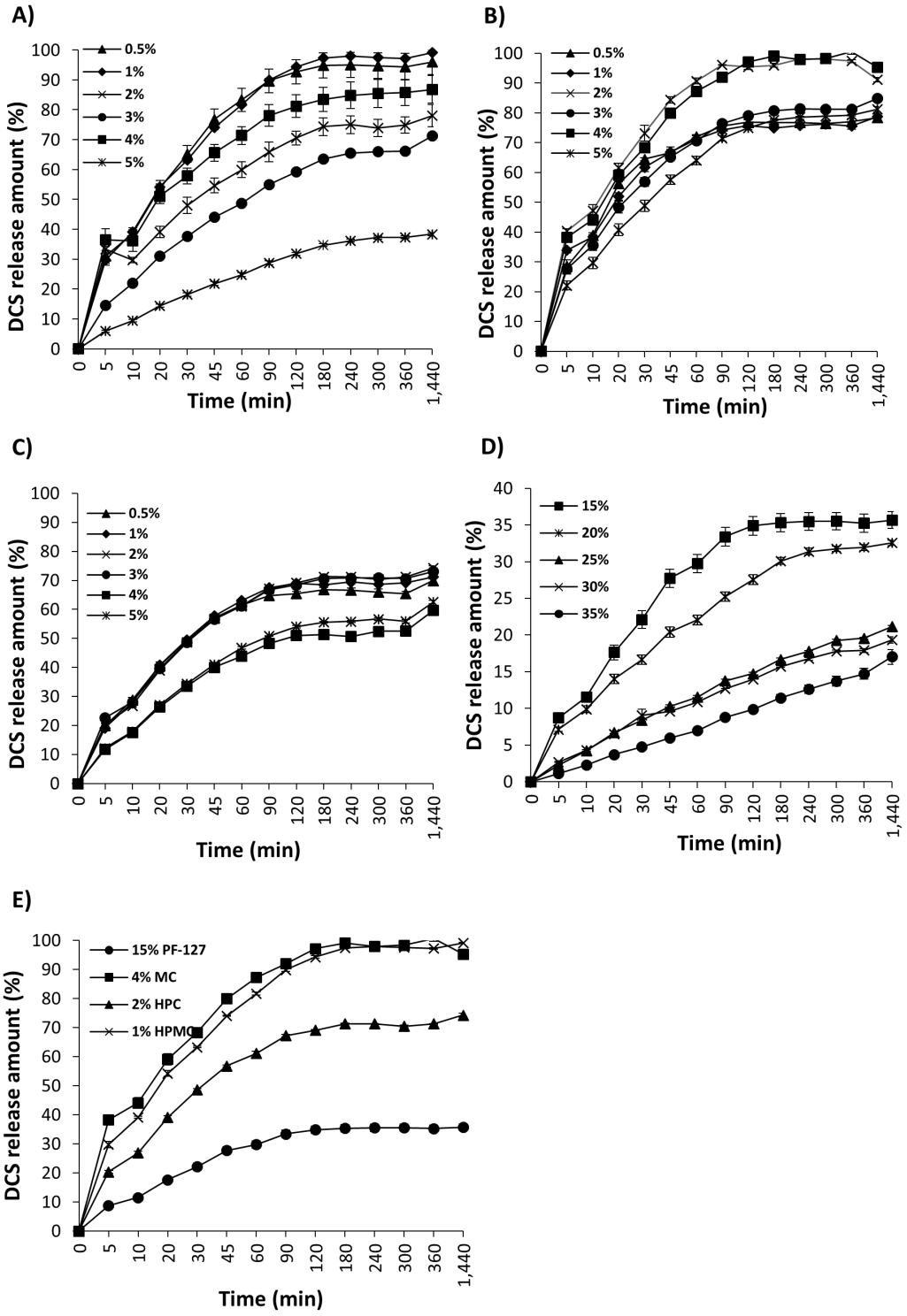

Figure 3. Dissolution of DCS 40 mg/2 mL from polymeric gels. *In vitro* DCS release behavior from **(A)** HPMC, **(B)** MC, **(C)** HPC and **(D)** PF-127 gel was analyzed with the Distek dissolution apparatus and HPLC. (E) Comparison of *in vitro* DCS release behavior of four polymeric gels (HPMC, MC, HPC and PF-127). The data are expressed as a function of time (min) versus cumulative DCS release amount (%). Data are expressed as mean ± standard deviation (n = 3).

4% HPMC were able to release 71, 59, 81% of DCS in two hours and 78, 71, and 87% in 24 hours. Therefore, there was a lack of correlation between the viscosity of HPMC and the drug release behavior. Five percent HPMC significantly retarded drug release to only 32% at 2 hours and 38% at 24 hours. The result may suggest that 5% HPMC is not suitable for the maximum release rate (Figure 3A).

All concentrations of MC gels could release > 50% of DCS at 1 hour and > 75% of DCS at 2 hours. 2 and 4% MC released > 90%, 3 and 5% released > 80%, and 0.5 and 1% > released 78% at 24 hours. Therefore, there was a lack of correlation between the viscosity and drug release behavior for MC as well (Figure 3B).

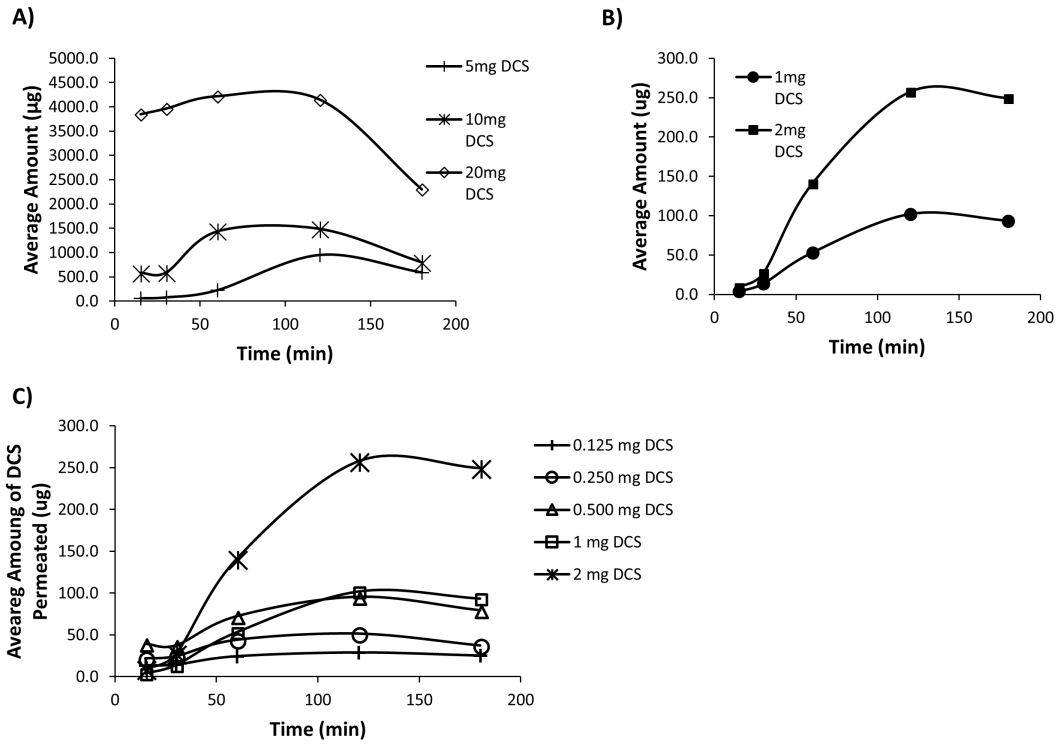

Figure 4. Drug Transport Assay on Calu-3 cells. (A) The analysis of the transport experiment using 5 mg, 10 mg, and 20 mg of DCS. **(B)** The analysis of the transport experiment using 1 mg and 2 mg of DCS. **(C)** The analysis of the transport experiment using 0.125 mg, 0.250 mg, 0.500 mg, 1 mg, and 2 mg of DCS.

The lowest concentration (0.5%), 1%, 2%, 3% of HPC gels could release 50% of DCS at 1 hour. 4% and 5% of HPC gels could release 50% of DCS at 2 hours. However, no HPC gels could release greater than 75% at 24 hours. Among 0.5-5% concentrations of HPC, 2% released the highest concentration (74%) while 4% released the lowest concentration (60%). Again, there was lack of correlation between the viscosity and drug release behavior for HPC gels (Figure 3C).

Unlike other cellulose derivative gels (HPMC, MC, HPC), there was inverse relationship between the concentration of PF-127 gels and DCS release kinetics. As concentration of PF-127 increased, the viscosity increased, and drug release amounts decreased. Moreover, 15% PF-127 was only able to release 36% from initial DCS amount (the highest in PF-127), and 35% PF-127 could release 17% from initial amount of DCS (the lowest in PF-127) (Figure 3D). Figure 3E shows the relative comparison of four polymeric gels with concentrations that showed the highest amount of DCS release. The rank order of the highest to the lowest DCS release profile among them was 1% HPMC > 4% MC > 2% HPC > 15% PF-127. Overall, the rank of polymeric gels that released the highest amount of DCS to the lowest was HPMC > MC > HPC > PF-127.

3.4. *Drug transport assay*

For the drug transport assay, Calu-3 cells which were continuously propagated, grew rapidly and consistently, and were subcultured about once a week. Calu-3 monolayers generally consisted of cuboidal and polygonal cells. Confluent monolayers were generally formed after about 3 days post-seeding when plated at 1×10^5 cells/cm^2 in 6-well cluster plates. Histological staining of cross sections (using a hemocytometer, Bright-Line (Horsham, PA) revealed that Calu-3 cells, when plated at 5×10^5 cells/cm^2, retained a predominant monolayer condition in Transwells.

The chromatogram of DCS standards showed a peak at retention time of 5.6 minutes. A blank sample was also injected to the HPLC system and no peak was observed from this sample. A good linearity was exhibited in the concentration range (1-1,000 µg/mL) by using the presently developed HPLC method. The average coefficient of determination of 0.99 was observed for the standard curve. The slopes of the curves illustrated an excellent agreement with coefficient of variability.

To determine the concentration of DCS that will be used in the study, several concentrations were evaluated in transport experiments: 0.125 mg, 0.250 mg, 0.500 mg, 1.00 mg, 2.00 mg, 5.00 mg, 10.0 mg, and 20.0 mg of DCS were mixed in a 1.5 mL solution. From the Figure 4A, one can see that there is not much of a correlation with the amount of DCS that passed through the monolayer over time. The amount of DCS that passed through the monolayer does however seem to go down after 2 hours of experiment. As a result, lower concentrations of DCS (< 5 mg) were also evaluated.

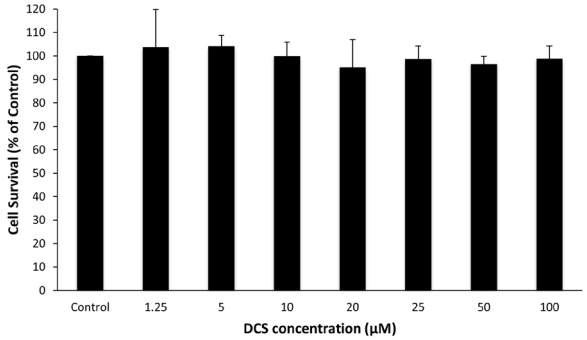

Figure 5. Cytotoxicity of DCS on RPMI 2650 cells. RPMI 2650 cells were treated with 1.25-100 µM concentrations of DCS and the cell viability was determined using the MTT colorimetric assay. Data are expressed as mean ± standard deviation (n = 3).

Figure 4B demonstrates that there is a closer correlation between the amounts of DCS that passed through the monolayer with time. Over the first 2 hours, there is an exponential increase in the amounts of DCS that penetrated through the monolayer, but after two hours, the graph plateaus. However, we further investigated DCS penetration at still lower concentrations (< 1mg). Figure 4C indicates that the lower the concentration the better the correlation between the passage of DCS over time. However, below 1mg of DCS, the correlation starts to deteriorate making 1mg a better choice over other concentrations.

3.5. *In vitro cytotoxicity test in RPMI 2650 cells*

The cytotoxicity of DCS was tested on RPMI 2650 nasal squamous cell carcinoma cells *in vitro*. The cell viability was determined using the MTT colorimetric assay after RPMI 2650 cells were treated with 1.25-100 µM concentrations of DCS. Since four polymeric gels: HPMC, HPC, MC and PF-127 are FDA approved non-toxic polymers, DCS dissolved in distilled water was used alone for the cell viability assay. Figure 5 shows the *in vitro* viability of RPMI 2650 cells after 72-hour treatment with 1.25-100 µM of DCS. Data were compared to the control (cells not treated with DCS) for the relative cell viability. There was no direct relationship between the dose and cell survival. p value of 0.51 indicates no statistically significant result.

4. Discussion

Nasal delivery of DCS, a partial agonist at NMDA receptors, *via* nasal passage could have important applications in many psychiatric disorders such as anxiety disorders (*2,4,5*). However, because DCS is only available in tablets for tuberculosis treatment, which requires higher dosage for longer period than anxiety disorders, the utilization of DCS to enhance exposure treatment in humans, is not firmly established. Besides, the hydrophilic nature of DCS and BBB limits the bioavailability of DCS at NMDA receptors. It could have important implication in anxiety disorders if DCS could be delivered directly into the brain. Nasal delivery system could be an attractive strategy as a means of delivering a drug into the brain due to direct contact of olfactory receptors to both the environment and the CNS. Additionally, the nature of highly vascularized nasal mucosa could provide rapid systemic effect and avoid hepatic first pass metabolism. Delivering DCS through nasal passage with polymeric gels, which bypass crossing BBB, may have advantages in terms of dosage requirements, bioavailability and onset time of action. A major barrier of nasal drug delivery is mucocilliary clearance which leads to low absorption of drugs. Using polymeric gels to prepare DCS nasal formulation could increase viscosity of the formulation, which would increase residence time in the nose thereby facilitating sustained release of DCS (*31*). Prolonging nasal residence time may lead to longer absorption time for the drug to be permeated through nasal mucosa and it could lead to better therapeutic effects. Some polymeric gels are suitable carriers to be used in nasal gel formulation because of their thermoreversible property and biocompatibility. Also, the nasal gel does not require a specialized administration device. In the present study, four polymeric gels with thermoreversible property were selected and screened for the feasibility of using these gels for future development of DCS nasal gel. HPMC, MC, HPC, and PF-127 are polymeric gels that are frequently used as a delivery vehicle for drugs due to their unique characteristics, including thermos-reversibility and safety (*37-41*). In this study, HPMC, MC, and HPC at 0.5-5% w/v and PF-127 at 15-35% w/v were prepared and screened for pH, viscosity and DCS release behavior *in vitro*. Additionally, the permeability and *in vitro* cytotoxicity of DCS in the nasal cells was also evaluated.

pH is a physical parameter that indicates the stability of the products as the change in pH can alter the property of solutions (*42*). "Average baseline human nasal pH is approximately 6.3". It is recommended to keep the final formulation at a pH of 4.5 to 6.5 in order to have the best efficacy (drugs are absorbed in the un-ionized form). The pH of a nasal formulation is also important to avoid irritation of nasal mucosa, prevent growth of pathogenic bacteria, and maintain functionality of excipients such as preservatives (*43*). The pH value ranged from 6.35 to 7.57 and could be easily adjusted with buffer (NaOH and HCl) to maintain proper pH. Furthermore, the prepared gels showed stability over three weeks (Table 1 and Figure 1).

The viscosity of a formulation is directly proportional to the nasal residence time. Ibrahim *et al*. have shown previously, with increasing pluronic concentration, the viscosity increases (*36*). In another study done by El-Kamel, the viscosity of PF-127 gel also increased as the

concentration increased (*35*). All four polymers tested in this study could enhance nasal residence time owing to increased viscosity in a concentration-dependent manner. The exponential increase in viscosity of all gels was observed with the increase in concentration with strong correlation between the concentration of gel and the viscosity (R^2 > 0.97-0.99). Viscosities of the gels ranged between 1.4 cP to 43,000 cP. A relatively large change in viscosity of gels was observed with HPMC and PF-127 compared to HPC and MC (Table 2 and Figure 2). HPMC, HPC, and MC showed exponential increase in viscosities with an increase in concentration and strong correlation between the concentration of gel and the viscosity could be established. Although viscosity of PF-127 could not be measured with the instrument available, other researchers reported that there is a direct relationship between PF-127 concentration and viscosity as well (*35,36*). Viscosities of HPMC and PF-127 were found to be in wider range compared to HPC and MC. This means if we need relatively higher viscosity formulation, we could manipulate HPMC and PF-127 concentrations for future development of DCS nasal gel as long as DCS release behavior is appropriate for anxiety disorders.

Furthermore, these polymeric gels showed that they release DCS in a sustained manner. The release of DCS was almost complete from 1% HPMC, 0.5% HPMC, and 4% MC (> 95%) within 24 hours, while 5% HPMC and PF-127 gels significantly retarded DCS release (< 40%) at 24 hours (Figure 3). In theory, DCS release rate should be decreased as polymeric gel concentrations increase. Overall, our results show that the rate of drug release decreased with increasing PF-127 viscosity (Figure 3D). However, there was a lack of correlation between the rate of drug release and the viscosity of cellulose derivative gels used in this study (Figures 3A-3C). Therefore, complex drug-polymeric gels-water interactions need to be investigated in the future. Currently, there is no defined or optimal viscosity that should be used in nasal formulation. It will depend on the patient's physiological condition. Therefore, the viscosity of the gel may be manipulated for different formulations as desired with the amount of drug that would want to be released to human nasal cells.

The permeability of drug is an important factor for BBB permeation to achieve desired and optimal therapeutic effects (*44*). D-Cycloserine is an ideal drug for intranasal administration as the drug's low molecular weight may provide good absorption of the drug regardless of its hydrophilicity and ionization state (*45*). Also, DCS should be absorbed well through the nasal cavity as DCS shows very good water solubility (*17*). In this project, along with the DCS release behavior of polymeric gels we also conducted *in vitro* permeation studies on Calu-3 cells. A close correlation with an exponential increase (for the first two hours) was observed between the amounts of DCS that passed through the monolayer over time. From Figure 4 we observed that the correlation deteriorates below 1 mg of DCS. However, the space between 2 mg and 1 mg curve was found to be relatively large compared to the space between the 1 mg curve and the lower DCS concentration curves, making the 1 mg concentration a better choice for future studies.

When the nasal formulation is applied to a patient, it should not cause any cell death in nasal cells. The potential toxicity of DCS was tested in RPMI 2650 nasal cells to partially improve the concept of the drug delivery *via* nasal passage. RPMI 2650 cells are derived from squamous cell carcinoma of the human nasal septum, and the cells resemble normal human nasal epithelium (*46*). RPMI 2650 cells are a valid model for an *in vitro* study of nasal drug absorption (*47,48*). No significant cytotoxicity occurred at any concentration between 1.25 µM - 100 µM of DCS in distilled water (p = 0.51). No dose dependent cell death occurred either (Figure 5). Therefore, in conclusion polymeric gels of DCS can be applied safely in nasal cells.

5. Conclusion

The optimal viscosity of the gel that should be used in nasal passage is not defined. Patient's physiological condition, the amount of drug needed, and the implication will define the type and the concentration of polymeric gels. This study was used as an initial screening of the feasibility of using polymeric gels for DCS delivery *via* nasal passage. Based on the overall results of this study and considering the data available in the literature, we firmly believe that HPMC, MC, HPC, and PF-127 could be used in a nasal gel formulation to increase the viscosity and release DCS from formulations in sustained manner at different rates. In the current study, we only looked at DCS release behavior of polymeric gels. In future, we also plan to alter and test the formulation after adding excipients such as buffer (NaOH and HCl) to maintain pH, sodium chloride to adjust osmolarity, and preservatives such as Parabens to prevent microbial growth. Additionally, we also plan to monitor the gelation temperature of the final formulation since it needs to be in the range of 25°C to 37°C to function as a thermoreversible gel. Finally, formulation with minimum concentration of polymeric gel showing acceptable gelation temperature will be selected for the animal studies.

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Original Article

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Clinical effect of long-term administration of tolvaptan in patients with heart failure and chronic kidney disease

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Summary **The effectiveness of long-term administration of tolvaptan in heart failure (HF) patients with chronic kidney disease (CKD) has not been fully studied. Hence, in this study, we investigated the effects of chronic administration of tolvaptan on patients with HF and CKD. We consecutively enrolled 31 patients with acute HF syndrome (AHFS) who were administrated tolvaptan as a long-term medication (TLV group). All patients had a history of prior HF admission and CKD. We also consecutively enrolled 27 patients with AHFS, a prior history of HF and CKD (conventional group). We compared renal function and outcomes between the two groups at discharge for AHFS and after 6 months of follow-up. The estimate glomerular filtration rate (eGFR) was maintained at approximately the same level in the TLV group exhibited approximately the same eGFR (–1.1 ± 8.3 mL/min/1.73 m^2) but decreased in the conventional group (–7.4 ± 10.4 mL/min/1.73 m^2). There was a significant difference in the changes observed in eGFR between the conventional and TLV groups (p = 0.01). There were no significant differences in the frequencies of rehospitalization and death. Long-term administration of tolvaptan may prevent increased renal dysfunction in HF patients with CKD. This conclusion should be confirmed in a large-scale prospective study.**

Keywords: Heart failure treatment, diuretic, renal dysfunction

1. Introduction

Diuretics are an important therapeutic tool for managing heart failure (HF) patients. In particular, loop diuretics are a mainstream therapy that act by reducing fluids in patients with acute HF by inhibiting sodium reabsorption in the loop of Henle. By increasing the distal tubular delivery of sodium, loop diuretics activate the renin-angiotensin system, which causes vasoconstriction of the afferent arteriole and a reduction in renal blood flow. Loop diuretics also activate the sympathetic nervous system, resulting in poor outcomes (*1-3*). Moreover, the use of loop diuretics can lead to serum potassium depletion, which can promote arrhythmias (*4,5*). Thus, loop diuretics are associated with poor outcomes that are broadly predictive of death and morbidity (*6*).

Tolvaptan is a selective vasopressin V_2 receptor antagonist that disturbs the movement of aquaporin 2 to the luminal side of cortical collecting duct cells by activating cyclic adenosine monophosphate (cAMP). In addition, tolvaptan inhibits the reabsorption of water and produces water diuresis through a relatively recently identified mechanism of action (*7,8*).

Tolvaptan is an alternative to the use of loop diuretics that is expected to slow the progression of renal failure and improve the prognosis of HF patients. Specifically, tolvaptan exerts a protective effect on the kidney by initiating a diuretic effect without activating the renin-angiotensin system (*1,2*). Additionally, it has been shown that renal blood flow and the glomerular filtration rate (GFR) are not reduced by tolvaptan (*9*). Hence, it has been suggested that tolvaptan administration reduces the risk of a decline in renal function in patients with acute HF syndrome (AHFS)

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(*10*). Tolvaptan has been shown to be quick-acting when used to treat HF and was used in ACTIVE (*11*) and EVEREST (a test of the efficacy of vasopressin antagonism in HF outcomes study) (*12,13*) studies. However, the primary mechanisms underlying the effects of tolvaptan on renal function have not been determined, and few reports have evaluated its efficacy when administered chronically. In the EVEREST, there was no significant improvement in two primary endpoints of all-cause mortality or in the rate of cardiovascular death or hospitalization for HF (*14*).

However, the results of post-marketing surveillance in Japan demonstrated that 30% of patients with HF were also administered tolvaptan for greater than 2 weeks (*15*). In clinical practice, some patients require chronic administration of tolvaptan. In this study, we investigated the effects of long-term administration of tolvaptan in patients with HF and chronic kidney disease (CKD).

2. Materials and Methods

2.1. *Study population*

This report is a retrospective observational study with no planned protocol. Thirty-one ADHF patients who were administered tolvaptan for 6 months or more from January 2013 to December 2016 were consecutively enrolled in the study. Twenty-seven HF patients with CKD and a past history of admission for HF from January 2013 to December 2016 were also consecutively enrolled. We compared the 31 patients with ADHF (TLV group) to the 27 patients with ADHF and CKD (conventional group).

2.2. *Data collection*

All data were collected retrospectively. Data from laboratory tests included serum creatinine (Cre) levels, serum concentrations of sodium, serum concentrations of potassium, and brain-type natriuretic peptide (BNP) levels. Tests were conducted at admission for ADHF (baseline), at discharge from ADHF and after a 6-months follow-up period. The estimate glomerular filtration rate (eGFR) was calculated using equation coefficients obtained from the modification of diet in renal disease (MDRD) study, which was performed in a Japanese population (*16*). CKD was defined as a syndrome consisting of a low eGFR (< 60 mL min^{-1}•1.73 m^{-2} for longer than 3 months (*17*). Based on the results of a previous study, we considered 20 mg furosemide to be equivalent to approximately 30 mg azosemide (*18*).

2.3. *Outcomes*

We evaluated renal function, dose changes in orally administered diuretics, New York Heart Association (NYHA) classification, ejection fraction (EF) and BNP before and at 6 months after discharge from ADHF.

2.4. *Statistical analysis*

All data were statistically analyzed using a standard statistical software package (StatMate IV ATMS Co., Ltd., Tokyo, Japan). All numerical data are expressed as the mean ± standard deviation. Unpaired Student's *t*-test or the Mann-Whitney U test was used to compare two groups. Categorical variables are expressed as a number (percent) and were compared by the chi-square or Fisher exact test. One-way analysis of variance (ANOVA) was used to detect significant factors among three or more groups. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. *Patients characteristics*

Table 1 shows the baseline clinical characteristics of the conventional and TLV groups. These data were collected at hospital admission for ADHF. There were no significant differences in age, medications, Cre, eGFR and echocardiographic data between the two groups. eGFR at baseline was equivalent in both groups. All patients had a previous history of HF and CKD.

The treatments administered during the acute phase are shown in Table 2. There were no significant differences in the daily dose of furosemide between the conventional and TLV groups. Similarly, there were no significant differences in the inotropic agents used between the two treatment groups.

No side effects were observed, and no patients discontinued tolvaptan in the TLV group. The treatments administered during the chronic phase are shown in Table 3. Concomitant medications included angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, β-blockers, calcium blockers, loop diuretics, spironolactone, thiazide diuretics, and inotropic agents and were not significantly different between the conventional and TLV groups.

3.2. *Changes in renal function*

The changes in renal function observed during the study are shown in Table 4. At the time of hospital discharge, eGFR and Cre had not significantly worsened since admission in either group. However, eGFR had significantly declined in the conventional group at 6 months after discharge (p = 0.001). A comparison of the changes observed in eGFR between discharge and 6 months follow-up in the conventional and TLV groups is shown in Figure 1. In the TLV group, eGFR remained approximately the same (–1.1 ± 8.3), whereas in the

Table 1. Comparisons of clinical characteristics, hemodynamics, laboratory data and underlying heart disease at baseline

Items	Conventional group, *n* = 27	TLV group, *n* = 31	*p*-value
Age (years)	78.4 ± 9.5	76.0 ± 14.2	0.445
Gender/male	12 (44%)	14 (45%)	1.000
BMI	22.4 ± 3.7	21.2 ± 3.7	0.220
HT	17 (63%)	17 (55%)	0.599
DM	14 (52%)	9 (29%)	0.108
DL	9 (33%)	7 (32%)	0.393
CKD	27 (100%)	31 (100%)	
Prior PCI	8 (30%)	6 (19%)	0.540
S/P CABG	2 (7%)	1 (3%)	0.593
Prior HF	27 (100%)	31 (100%)	
Hemodynamics			
SBP (mmHg)	140 ± 36	126 ± 25	0.095
DBP (mmHg)	74 ± 23	68 ± 17	0.258
HR (/bpm)	86 ± 28	80 ± 20	0.370
CS (1/2/3/4/5)	14/6/6/0/1	7/17/5/0/2	0.841
Nohria (A/B/L/C)	0/21/0/6	0/28/0/3	0.909
Killip (1/2/3/4)	0/4/22/1	0/11/16/4	0.878
NYHA (I/II/III/IV)	0/0/2/25	0/2/6/23	0.905
Laboratory data			
Hb (g/dL)	10.8 ± 1.5	10.8 ± 2.1	0.949
Alb (g/dL)	3.6 ± 0.5	3.5 ± 0.6	0.855
T-bil (mg/dL)	0.9 ± 0.5	1.0 ± 0.9	0.644
Na (mEq/L)	140 ± 4	138 ± 6	0.102
K (mEq/L)	4.3 ± 0.8	4.6 ± 0.7	0.152
BUN (mg/dL)	28.4 ± 12.5	36.1 ± 14.6	0.035*
Cre (mg/dL)	1.44 ± 0.50	1.72 ± 0.83	0.133
eGFR (mL/min/1.73m^2)	36.1 ± 12.0	34.2 ± 15.0	0.598
BNP (pg/mL)	873 ± 712	1260 ± 970	0.093
Echocardiographic data			
LVDd (mm)	55.6 ± 11.5	56.4 ± 11.7	0.788
LVDs (mm)	40.7 ± 11.5	42.1 ± 13.2	0.670
EF (%)	50.2 ± 13.6	50.9 ± 15.2	0.842
Underlying Heart Disease			0.684
Ischemic heart disease	10 (37%)	4 (13%)	
Hypertensive heart disease	9 (33%)	11 (36%)	
Cardiomyopathy	2 (7%)	9 (29%)	
Valvular heart disease	6 (22%)	7 (23%)	
HFpEF (≥ 50%)	16 (59%)	20 (65%)	0.788
Medications at admission			
ACE-I/ARB	24 (89%)	27 (87%)	1.000
Beta-blocker	15 (56%)	19 (61%)	0.790
CCB	9 (33%)	12 (39%)	0.786
Loop diuretic	26 (96%)	31 (100%)	0.466
Spironolactone	14 (52%)	15 (48%)	1.000
Thiazide	5 (19%)	9 (29%)	0.378
Tolvaptan	0 (0%)	0 (0%)	1.000
AAD	5 (19%)	5 (16%)	1.000
Digoxin	3 (11%)	6 (19%)	0.481

BMI, body mass index; HT, hypertension; DM, diabetes mellitus; DL, dyslipidemia; CKD, chronic kidney disease; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting; HF, heart failure; SBP, systolic blood pressure; DBP, diastolic blood pressure; Pulse P, pulse pressure; HR, heart rate; CS, clinical scenario; NYHA, New York Heart Association; BNP, brain natriuretic peptide; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter; EF, ejection fraction; MR, mitral regurgitation; HFpEF, heart failure with preserved ejection fraction; ACE-I, angiotensin converting enzyme inhibitor; ARB, angiotensinII receptor blocker; CCB, calcium channel blocker; AAD, anti-arrhythmic drugs.

conventional group, it had declined at discharge (–7.4 ± 10.4).

3.3. *Changes in medication dose*

The dose of loop diuretics was higher (+17.8 ± 12.4) at 6 months after discharge in the TLV group than in the conventional group ($p = 0.02$) (Table 4). There were no significant differences in cardio-protective medications, such as angiotensin-converting enzyme inhibitors and, beta-blockers, between admission and 6-months after discharge in either group (Table 3).

3.4. *Changes in clinical data*

The observed changes in clinical data are shown in Table 4. NYHA classification improved between baseline and discharge in both groups. There were

Table 2. Treatment during the acute phase

Items	Conventional group, $n = 27$	TLV group, $n = 31$	p-value
NIPPV	3 (11%)	5 (16%)	0.712
Carperitide	4 (15%)	2 (6%)	0.402
Nitrate	8 (30%)	6 (19%)	0.540
Nicorandil	1 (4%)	1 (3%)	1.000
Catecholamine	2 (7%)	8 (26%)	0.087
Furosemide infusion	27 (100%)	31 (100%)	1.000

NIPPV, non-invasive positive airway pressure ventilation.

Table 3. Medications used after 6 months of follow-up

Items	Conventional group, $n = 27$	TLV group, $n = 31$	p-value
ACE-I/ARB	24 (89%)	27 (87%)	1.000
Beta-blocker	15 (56%)	19 (61%)	0.790
CCB	9 (33%)	12 (39%)	0.786
Loop diuretic	26 (96%)	31 (100%)	0.466
Spironolactone	14 (52%)	15 (48%)	1.000
Thiazide	5 (19%)	9 (29%)	0.378
Tolvaptan	0	31 (100%)	0.000*
AAD	5 (19%)	5 (16%)	1.000
Digoxin	3 (11%)	6 (19%)	0.481

ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CCB, calcium channel blocker; AAD, anti-arrhythmic drugs.

Table 4. Changes in clinical data

Conventional group	Baseline	At discharge	6 M
eGFR (mL/min/1.73m^2)	36.1 ± 12.0	37.7 ± 16.5	30.3 ± 9.2*
Tolvaptan (mg)	0	0	0
Dose of loop diuretics (mg)	24.4 ± 21.9	45.9 ± 30.9	43.3 ± 29.0
NYHA (I/II/III/IV)	0/0/2/25	21/6/0/0	24/2/1/0
EF (%)	50.2 ± 13.6	-	52.3 ± 22.4
BNP (pg/mL)	873 ± 712	409 ± 444	504 ± 335
TLV group	**Baseline**	**At discharge**	**6 M**
eGFR (mL/min/1.73m^2)	34.2 ± 14.9	36.1 ± 14.9	35.1 ± 16.9
Tolvaptan (mg)	0	6.4 ± 3.8	8.7 ± 5.0
Dose of loop diuretics (mg)	38.1 ± 28.9	50.3 ± 29.6	68.1 ± 42.0*
NYHA (I/II/III/IV)	0/2/6/23	23/7/1/0	24/6/1/0
EF (%)	50.9 ± 15.2	-	51.8 ± 17.9
BNP (pg/mL)	1,260 ± 970	680 ± 489	530 ± 500

NYHA, New York Heart Association; EF, ejection fraction; BNP, brain natriuretic peptide; *$p < 0.05$ versus at discharge in the same group.

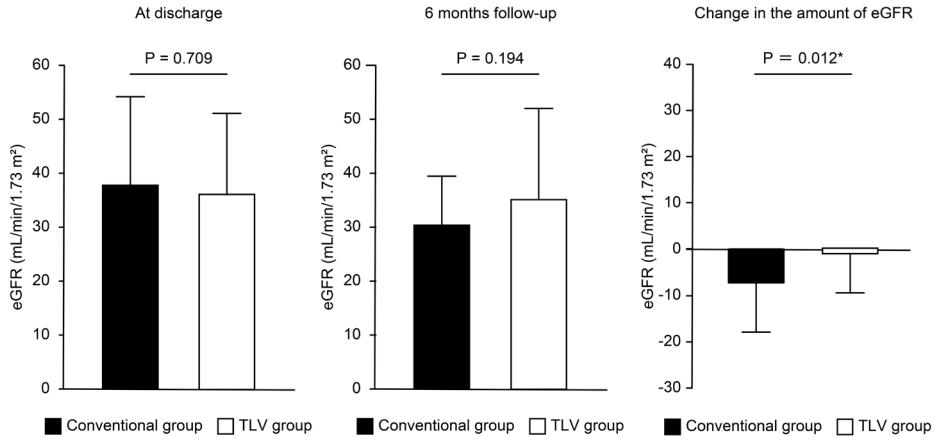

Figure 1. Changes in eGFR between the time of hospital discharge and 6 months later. During this period, there were no significant differences in eGFR in the TLV group, whereas eGFR significantly declined in the conventional group after 6 months of follow-up. The right graph shows a comparison of the changes observed in eGFR between the Conventional and TLV groups. The TLV group exhibited approximately the same eGFR (–1.1 ± 8.3), while eGFR declined in the conventional group (–7.4 ± 10.4).

Table 5. Clinical outcomes

Items	Conventional group	TLV group	*p*-value
All-cause death	0 (0%)	0 (0%)	1.000
Cardiac death	0 (0%)	0 (0%)	1.000
Heart failure hospitalization	8 (30%)	9 (29%)	1.000

no significant differences between the two groups in the changes in clinical data that occurred between discharge and 6 months later. There were no significant differences in EF between baseline and after 6 months of follow-up. BNP was improved in both groups between baseline and discharge. There were no significant differences in the changes that occurred from discharge to 6 months later between the groups. The observed clinical outcomes are shown in Table 5. The frequency of hospitalization for HF was not significantly different between the groups.

4. Discussion

This study investigated the clinical effect of long-term administration of tolvaptan in HF patients with CKD. All patients in this study were dependent on high doses of diuretics for long-term periods of time. Although dose of loop diuretics was increased at 6 months follow-up, renal function did not worsen in TLV group.

Several studies have shown that renal function is an important factor when considering a prognosis for HF (*19,20*). Consequently, preserving renal function is a primary objective in patients with HF. The protective effects exerted by tolvaptan on renal function are likely attributable to several mechanisms. Specifically, Costello-Boerrigter *et al*. reported that renal blood flow and GFR were not reduced by tolvaptan (*21*). In addition, the diuretic effects of tolvaptan may prevent the deterioration of eGFR by ameliorating congestive kidney failure without activating the renin-angiotensin system (*22-24*). In this study, the dose of furosemide was higher in the TLV group. Tolvaptan may therefore exert its renal-protecting effect even in patients administered high doses of furosemide. Renal congestion leads to increased renal interstitial pressure, which affects the entire capillary bed and tubules and can potentially induce local hypoxia. Tubular compression raises luminal pressure, further attenuating the transglomerular pressure gradient, and lowering the GFR (*25*). Tolvaptan is thought to improve renal congestion without promoting renal failure because it affects water diuresis from interstitial tissue. Unfortunately, there is no direct method to assess renal congestion, and causal relationships between chronically administered of tolvaptan and improvements in renal congestion were therefore not investigated in this study.

As no side effect and electrolyte imbalance were observed after chronic administration of tolvaptan, chronic administration of tolvaptan could be a safe treatment for HF patients with CKD.

Several studies have reported that long-term administration of tolvaptan reduced the frequency of admission for HF (*26,27*). However, the frequency of hospitalization for HF was not significantly different between the groups in this study. The dose of loop diuretics is considered a predictor of hospitalization for HF and was significantly higher in the TLV group, which may have affected this outcome (*28,29*). All patients had previously been hospitalized for ADHF and CKD. Previous studies have shown that a past history of hospitalization for HF is an independent risk factor for cardiovascular death in HF patients (*30*). Additionally, CKD is reportedly an independent risk factor for adverse outcomes in HF patients (*19*). As mentioned above, the patients in this study were considered to have poor prognoses. Moreover, indications for administration of tolvaptan were left to the discretion of the physician. The TLV group might have had a more severe background. This outcome should be confirmed in a further prospective large-scale study.

Our study has several limitations. *1*) This was a retrospective observational study, and a small number of patients in a single center were included. *2*) Patient characteristics were not identified. *3*) The indications for long-term administration of tolvaptan were left to the discretion of the physician.

In conclusion, the results of our study suggest that chronic administration of tolvaptan may prevent increased renal dysfunction in HF patients with CKD and prior history of HF. Tolvaptan could be a safe and useful diuretic for HF patients with CKD. This conclusion should be confirmed in a future prospective study.

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Original Article

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Characteristics of gut microbiota and its response to a Chinese Herbal Formula in elder patients with metabolic syndrome

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Summary **Alterations in gut microbiota have been known to play a critical role in metabolic syndrome. However, the microbial features in elderly patients with metabolic syndrome remain unclear. A traditional Chinese Herbal Formula, Yangyin Tiluo Decoction (YTD), can alleviate metabolic syndrome and cardiovascular disease. To characterize gut microbiota in elder patients and effects of YTD on gut microbiota during treatment of metabolic syndrome, 11 healthy elderly persons and 12 elderly persons (aged 60-90 years) with metabolic syndrome were enrolled. The patients were randomly assigned to receive YTD for 4 weeks (200 mL of the decoction two times daily). The microbial composition in healthy control, pre- and post-YTD treatment group were analyzed by 16S rRNA sequencing of fecal DNAs. Biochemical measurements were conducted for elderly patients. The results showed a high inter-individual variation of gut microbiota in elderly persons. The gut microbiota was dominated by phylum Firmicutes and Actinobacteria, which was distinct from the previously defined microbiota in Irish elderly persons. The elderly patients with metabolic syndrome had higher proportions of *Lactobacillus* and *Bifidobacterium*, and lower proportions of *Anaerostipes*, *Coprococcus*, *Ruminococcus* than healthy controls. YTD treatment reduced the abundance of genus Bacteroidales Incertae Sedis and species Enterobacteriaceae Incertae Sedis. The concentration of plasma lipoprotein (a) was also reduced, which was negatively correlated with the abundance of an *Acinetobacter* species. These results reveal a remarkable dominance of Firmicutes and Actinobacteria, and highlight the distinct gut microbiota in elderly patients with metabolic syndrome, which may be involved in pathogenesis. Furthermore, the benefits of YTD treatment were observed, providing an approach to improve metabolic syndrome in elderly patients.**

Keywords: *Lactobacillus,* metabolic syndrome, elderly persons, Chinese Herbal Formula treatment

1. Introduction

Metabolic syndrome (visceral obesity, dyslipidemia, hyperglycemia, and hypertension), has become one of the major public-health challenges worldwide (*1*), especially in elderly persons. The pathogenesis, unified by the putative mechanism of insulin resistance, was thought to be related to interactions between sedentary lifestyle, diet, and genetic factors (*2*). Due to the decline in body function, elderly persons are vulnerable to cardiovascular disorders. Therefore, the etiology of metabolic syndrome can be more complex in elderly persons.

Gut microbiota may have a vital role in metabolic syndrome. The alteration in composition and activity of gut microbiota affects the pathogenesis of obesity and related disorders (*3*). Accumulating evidence suggests that dysbiosis of gut microbiota induced by a high fat/high calorie diet has a key role in the development

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of obesity, insulin resistance and other hallmarks of metabolic syndrome (*4*). Furthermore, reductions in *Bifidobacterium*, butyrate-producing bacteria and increases in pro-inflammatory/pathogenic bacteria are always associated with the development of metabolic syndrome in humans (*5*). Defining the gut microbiota in elder persons with metabolic syndrome can provide reference for possible therapies that target gut microbiota. However, the composition of gut microbiota in elder patients with metabolic syndrome remains unclear.

To improve the quality of life in elderly persons with metabolic syndrome, drugs that treat cardiovascular diseases may be useful. Among the treatments, Chinese Herbal Formulas (TCMs) can serve as valuable therapeutic strategies and drug discovery resources (*6*). TCMs, which are forms of polypharmacy, have been clinically used in China for treatment of many diseases for thousands of years (*7*).

Yangyin Tiluo Decoction (YTD) is used to treat cerebral infarction in elders in China. Clinical studies found that YTD could alleviate metabolic syndrome in elderly persons (*8*). YTD treatment could also downregulate the expression of tumor necrosis factor α and interleukin 6 in lipopolysaccharide-treated human venous endothelial cells (*9*), supporting its function in alleviating cardiovascular diseases. It also suggested that the excess caloric consumption driving the current epidemic of metabolic syndrome may be caused by alterations in host-microbiota interactions (*10*). Even though, whether YTD could modulate gut microbiota during treatment of metabolic syndrome is still unknown.

In this study, we conducted a controlled, pre- and post-treatment comparison trial to define the characteristics of gut microbiota related to metabolic syndrome in elderly persons. Furthermore, the structural alterations of gut microbiota in response to YTD were also investigated. These results provide reference for understanding gut microbiota in elderly persons with metabolic syndrome.

2. Materials and Methods

2.1. *Ethics, consent and permissions*

The study was approved by the Ethics Committee of Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (No. 2013NL-029-02, registered 19 April 2013), and written informed consent was obtained from each participant before their admission to the protocol.

2.2. *Study design*

The study was a 4-week, pre- and post-treatment comparison trial with healthy controls. Participants were recruited by Geriatrics Department of Jiangsu Province Hospital of Traditional Chinese Medicine from July 2013 to June 2015, during which the samples were collected. The authors had access to information that could identify individual participants during or after data collection.

Patient enrollment was conducted according to inclusion and exclusion criteria. The inclusion criteria were: *i*) previously diagnosed metabolic syndrome patients; *ii*) age of 60-89 years; *iii*) good compliance; *iv*) signed informed consent. Volunteers who met all four criteria were included as disease group, and those who met *ii*)-*iv*) were included as the healthy control group. Exclusion criteria were: *i*) the etiology was too complex to obtain clear evaluation about the efficiency and safety of the drug; *ii*) disability and psychiatric disturbance; *iii*) presence of cancer; *iv*) history of abdominal operation or intestinal organic lesion; *v*) allergic persons; *vi*) treatment with antibiotics, probiotics, antidiarrheal agent, or swelling agent within the past 1 month; *vii*) unitary or inappropriate diet structure, dietary intake much higher or lower than normal elder persons.

Metabolic syndrome was defined using the 2004 Chinese Diabetes Society criteria, according to the presence of three or more of following aspects: *i*) overweight and/or obesity: BMI ≥ 25.0 kg/m^2; *ii*) hyperglycemia: fasting plasma glucose ≥ 6.1 mmol/L and/or 2 h plasma glucose ≥ 7.8 mmol/L, or previously diagnosed type 2 diabetes and receiving treatment; *iii*) systolic blood pressure/diastolic blood pressure ≥ 140/90 mmHg, or previously diagnosed hypertension and receiving treatment; *iv*) dyslipidemia: fasting plasma triglyceride ≥ 1.7 mmol/L (150mg/dL), and/or fasting plasma HDL-c < 0.9 mmol/L (men) or < 1.0 mmol/L (women) (*10*).

Using the screening criteria, 11 healthy elderly people were assigned as the healthy control group, while 12 patients were treated with YTD for four weeks. The information of selected subjects can be found in Table 1. No missing data were observed for each variable of interest. All the participants were advised to take low-salt, low-fat diabetic diet, and to avoid taking medication that may affect the gut microbiota, such as prebiotics and probiotics. The variables were included in data analysis to avoid biases. The study size was chosen based on the numbers of patients passing the inclusion criteria and the requirement for statistical analysis.

2.3. *Drug administration*

The Yangyin Tiluo Decoction (YTD) is a TCM composed of *Polygonatum sibiricum* (20 g), *Lycium barbarum* (15 g), *Rehmannia glutinosa Libosch* (12 g), *Rhodiola rosea* (20 g), *Panax notoginseng* (5 g), *Ligusticum chuanxiong Hort* (10 g), *Lumbricus* (10 g), *Radix puerariae* (20 g), and *Folium nelumbinis* (20 g). The preparation of YTD was conducted based on the Standard Operating Procedure of Jiangsu Province Hospital of Traditional Chinese Medicine. Briefly, all the components were put

Table 1. Characteristics of the study subjects

Items	Healthy control	Patients with metabolic syndrome
Age	71.8 ± 11.5	82.9 ± 3.3
n	11	12
Gender (male/female)	6/5	8/4
Height (cm)	1.66 ± 0.05	1.68 ± 0.04
Weight (kg)	65.9 ± 5.0	72.2 ± 8.3
BMI (kg/m^2)	24.1 ± 1.5	25.6 ± 2.7
> 25	4	7
< 25	7	5

Values of age, height, weight, and BMI are expressed as mean ± SD.

into a ceramic pharmacy pot, and 300 mL cold drinking water was added to soak for 1 hour. After boiling with a raging blaze for 10 min, it was kept boiling for 20 min with small fire. The decoction was filtered into 200 mL bags (part 1) while the decoction was hot. Then another 300 mL cold drinking water was added to the residue, and the boiling process was repeated. The decoction was filtered into another set of 200 mL bags (part 2). Part 1 and 2 were mixed homogeneously, then divided into two equal parts, and each part was taken after meals in the morning and evening respectively for 4 weeks.

2.4. *Sample collections*

For healthy groups, the first feces portion at morning was collected at the beginning of the experiment. For disease group, the first feces portion and fasting venous blood were sampled at the beginning of the experiment (before YTD treatment) and after YTD treatment for 4 weeks. Samples were kept at -80°C before further analysis.

2.5. *Fecal DNA extraction and pyrosequencing*

All fecal samples from healthy and diseased persons were used for fecal DNA extraction, including 11 samples of healthy control, 12 samples of patients before YTD treatment, and 12 samples after YTD treatment. Total genomic DNA in feces were extracted from 0.3 g of sample using bead-beating and phenol-chloroform extraction according to Zoetendal *et al*. (*11*). The quality and concentration of DNA were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA).

Amplification of genomic DNA was performed using barcoded primers that targeted the V3 to V4 regions of the bacterial 16S rRNA gene, with universal bacterial primers (forward 5'-barcode-TAC GGR AGG CAG CAG-3' and reverse 5'-AGG GTA TCT AAT CCT-3'), where barcode is an eight-base sequence unique to each sample. Procedures of amplification, amplicon purification, sequencing, and basic analysis were conducted according to previous study (*12*). Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (accession SRP118482).

Raw fastq files were demultiplexed, quality-filtered using QIIME version 1.17 (*13*) with the following criteria: *i*) The 250 bp reads were truncated at any site receiving an average quality score < 20 over a 10 bp sliding window, discarding the truncated reads that were shorter than 50 bp; *ii*) exact barcode matching, 2 nucleotide mismatch in primer matching, reads containing ambiguous characters were removed; *iii*) Only sequences that overlaped longer than 10 bp were assembled according to their overlap sequence. Reads which could not be assembled were discarded. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE (version 7.1 *http://drive5.com/uparse/*) and chimeric sequences were identified and removed using UCHIME. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by Ribosomal Database Project Classifier (*http://rdp.cme.msu.edu/*) against the Silva (SSU123) 16S rRNA database using a confidence threshold of 70% (*14*). The rarefaction analysis, alpha diversity, and taxonomic assignment were also analyzed in QIIME. Unweighted Unifrac principal coordinate analysis (PCoA) (*15*) based on OTUs was performed to provide an overall view of the microbial structures.

2.6. *Biochemical measurements*

During the experiment, nine patients after YTD treatment finished the essential procedures for sampling fasting venous blood. Biochemical measurements of plasma glucose, total cholesterol, triglyceride, lipoprotein (a), low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and uric acid were conducted in the central laboratory of Jiangsu Province Hospital of Traditional Chinese Medicine (Nanjing, China). The biochemical parameters were analyzed using an automatic analyzer (Beckman AU5800; Beckman Coulter, USA). Lipoprotein (a) is a useful indicator in patients with cerebrovascular disease and atherosclerosis.

2.7. *Statistical analysis*

Homogeneity of variance was tested for all measures.

To characterize the gut microbiota in elder persons with metabolic syndrome, microbiome data from patients before YTD treatment were analyzed against healthy persons and submitted for LEfSe analysis. LEfSe algorithm can identify differentially abundant features between two or more biological conditions (*16*). This method defines discriminative features based on the following conditions: *i*) the alpha value for the factorial Kruskal–Wallis test among classes is < 0.05 and *ii*) the threshold on the logarithmic linear discriminant analysis (LDA) effect sizes for is > 2.0. LEfSe was performed on the website *http:// http://huttenhower.org/galaxy*.

To compare the effect of YTD treatment, biochemical measurement data and microbiome were analyzed using paired Wilcoxon test with modules implemented in MetaboAnalyst version 3.0 (*17*). Significance was considered at $p < 0.05$. In case of multiple comparisons, *p*-values were adjusted with a false discovery rate (FDR) analysis (*18*), limiting the overall false discovery rate to 5% ($q < 0.05$). Adjustments of comparisons for age and gender were performed using analysis of covariance (SPSS verson 20.0; SPSS Inc., Chicago, Illinois).

Correlations between microbial composition with biochemical parameters were further analyzed using Spearman's rho correlation analysis (GraphPad Prism version 6.0, GraphPad Software, San Diego, CA). Correlation was considered significant when the absolute value of Spearman's rank correlation coefficient (Spearman's rho) was above 0.6 and statistically significant ($p < 0.05$).

3. Results

3.1. *Summary of pyrosequencing data*

In total 1,281,404 qualified bacterial 16S rRNA gene reads were obtained from 35 fecal samples (36,611 reads per sample) after pyrosequencing and used for subsequent analysis. The rarefaction analysis including all samples revealed a curve approaching saturation (Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=25*), indicating that the measurement at the threshold level covers almost the full extent of the microbial diversity. The abundance-based coverage was higher than 0.99 for all three groups (Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=25*). The estimators of microbial diversity and richness, including Chao1 index, and Shannon and Simpson diversity index, was not significantly different between groups ($p > 0.05$, Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=25*).

3.2. *High inter-individual variation of the elder gut microbiota*

OTU-based unweighted principal coordinates analysis showed that the overall structures were not significantly different between groups ($p > 0.05$). We noted the scattered distribution of different samples in healthy elderly persons (Figure 1A). Taxonomy-based analysis showed that the relative abundance of Bacteroidetes ranged from 0.27% to 50.9% and that the relative abundance of Actinobacteria ranged from 0.44% to 31.4%, which indicated a high inter-individual variation of gut microbiota in elderly persons (Figure 1B).

The unweighted PCoA showed that the response to YTD treatment varied between patients. Sample IDs 2, 6, 7, and 10 showed a huge shift in microbial composition while others showed a slight shift (Figure 1A), suggesting an individual-specific response to YTD treatment.

3.3. *Firmicutes- and Actinobacteria-dominant gut microbiota in the elder cohort*

At the phylum level, a total of 14 phyla were observed. In the cohort, Firmicutes was the most dominant phylum with an average abundance range from 52.3% to 72.8% (Figure 2A). In patients before and after YTD treatment, Actinobacteria was also dominant, which contributed 22.2% and 22.4% of gut microbiota before or after YTD treatment respectively. Bacteroidetes

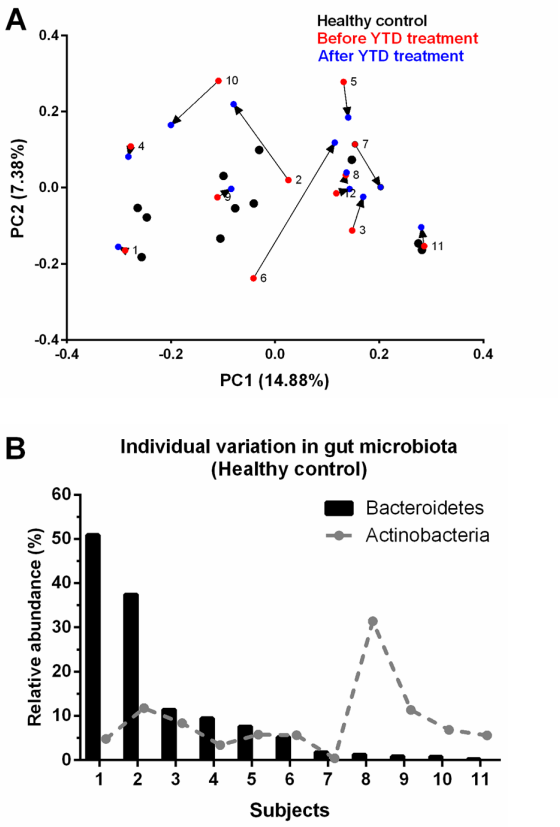

Figure 1. Principle coordinate analysis by unweighted Unifrac distance (A) and individual variation in the abundances of Bacteroidetes and Actinobacteria in healthy controls (B). Abbreviations used: YTD, Yangyin Tiluo Decoction.

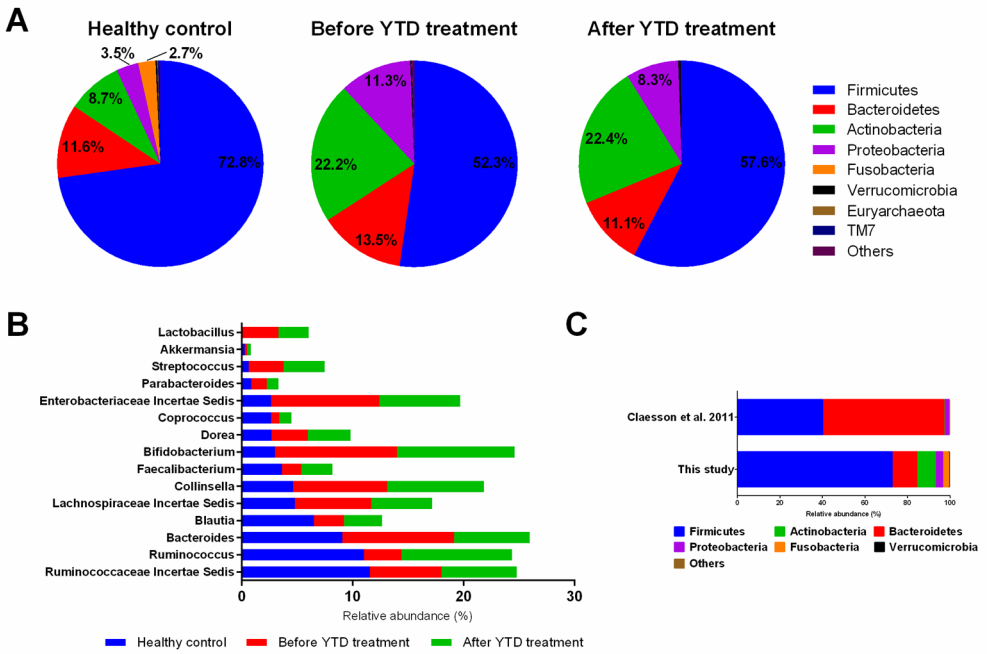

Figure 2. Fecal microbiota composition at phylum (A) and genus (B) level from elderly persons. (C) Comparison of microbiota composition with studies of Irish elderly population. Abbreviations used: YTD, Yangyin Tiluo Decoction.

constituted the next dominant phylum with the average abundance range from 11.1% to 13.5%. The Firmicutes- and Actinobacteria- dominant microbiota were also apparent at the genus level, as reflected by the dominant genus Ruminococcaceae Incertae Sedis, *Ruminococcus*, *Bifidobacterium*, and *Collinsella* (Figure 2B). When comparing the microbial composition between studies (Figure 2C), the dominant phylum was Firmicutes (average abundance 57%) followed by Bacteroidetes (average abundance 42%) in an Irish cohort (*19*), which was quite different from the healthy control group and patient group in the present study.

3.4. *Characteristics of fecal microbiota in elder patients with metabolic syndrome*

By using LEfSe analysis, a total of 24 microbial features were found to be different between healthy controls and elderly patients with metabolic syndrome before YTD treatment, with nine enriched in metabolic syndrome patients and 15 enriched in healthy controls (Figure 3). The fecal microbiota of untreated metabolic syndrome patients exhibited a relative abundance of Actinobacteria (Bifidobacteriaceae, *Bifidobacterium*, Bifidobacteriaceae Incertae Sedis), Lactobacillaceae (*Lactobacillus*), and *Megasphaera* compared to healthy controls. Interestingly, the relative abundance of *Lactobacillus* was increased by 187-fold in untreated metabolic syndrome patients (3.28%) compared to untreated metabolic syndrome patients (0.018%). On the contrary, metabolic syndrome patients had a lower abundance of phylum Firmicutes, families Erysipelotrichaceae, Clostridiaceae, and Turicibacteraceae, genus

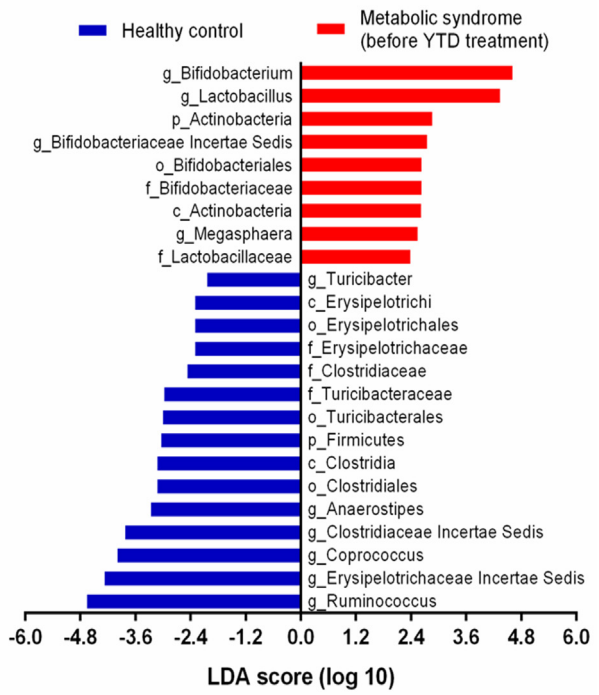

Figure 3. Differences in the gut microbiota composition between healthy controls and elderly patients with metabolic syndrome (before YTD treatment). Abbreviations used: YTD, Yangyin Tiluo Decoction.

Turicibacter, *Anaerostipes*, *Ruminococcus*, *Coprococcus*, Clostridiaceae Incertae Sedis, and Erysipelotrichaceae Incertae Sedis than healthy controls. The detailed results of relative abundances and LDA scores can be found in Table S2 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=25*).

3.5. *Effects of YTD treatment on gut microbiota and biochemical parameters*

Compared with the untreated patients, YTD treatment increased the relative abundance of Moraxellaceae, *Acinetobacter*, species *Acinetobacter* Incertae Sedis and Erysipelotrichaceae Incertae Sedis, while it decreased the relative abunance of Alphaproteobacteria, Rhizobiales, genus Bacteroidales Incertae Sedis, and species Enterobacteriaceae Incertae Sedis ($p < 0.05$, Figure 4A). The abundance of species *Acinetobacter* Incertae Sedis and Erysipelotrichaceae Incertae Sedis was increased by 18.9-fold and 86.7 fold, respectively. The abundance of genus Bacteroidales Incertae Sedis and species Enterobacteriaceae Incertae Sedis were decreased by 21.5-fold and 2.47-fold, respectively.

Analysis of plasma parameters indicated that YTD treatment reduced the concentrations of lipoprotein (a) ($p < 0.05$, Figure 4B, Table 2), while it did not affect the concentrations of glucose, cholesterol, triglyceride, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and uric acid ($p > 0.05$, Table 2). Correlation analysis identified the significant negative correlation between the concentrations of lipoprotein (a) with species *Acinetobacter* Incertae Sedis (Figure 4C).

4. Discussion

Changes in gut microbiota have been associated with the presence of metabolic syndrome. In the present study, by analyzing microbiota composition in a Chinese elderly population, we found that Chinese Herbal Formula YTD altered microbial composition and decreased metabolic markers of cerebrovascular disease in metabolic syndrome patients. These results are of great significance for targeting gut microbiota to improve health in elderly persons.

4.1. *Characteristics of gut microbiota in the Chinese elderly populations: inter-individual variability*

Employing the Chinese elderly population, we clearly showed high inter-individual variability of gut microbiota between subjects. These features have also been found in healthy Irish elderly populations (*19*) or hospitalized Italian elderly populations (*20*). Multiple factors, such as diet, physiological condition, and age, can increase the inter-individual variability of gut microbiota. Accompanying the individualized microbiota observed, the response of gut microbiota to YTD treatment was also individual-specific. It cannot be determined which factor contributes to the outcome. However, this

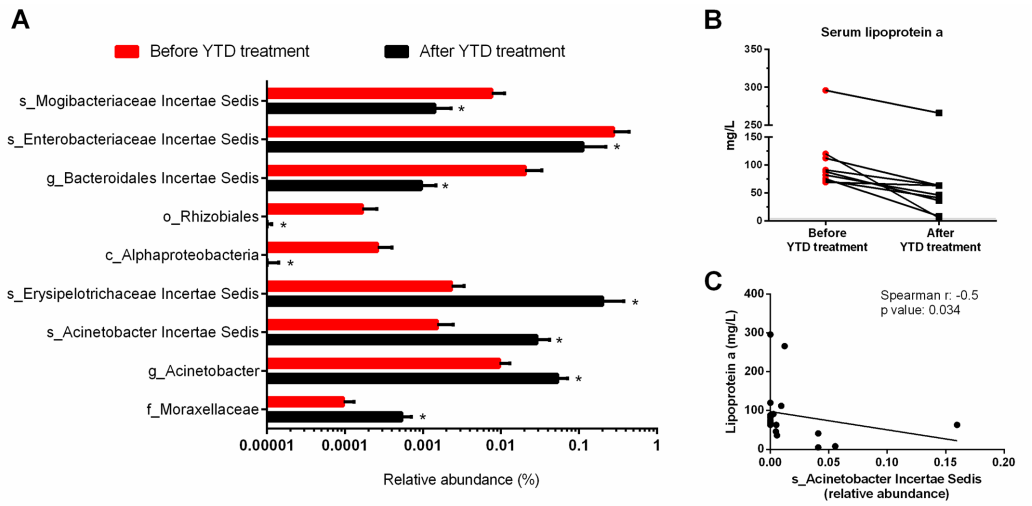

Figure 4. Effects of YTD treatment on the microbial composition (A) and plasma lipoprotein (B). (C) Correlations between significantly changed taxa with lipoprotein (a). Abbreviations used: YTD, Yangyin Tiluo Decoction.

Table 2. Plasma biochemical parameters in elder patients

Items	Before YTD treatment	After YTD treatment	*p*-value
Glucose (mmol/L)	5.96 (4.54-8.03)	6.98 (3.66-10.18)	0.49
Cholesterol (mmol/L)	3.41 (2.22-4.96)	3.22 (2.38-5)	0.747
Triglyceride (mmol/L)	1.05 (0.65-2.13)	0.98 (0.65-2.16)	0.741
Lipoprotein (a) (mg/L)	88 (69-296)	46 (5-266)	0.002
Low-density lipoprotein cholesterol (mmol/L)	1.95 (1.08-2.97)	1.97 (1.32-2.93)	0.848
High-density lipoprotein cholesterol (mmol/L)	1.03 (0.74-1.36)	0.89 (0.72-1.41)	0.855
Uric acid (μmol/L)	348.3 (214.2-462.9)	285.4 (217.8-394.5)	0.134

Abbreviations used: YTD, Yangyin Tiluo Decoction.

phenomenon highlights the importance of considering individualized treatment for modulating gut health.

4.2. *Increase of lactic acid bacteria and decrease of butyrate-producing bacteria in elderly patients with metabolic syndrome*

A novel finding of the present study was that the elderly patients with metabolic syndrome had higher proportions of *Lactobacillus* and *Bifidobacterium*, and lower proportions of butyrate-producing bacteria than healthy controls. *Lactobacillus* and *Bifidobacterium* are generally considered to be health-promoting, and some species are widely used as probiotics (*21*). The abundances of *Lactobacillus* were also increased in type 2 diabetic patients (60 ± 8 years old) compared with healthy controls (40 ± 8 years old) in a South China population (*22*). An increase in *Lactobacillus* and a decrease in butyrate-producing bacteria have been also observed in type 2 diabetes patients (*23,24*), inflammatory bowel disease patients (*25*), and high-fat diet-induced fatty liver mice (*26*). Interestingly, both diabetes and inflammatory bowel disease patients had an elevated pro-inflammation response *in vivo*. In elderly persons, the predisposition to pro-inflammation response can be easier in gut due to the aging process (*27*). *Lactobacillus* species, such as *Lactobacillus plantarum* could induce interleukin 1β production and increase the inflammatory response (*28*). A previous study suggested that in metabolic syndrome patients, an increase in intestinal glucose availability may raise the substrate for *Lactobacillus* (*23*). Therefore, whether the boost of lactic acid bacteria contributes to the immunological response in the elderly metabolic syndrome is interesting to be studied in the future.

4.3. *YTD modulated gut microbiota and reduced plasma lipoprotein (a)*

YTD also decreased the concentration of lipoprotein (a), a well-established risk factor of cardiovascular disease and atherosclerosis. Decrease of lipoprotein (a) reflected the clinical effect of alleviating metabolic syndrome in elderly persons by YTD. Interestingly, a negative correlation was identified between lipoprotein (a) and an *Acinetobacter* species, suggesting a possible linkage between gut microbes and biochemical parameters. The metabolic product of *Acinetobacter*, an endophytic bacteria of *Lycium barbarum* (a major component of YTD), has antimicrobial activity against *Staphylococcus aureus* (*29*). These results further support the benefit of YTD to alleviate metabolic syndrome.

4.4. *Which component of YTD causes changes in gut microbiota*

Recent studies have found that a considerable number of Chinese herbs can directly or indirectly affect gut microbes. Coincidentally, *Polygonatum sibiricum*, *Rehmannia glutinosa Libosch*, *Lycium barbarum* and *Radix puerariae* in YTD have been proved to significantly change the intestinal microbial structure of patients with metabolic diseases in multiple studies.

Polygonatum sibiricum contains a variety of beneficial elements, such as polysaccharides, steroidal saponins, anthraquinones, alkaloids, cardiac glycosides, lignin, vitamins and amino acids. Wang YF (*30*) used the polysaccharide of *Polygonatum sibiricum* to interfere with the intestinal flora of rats with lipid metabolism disorder. Yun Nan rhizoma polygonati polysaccharide can reverse Firmicutes and Bacteroidetes, reduce the relative abundance of Proteobacteria, *Lactobacillus* and *Psychrobacter*, and show favorable effects on the composition of gut microbiota.

One of the main components of *Rehmannia glutinosa* Libosch is stachyose. Stachyose addition affects gut microbiota of STZ induced diabetic mice (31). Compared with the normal group, in the stachyose group, Proteobacteria ($p < 0.01$), but decreased significantly in Firmicutes ($p < 0.01$) and decreased slightly in Bacteroidetes; the numbers of bacteria significantly decreased in *Lactobacillus* and *Bacteroides* ($p < 0.01$) but significantly increased in *Helicobacterium* and *Mycoplasma* ($p < 0.05$).

The main ingredients of *Lycium barbarum* are lyceum barbarum polysaccharide, betaine, riboflavin, sterol, rutin and lycium, and various amino acids and trace elements (*32*). Liu YT (*33*) found that low concentration extracts of Fructus Lycii showed promoting effects on *Bifidobacterium* and *Lactobacillus*, which was better than that of the high concentration group. However, the promoting effects on enterobacteria and enterococcus were not obvious.

The main active components of Radix Puerariae are pueraria isoflavones, daidzein and puerarin 7-xyloside. Xu J (*34*) examined the effect and mechanism of Gegen Qinlian decoction (GQD, a traditional Chinese herbal formula containing pueraria as the main component) on the treatment of type 2 diabetes by investigating intestinal flora composition. They found that patients who were given a high dose of GQD could significantly recover their blood sugar levels, as well as the levels of HbA1c. Analysis of gut microbiota composition found that GQD treatment changed the abundance of 47 phylotypes, 17 of which are negatively correlated with blood glucose levels, and 9 that are negatively correlated with HbA1c levels. The study confirmed that GQD could change the intestinal flora by regulating the number of probiotics in the intestinal tract, thus achieving the function of treating type 2 diabetes.

Therefore, we hypothesized that YTD may regulate the gut microbiota through one or more active ingredients of these Chinese herbs or ingredients, thus contributing to the clinical efficacy of alleviating metabolic syndrome.

In conclusion, by analyzing the gut microbiota in elderly persons, a high inter-individual variation in gut microbiota was observed. The Chinese elderly populations employed in this study had a gut microbiota dominated by Firmicutes and Actinobacteria. Our result further highlights the distinct fecal microbiota in elderly patients with metabolic syndrome compared with healthy controls. Metabolic syndrome patients had higher relative abundance of *Lactobacillus* and *Bifidobacterium* and lower *Anaerostipes* and *Coprococcus* than healthy controls. The Chinese herbal formula Yangyin Tiluo Decoction could reduce the abundance of potentially pathogenic bacteria and lipoprotein (a), which provides an approach to treat elderly patients with metabolic syndrome. Collectively, these findings gain insights into the gut microbiota of elderly patients with metabolic syndrome, and the role of YTD in modulating gut microbiota during metabolic syndrome treatment.

Acknowledgements

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Availability of data and materials

All data generated and analyzed during this study are included in this published article. The raw sequence reads were deposited into NCBI Sequence Read Archive (SRA) database (accession SRP118482).

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Original Article

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Exploring the causes of peripheral intravenous catheter failure based on shape of catheters removed from various insertion sites

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Summary **The risk of peripheral intravenous catheter failure varies according to the insertion site. This study examined catheter shape just after removal to evaluate the causes of catheter failure according to site. This study was a secondary analysis of previous study data. Our observational study was conducted during a 6-month period at The University of Tokyo Hospital. Participants were hospitalized adults who received infusion therapy *via* a short peripheral catheter. We acquired ultrasound images of blood vessels and surrounding tissues at the catheter insertion site before catheter removal and clinical images of the removed catheters. We analyzed 184 catheters from 142 participants. There were no significant differences in the catheter failure rate (29.9%) among insertion sites. Curvature in the middle of the catheter was present in 9.2% of cases; the median bend angle at the catheter base was 9.1° (range: 0.0°-68.3°). The bend angle of catheters inserted in the upper arm was significantly greater than of catheters in the forearm (p = 0.013). Catheter curvature was related to catheter failure (14.8% of failed catheters had curvature; p = 0.035) and occlusion (35.3% of occluded catheters had curvature; p = 0.008) in upper arm and forearm placements. The median distance from the elbow to the insertion site was shorter for failed catheters than for surviving catheters. To prevent catheter failure, especially occlusion resulting from catheter curvature, a catheter should be inserted at an appropriate insertion site far from the antecubital fossa.**

Keywords: Catheter deformation, peripheral intravenous catheterization, peripheral venous access, short peripheral intravenous catheter

1. Introduction

Hospitalized patients often require intravenous therapy. Approximately 300 million peripheral intravenous catheters (PIVC) are used annually in the United States (*1*) and 59% of hospitalized patients have at least one PIVC in place, according to international data (*2*). Regardless of the reason for use, it is important that healthcare providers use an appropriate vascular access device and vein to prevent catheter failure, which can result in adverse events or occlusion (*3-5*).

We previously recommended selecting a vein that is approximately three times as large as the outer diameter of the PIVC to prevent catheter failure (*6*). Large veins are preferable; for example, veins in the upper arm are larger than those in the forearm (*7*). If an appropriately sized vein cannot be found in the forearm, then a suitable vein in the upper arm should be sought. However, it is reported that catheter insertion in the hand, antecubital fossa, or upper arm is associated with higher rates of occlusion (defined as any circumstance

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in which the PIVC remains in place but catheter flushing and fluid infusion are not possible) than catheter insertion in the forearm (*8*). It is possible that re-expansion of subcutaneous fat in the upper arm after catheter insertion distorts the catheter track, potentially dislodging or kinking the catheter (**9**). The current guidelines recommend limiting catheter sites to the upper extremities, with a preference for distal locations that avoid the wrist (*10,11*). Although the median cubital vein is generally an easy choice because of its large size, catheters at that site may occlude because of kinking resulting from movement of the elbow and/or a loose attachment. Our previous study revealed that the height-adjusted distance between the puncture point and the antecubital fossa was related to "thrombus with subcutaneous edema related to PIVC failure" (*12*). Movement in the arm joints (*i.e*. shoulder, elbow, and wrist) can place mechanical stress on the catheter. If an external force is applied (*e.g*. elbow flexion causing catheter hub movement), the catheter may bend or otherwise change shape. However, we were unable to find publications to support this assumption.

Indwelling catheters can continuously stimulate vessel walls and subcutaneous tissue, resulting in thrombus formation or subcutaneous edema (*13*). Clarification of these relationships might improve the choice of insertion sites and appropriate catheters to prevent PIVC failure. Therefore, we analyzed information from clinical ultrasound and digital camera images acquired in a previous observational study (*12*) to investigate the questions. In the previous study, nurses and physicians used short peripheral catheters (SPCs) made of Teflon® (ethylene tetrafluoroethylene), which is a plastic. Almost all plastics subjected to a continuous load experience deformation over time (*14*). We thought that the SPCs used in that study would show deformation at removal.

The aim of this study was to examine the causes of PIVC failure associated with catheter location by evaluating catheter shape just after removal by using data from our previous study (*12*).

2. Materials and Methods

2.1. *Study design and setting*

This study was a secondary analysis of data from our previous study (*12*). Our observational study was conducted at The University of Tokyo Hospital between January and June 2014. Participants were adult patients who received infusion therapy *via* SPCs during medical ward hospitalization. Patients with poor cognitive ability were excluded from the study.

2.2. *Study procedure*

All SPCs (Surshield® Surflo®2; Terumo Corp., Tokyo, Japan) were made of Teflon® (ethylene tetrafluoroethylene). One of three catheter sizes (length, outer diameter) was chosen: 20 gauge (32 mm, 1.1 mm), 22 gauge (25 mm, 0.9 mm), or 24 gauge (19 mm, 0.7 mm). All PIVCs were fixed with dressing films and tape or bandage after insertion.

Risk management for each catheter was based on the hospital's policies. Researchers were present when catheters were removed because of completion of fluid therapy, routine replacement, or catheter failure with adverse symptoms (such as swelling, redness, pain, or occlusion). Nurses assessed catheter failure, which was defined as interruption of fluid therapy associated with signs and symptoms such as occlusion, swelling, redness, and pain. If nurses confirmed that the infusion volume was insufficient or stopped, the case was defined as "occlusion".

Researchers obtained clinical images of the insertion site with a digital camera and ultrasound. Images of the removed catheters were acquired from different angles. Background demographic data, such as age, sex, and duration of catheter insertion, were recorded from medical charts.

2.3. *Ultrasound scanning technique*

B-mode ultrasonography is a real-time, noninvasive method for exploring subcutaneous tissues and blood vessels (*15*). Ultrasound diagnostic equipment (Hitachi Healthcare Manufacturing, Tokyo, Japan) with linear-array transducers (5-18.0 MHz) was used to observe blood vessels and subcutaneous tissues in this study. The image depth was set at 1.5-2 cm. Echo gain was set at 25 and the dynamic range at 65. Because the pressure of the transducer compresses veins, we used ultrasound gel (Aquasonic 100; Parker Laboratories, Fairfield, NJ) and also placed gel pads (Sonar Pad; Nippon BXI, Tokyo, Japan) over the transparent dressing (*12*).

2.4. *Data analysis*

2.4.1. *Insertion sites*

A total of 184 catheter insertion sites were classified into four groups: upper arm (n = 9), forearm (n = 167), antecubital fossa (n = 3), and dorsum of hand (n = 5). Parameters were compared among all four groups and between upper arm and forearm. Forearm placements were further classified as cephalic vein (n = 107), median vein (n = 30), and basilic vein (n = 30); differences among these veins were analyzed.

2.4.2. *Definitions of catheter bend angle and catheter curvature*

We found two types of catheter shape. The first was a bend at the base of the catheter and the second was

a curve or kink in the middle of the catheter. The bend angle was measured on a photograph by a single researcher blind to the site of catheter insertion. First, a line passing through the center of the catheter hub was drawn; next, a line was drawn along the catheter. The angle between these lines was measured with open-source software ImageJ (Figure 1B) (*16*). The researcher selected the greatest angle among several pictures taken from different angles. Catheter curvature was defined as a clear curve or kink in the middle of the catheter (Figure 1C).

2.4.3. *Ultrasonography images*

All images were assessed by a certified sonographer with over 10 years' experience who was blind to the catheter insertion site.

The definitions of thrombus formation and subcutaneous edema were based on our previous study (*15*). Intravenous thrombus was defined as a marked echogenic mass with an uneven surface. Subcutaneous edema was defined as a homogeneous cobblestone appearance of the subcutaneous fat layer resulting from excessive fluid in the interstitium. Presence or absence of subcutaneous edema and intravenous thrombus were assessed on both transverse and longitudinal ultrasound images (Figures 1D and 1E).

2.4.4. *Statistical analysis*

Data are presented as mean with standard deviation (SD) or median with range. Chi-square tests and Fisher's exact test were used for categorical data; Mann-Whitney *U* tests were used for quantitative data. A two-tailed *p*-value < 0.05 was considered statistically significant. Data were analyzed with Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, ver. 22.0; IBM Corp., Armonk, NY).

2.5. *Ethical considerations*

All patients who had planned medical treatment with fluid therapy were informed about the purpose and methods of the study, safety considerations, and right to revoke consent at any time. Patients were enrolled after providing written informed consent. The study was approved by the Research Ethics Committee of the Graduate School of Medicine at The University of Tokyo (#10348).

3. Results

3.1. *Participant characteristics*

Images of 217 removed catheters were taken with a digital camera. Thirty-three catheters were excluded because ultrasound images were not obtained before catheter removal. The remaining 184 catheters from 142 participants were analyzed. Mean patient age (SD) was 69.6 (12.7) years; 84 participants (59.2%) were male (Table 1).

3.2. *Insertion site, catheter characteristics, and catheter failure*

SPCs were frequently inserted into the forearm (90.8%). The most common catheter size was 22 gauge (81.5%). Mean indwelling time was 45.7 h.

The median bend angle, measured at the base of the catheter, was 9.1° (range: 0.0°-68.3°). The bend angle was significantly greater in upper arm placements than in forearm placements ($p = 0.013$). The distance from the joint was shorter in upper arm placement than in forearm placement ($p < 0.001$). All three catheters inserted in the

Table 1. Patient characteristics, catheter shape, and ultrasound findings

Items	Total (n = 184)	Upper arm (n = 9, 4.9%)	Forearm (n = 167, 90.8%)	Antecubital fossa (n = 3, 1.6%)	Hand (n = 5, 2.7%)
Age (years), mean (SD)	70.2 (12.6)	69.3 (18.9)	70.2 (12.5)	72.7 (3.8)	72.4 (11.1)
Sex, *n* (%)					
Male	110 (59.8)	6 (66.7)	99 (59.3)	1 (33.3)	4 (80.0)
Female	74 (40.2)	3 (33.3)	68 (40.7)	2 (66.7)	1 (20.0)
Catheter gauge, *n* (%)					
20-gauge	3 (1.6)	0 (0.0)	2 (1.2)	0 (0.0)	1 (20.0)
22-gauge	150 (81.5)	8 (88.9)	140 (83.8)	1 (33.3)	1 (20.0)
24-gauge	31 (16.8)	1 (11.1)	25 (15.0)	2 (66.7)	3 (60.0)
Indwelling time (hours), mean (SD)	45.7 (27.3)	60.1 (40.4)	44.5 (26.4)	72.7 (3.8)	44.3 (28.6)
Bend angle[a] (°) , median (range)	9.1 (0.0 - 68.3)	18.9 (1.3 - 68.3)*[c]	8.5 (0.0 - 54.0)*[c]	18.1 (12.1 - 30.6)	4.6 (4.1 - 30.9)
The distance from the joint[b] (cm), median (range)	10.7 (2.5 - 25.8)	6.0 (3.2 - 9.4)**[c]	11.5 (2.8 - 25.8)**[c]	–	3.3 (2.5 - 6.2)
Catheter curvature, *n* (%)[c]	17 (9.2)	1 (11.1)	13 (7.8)	3 (100.0)	0 (0.0)
Catheter failure, *n* (%)	55 (29.9)	3 (30.3)	51 (30.5)	0 (0.0)	1 (20.0)
US images, *n* (%)					
Subcutaneous edema	81 (44.0)	5 (55.6)	73 (43.7)	1 (33.3)	2 (40.0)
Thrombus[d]	106 (60.6)	6 (66.7)	94 (59.1)	2 (66.7)	4 (100.0)

[a]Bend at catheter base. [b]Upper arm: from antecubital fossa, Forearm: from wrist joint. [c]Curve or kink in the middle of the catheter. [d]Nine images were excluded because they were unclear. [c]Comparison between forearm placement and upper arm placement, Mann-Whitney *U* test, $*p = 0.013$, $**p < 0.001$.

Table 2. Catheter shapes and ultrasound findings in surviving *vs.* failed catheters

Items	Total (n = 176)			Upper arm (n = 9)			Forearm (n = 167)		
	CF (n = 54)	Non-CF (n = 122)	p-value	CF (n = 3)	Non-CF (n = 6)	p-value	CF (n = 51)	Non-CF (n = 116)	p-value
Age (years), mean (SD)	71.9 (14.4)	69.4 (12.0)	0.056[c]	56.3 (30.4)	75.8 (7.3)	0.548[c]	72.8 (12.9)	69.1 (12.2)	0.022[c]*
Indwelling time (hours), mean (SD)	36.0 (26.6)	49.3 (26.8)	< 0.001[c]**	25.0 (20.3)	77.7 (36.5)	0.095[c]	36.7 (26.9)	47.9 (25.5)	0.002[c]
Bend angle[a] (°), median (range)	7.9 (1.0 - 54.0)	9.6 (0.0 - 68.3)	0.875[c]	22.1 (1.3 - 24.8)	18 .2 (9 . 0 -	0.905[c]	7.7 (0.1 - 54.0)	9.2 (0.0 - 37.8)	0.971[c]
Catheter curvature[b], n (%)	8 (14.8)	6 (4.9)	0.035[d]*	0 (0.0)	68.3) 1 (16.7)	1.0[d]	8 (15.7)	5 (4.3)	0.023[d]*
The distance from the joint[c] (cm), median (range)	12.5 (3.2 - 25.8)	10.3 (2.8 - 23.1)	0.139[c]	3.3 (3.2 - 6.0)	7.2 (5.3 - 9.4)	0.095[c]	12.6 (3.5 - 25.8)	10.9 (2.8-23.1)	0.068[c]
US images, n (%)									
Subcutaneous edema	42 (77.8)	36 (29.5)	<0 .001[d]**	2 (66.7)	3 (50.0)	1.0[d]	40 (78.4)	33 (28.4)	< 0.001[d]**
Thrombus[d]	39 (76.5)	61 (52.1)	0.004[d]**	2 (66.7)	4 (66.7)	1.0[d]	37 (77.1)	57 (51.4)	0.003[d]**
Symptoms, n (%)									
Occlusion	22 (40.7)	0 (0.0)		2 (66.7)	0 (0.0)		20 (39.2)	0 (0.0)	
Swelling	32 (59.3)	13 (10.7)		2 (66.7)	1 (16.7)		30 (58.8)	12 (10.4)	
Redness	24 (44.4)	31 (25.6)		1 (33.3)	1 (16.7)		23 (45.1)	30 (26.1)	
Pain	30 (55.6)	10 (8.2)		2 (66.7)	0 (0.0)		28 (54.9)	10 (8.6)	

[a]Bend at catheter base. [b]Curve or kink in the middle of the catheter. [c]Upper arm: from antecubital fossa, Forearm: from wrist joint. [d]Eight images were excluded because they were unclear. [e]Mann-Whitney U test, [f]Fisher's exact test,, *p < 0.05, **p < 0.01.

Figure 1. Definition of bend angle, catheter curvature, and ultrasound findings. (A) Photograph of forearm insertion site. **(B)** Photograph of removed catheter, showing bend angle (angle between a line passing through the center of the catheter hub and a line along the catheter). **(C)** Catheter curvature is defined as presence of a curve or kink in the middle of the catheter. **(D)** Ultrasound image before catheter removal (transverse image). **(E)** Ultrasound image (longitudinal image). Ultrasound images showing vessel wall (arrows) catheter tip (arrowheads), subcutaneous edema (circle), and thrombus (dotted circle).

antecubital fossa had a curve or kink in the middle of the catheter. In contrast, none of the catheters inserted in the dorsum of the hand were curved or kinked in the middle of the catheter.

Catheter failure occurred in 29.9% of all catheters. There was no significant difference in catheter failure rate among insertion sites (Table 1).

Table 2 shows catheter shapes and ultrasound findings in surviving *vs.* failed catheters inserted in the upper arm and forearm. Bend angle at the base of the catheter was not related to catheter failure (CF). However, curving or kinking in the middle of the catheter was related to CF (14.8% of failed catheters had curving, p = 0.035). Furthermore, catheter curving was related to occlusion (35.3% of occluded catheters had curving, p = 0.008; data not shown). The median distance between the insertion site and the elbow joint was shorter in CF cases (median, range: 3.3, 3.2-6.0) than in non-CF cases (7.2, 5.3-9.4) in the upper arm. Thrombus formation or subcutaneous edema was seen on US images in 75% of CF cases (Table 2).

In forearm placement, catheter curving was related to CF (p = 0.023), occlusion (46.2% of occluded catheters were curved, p = 0.001), and thrombus formation (90.9% of catheters with thrombi were curved, p = 0.028; data not shown). Half of catheters inserted in the median vein failed (53.3%, p = 0.007; data not shown). The median distance (range) between the joint and catheter insertion site in the cephalic vein, median vein, and basilic vein was 9.6 mm (2.8-24.1), 13.4 mm (7.5-25.8), and 12.3 mm (4.2-21.7), respectively. Median bending angle (range) of catheters in the cephalic vein, median vein,

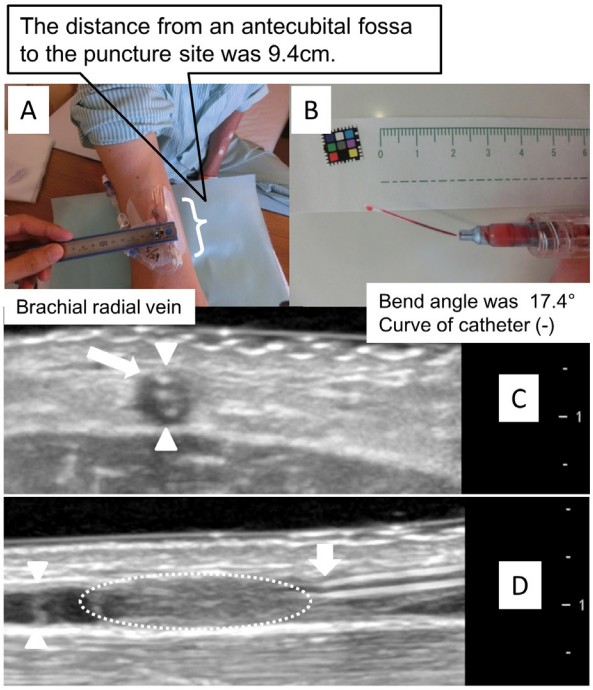

Figure 2. Upper arm vein (non-CF): Case 16202. (A) Upper arm insertion site. **(B)** Bend angle. **(C)** Ultrasound image before catheter removal (transverse image). **(D)** Ultrasound image (longitudinal image). Ultrasound images showing vessel wall (arrowheads), catheter tip (arrows), and thrombus (dotted circle).

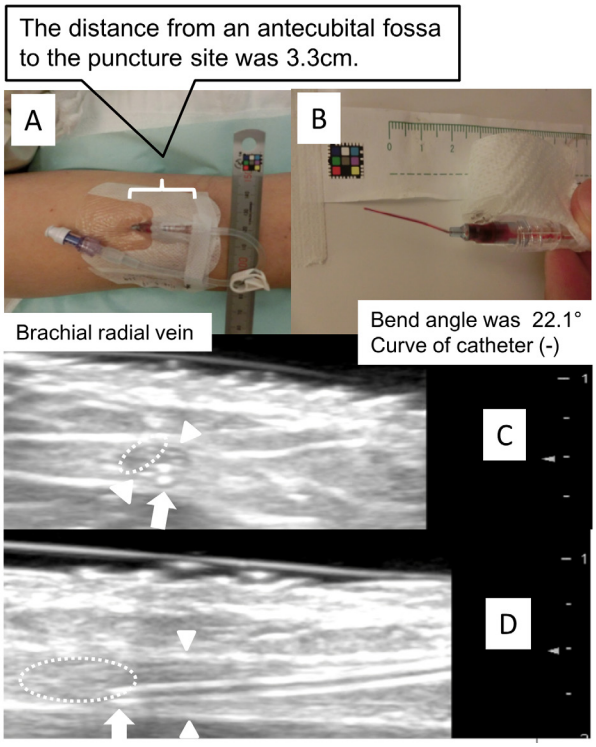

Figure 3. Upper arm vein (CF): Case 22302. (A) Upper arm insertion site. **(B)** Bend angle. **(C)** Ultrasound image before catheter removal (transverse image). **(D)** Ultrasound image (longitudinal image). Ultrasound images showing vessel wall (arrowheads), catheter tip (arrows), and thrombus (dotted circle).

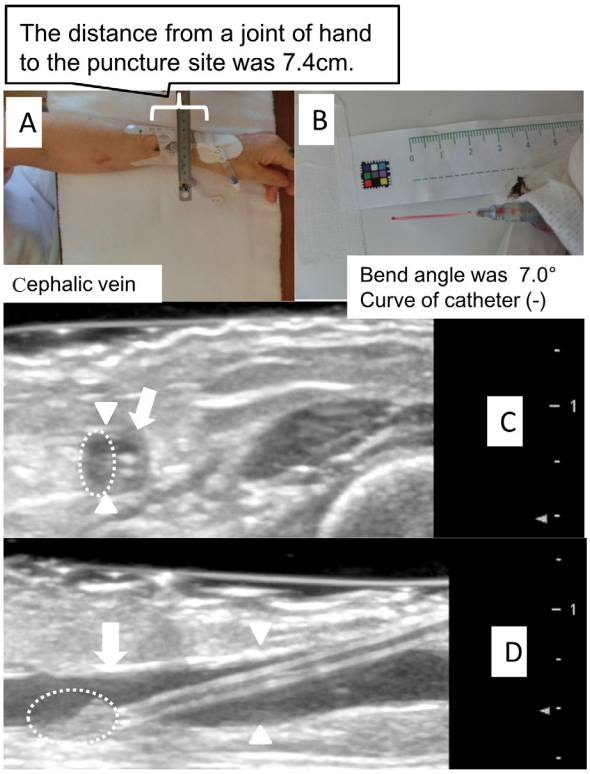

Figure 4. Forearm vein (non-CF): Case 20201. (A) Forearm insertion site. **(B)** Bend angle. **(C)** Ultrasound image before catheter removal (transverse image). **(D)** Ultrasound image (longitudinal image). Ultrasound images showing vessel wall (arrowheads), catheter tip (arrows), and thrombus (dotted circle).

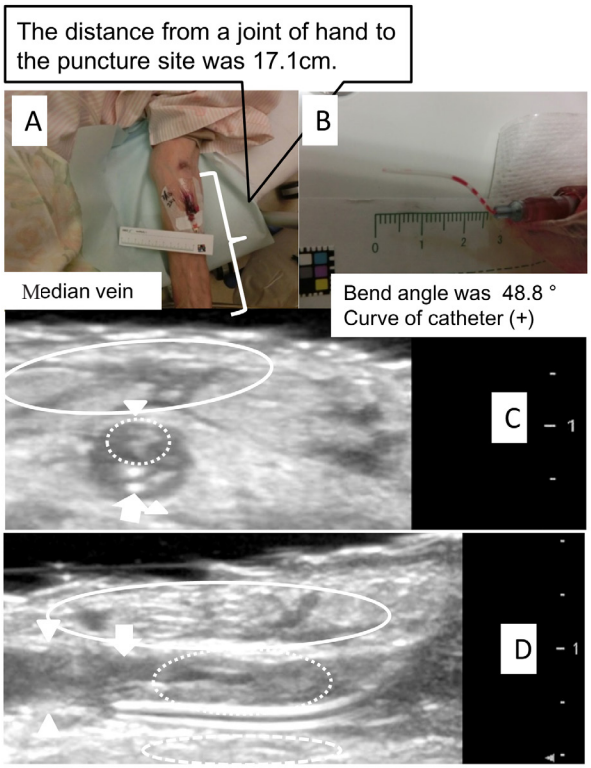

Figure 5. Forearm vein (CF): Case 16701. (A) Forearm insertion site. **(B)** Bend angle. **(C)** Ultrasound image before catheter removal (transverse image). **(D)** Ultrasound image (longitudinal image). Ultrasound images showing vessel wall (arrowheads), catheter tip (arrows), subcutaneous edema (circle), and thrombus (dotted circle).

and basilic vein were 9.3° (0.0°-54.0°), 6.9° (0.2°-48.8°), and 8.4° (0.0°-29.1°), respectively (data not shown).

3.3. *Clinical observations from digital camera and ultrasound images*

Below we describe characteristic non-CF and CF cases of upper arm and forearm placements (Figures 2-5).

Figure 2 and 3 shows cases of catheter insertion in the upper arm. Figure 2 shows non-CF in a 67-year-old man (Case 16202). Indwelling time was 94 h, total infusion volume was 4490 mL, flow rates were 60-250 mL/h, and intravenous lock was performed three times. This patient received an intravenous anticancer agent and a high pH (> 8) agent. The catheter was 22 gauge. The removed catheter had a bend angle of 17.4°; the distance from the antecubital fossa to the puncture site was 9.4 cm. Although thrombus was observed, the patient did not complain of symptoms and medical treatment was completed.

Figure 3 shows CF in a 51-year-old man (Case 22302). Indwelling time was 9.5 h, total infusion volume was 920 mL, flow rates were 20-100 mL/h, and intravenous lock was not used. This patient received antibacterial agents. The catheter was 22 gauge. The removed catheter had a bend angle of 22.1°; the distance from the antecubital fossa to the puncture site was 3.3 cm. Thrombus was observed. The reason for removal was occlusion.

Figures 4 and 5 show cases of forearm vein placement. Figure 4 shows non-CF in a 79-year-old woman (Case 20201). Indwelling time was 70.5 h, total infusion volume was 2150 mL, flow rates were 80-100 mL/h, and intravenous lock was performed twice. This patient received antibacterial agents. The catheter was 22 gauge. The removed catheter had a bend angle of 7.0°; the distance from the wrist to the puncture site was 7.4 cm. Thrombus was observed and medical treatment was completed.

Figure 5 shows CF in an 81-year-old woman (Case 16701). Indwelling time was 11.5 h, total infusion volume was 252 mL, flow rates were 50-100 mL/h, and intravenous lock was not used. This patient received antibacterial agents and a high pH agent. The catheter was 22 gauge. The removed catheter had a bend angle of 48.8°. The distance from the wrist to the puncture site was 17.1 cm. Thrombus and subcutaneous edema was observed; the reason for removal was occlusion. Furthermore, swelling and redness were observed, and the patient had pain.

4. Discussion

To investigate the associations between PIVC failure and catheter location, we acquired ultrasound images of blood vessels and subcutaneous tissues and clinical images of removed catheters. We found that catheters inserted in the upper extremities had bending at the base of the catheter and curving or kinking in the middle of the catheter. The bend angle of catheters inserted in the upper arm was greater than that of catheters inserted in the forearm. The angle of insertion of needles in upper arm veins is greater, because upper arm veins are deeper than those of the forearm, and the subcutaneous fat of the upper arm is thicker than that of the forearm or hand (*17*). Therefore, healthcare provider must insert SPCs at a greater angle through this subcutaneous fat to prevent the catheter from pulling out of the blood vessel (*9*). Furthermore, the cephalic vein in the upper arm runs along the biceps muscle. Contraction of the biceps muscle flexes the elbow and might exert external force, bending the catheter (Figures 2 and 3). If the catheter hub moves away from the insertion site, the base of the catheter bends easily. These conditions may explain why catheters inserted in the upper arm had a greater bend angle than those inserted in the forearm. However, in this study, the bend angle was not related to CF. This finding might have resulted from the comparatively low number of catheters inserted in the upper arm.

Curving or kinking in the middle of the catheter was related to CF, occlusion, and thrombus formation in the forearm. It may be unusual to find bending or curvature in a removed catheter because the malleability of intravenous catheters has improved in recent years (*18*). There is an association between the materials from which catheters are made and the incidence of thrombophlebitis (*19,20*). Blood vessels tend to be soft and bend easily under pressure, which may result in intravenous catheter bending (*21*). If a catheter is bent within a blood vessel, it might develop a curve or kink. This curving may predispose to phlebitis if excessive movement causes vessel wall trauma (*22*). In the current study, catheter insertion in the median vein was related to CF (Figure 5). The distance from the elbow joint to catheters inserted in the median vein was shorter than to catheters in the cephalic vein, which might have affected CF. Elbow joint flexion can exert external forces that affect the vein and the indwelling catheter.

To prevent catheter failure, especially occlusion resulting from catheter curvature, an appropriate insertion site should be selected. It is important to consider both the method of catheter fixation and the insertion angle to avoid mechanical stimulus from external forces on the catheter hub. If the PIVC is inserted into the upper arm, a suitable catheter length should be used to allow a smaller insertion angle to prevent excessive bending at the base of the catheter; insertion should be as far as possible from the elbow joint. Furthermore, the catheter hub must be fixed rigidly because the base of the catheter may bend with forward movement beyond the insertion site during elbow flexion. Although all catheters were fixed with dressing films and tape or bandage after placement, these methods might not be enough as the catheter hub

fixation.

Most plastic catheters are flexible and can bend with the shape of the blood vessel. However, catheters can deform over time (*12*). Catheters inserted at the antecubital fossa all showed deformation in the middle of the catheter. Avoiding curvature of the catheter could reduce the mechanical stimulus that affects the vascular endothelium. Further research to examine the difference between catheter materials and removed catheter shapes is needed.

This study was limited by the small number of catheters inserted in the upper arm, antecubital fossa, and hand compared with the forearm. We could not obtain and analyze detailed data on intravenous fluid therapy conditions. Further observational studies with more catheters inserted in the upper arm and using catheters of the different length are required.

In conclusion, although insertion site was not directly associated with CF, curving or kinking in the middle of the catheter was related to CF, occlusion, and thrombus formation in the forearm. Severe catheter bending at the base may result in occlusion. External forces that deform the catheter could affect blood vessels and subcutaneous tissues by mechanical stimulus.

To prevent catheter failure, especially occlusion resulting from catheter curvature, an appropriate insertion site far from the elbow should be selected. Furthermore, it is important that the catheter is fixed rigidly to prevent movement of the catheter hub.

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Conflict of Interest

This study was a joint project with the Terumo Corp., which provided sponsorship.

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Brief Report

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A study on yogurt consumption: A case of industry-academia collaboration in Fukushima and Tokyo

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Summary **This paper proposes a new product development of yogurt project based on industry-academia collaboration between Teikyo University and Tohoku Kyodo Milk Industry and discusses the possible economic impact of this project on the Tohoku region as well as the Tama area in Tokyo. We also introduce a preliminary survey to partially clarify the consumption patterns for yogurt among students at Teikyo University. The survey reveals that most of our respondents consume yogurt regularly. The stated reason for yogurt consumption is to enjoy yogurt as a dessert rather than as a health food. We also find that the most significant determinant factors for purchasing yogurt are taste, price, and quantity. Based on the data, respondents are willing to pay between 100 JPY and 145 JPY for yogurt. In response to these findings, we discuss some additional surveys that need to be conducted in the future.**

Keywords: Innovation, industry-academia collaboration, consumer analysis

1. Introduction

Approximately one century has passed since Schumpeter (1912) started discussing the importance of innovation (*1*). The manner by which innovation is carried out has changed throughout the century, but regardless, innovation is necessary for nations and companies. In recent years, innovation has been carried out through collaboration between universities, which require academic success, and companies, which demand economic performance. Universities and companies aim to put this type of innovation into practical use by formalizing industry-academia collaboration. This paper investigates the current state of industry-academia collaboration from economic and business perspectives based on the case example of the "Yogurt Project Launched by Teikyo University," which was conducted by Teikyo University in Tokyo and Tohoku Kyodo Milk Industry in Fukushima.

The "Yogurt Project Launched by Teikyo University" aims to commercialize yogurt made with Leuconostoc, which has a high natural immunity, developed and discovered primarily by Professor Kazuhisa Sekimizu at the Teikyo University (professor emeritus at the University of Tokyo), as "Teikyo University Yogurt" (See Ishii *et al*. (2017) for further details (*2*)). Specifically, "Genome Pharmaceuticals Institute" established by Professor Sekimizu uses a unique technique to produce and supply Leuconostic to Tohoku Kyodo Milk Industry. Leuconostic was developed by conducting a muscle contraction assay on silkworms. This lactobacillus is used to produce yogurt. Teikyo University decided to name the product "Teikyo University Yogurt". The Faculty of Economics at Teikyo University including the author and the author's seminar students (the "Mitsunami Seminar") are currently discussing and creating business models for marketing and package design for the yogurt. This is therefore a planning and development project to produce yogurt by collaboration between Teikyo University, Tohoku Kyodo Milk Industry, and Genome Pharmaceuticals Institute. Since Tohoku Kyodo Milk Industry is located in Koriyama City in Fukushima Prefecture, this project is expected to contribute to recovery efforts following the March 2011 earthquake in Japan. Based on previous results, the author and his seminar students determined the following two purposes of this project. The first purpose is to provide assistance to recovery efforts for the Tohoku region

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and Fukushima Prefecture. The earthquake, which occurred on March 11, 2011, brought about radioactive contaminants resulting from the Fukushima Daiichi nuclear disaster caused by earthquake and tsunami damage. According to Tohoku Kyodo Milk Industry, the reputation of milk produced in Fukushima has still not recovered even after seven years since the earthquake disaster, despite the fact that the milk is now safe to consume. Given this situation, we expect to directly contribute to recovery efforts following the earthquake disaster by encouraging consumers to buy this yogurt, since yogurt is widely believed to be less harmful than milk, and therefore, might soften consumers' attitudes. The second purpose of this project is regional development in the Tama area where Teikyo University is located. The Tama area has seen a declining economy as compared to central Tokyo, although it is located within the Tokyo metropolitan area. Based on an overall view of the industry, however, the Tama area seems to have considerable potential not only because of agriculture but also due to an active brewing industry, as well as many tourist attractions. Therefore, if the Tama area, which has such facets, can collaborate with Teikyo University to produce "Teikyo University Yogurt," the product is expected to connect consumers with the industry and sources in the area, thereby contributing to regional development. The project has the potential to activate regional economies not only in the Tama area but also in the Tohoku region and Fukushima Prefecture.

2. Materials and Methods

2.1. *Survey method*

In January 2018, the author's seminar students conducted a survey aimed at university students belonging to the Faculty of Economics at Teikyo University, pertaining to consumers' lifestyle and yogurt consumption. The aim of this survey is to conduct a preliminary study regarding commercialization of yogurt and a trial sale (planned at Teikyo University), and to collect basic information for a subsequent study on yogurt consumption. Specifically, the survey was conducted from January 9 to 15, 2018 for 754 students at Teikyo University (male 561; female 193) who attended the author's lectures.

2.2. *Survey design*

The survey consists of two parts: (*i*) the university students' life-style and (*ii*) their yogurt consumption behavior. The first part collected the following items: family structure, place to buy food, dietary habits, use of SNS, and recognition of tourist attractions at the university's location, and so on. The second part collected the following items: frequency and timing of yogurt purchases, the brand name of the yogurt, actual purchaser, purchase reason, willingness to pay, and determinant of purchase. Yogurt consumption is not determined only by preference or frequency, but rather it involves the individual's lifestyle as a subject of consumption. For example, some university students who live with his/her parents may consume more yogurt rather than others living alone because their parents habitually purchase yogurt. Thus, since some university students living with their parents have no custom of buying yogurt themselves, they are regarded as having no potential to be customers, even if yogurt is sold at the university. Therefore, because various kinds of individual attributes affect yogurt consumption behavior, the first part of the survey includes not only a decision factor for buying yogurt, but also individual lifestyle.

In the second part, we asked about the frequency and timing of yogurt purchases, the brand name of the yogurt, actual purchaser, purchase reason, willingness to pay, and determinant of purchase. In terms of yogurt product marketing, the price that a customer must pay to purchase yogurt, that is, their willingness to pay and the determinant of purchase action is critical information. Regarding the determinant of purchase action, we asked the following items in a ranking format based on priority to collect the data that are most important for the university students to buy yogurt: reputation, price, quantity, taste, ingredient, and package design. For example, when many students respond with "quantity," the "quantity" is regarded as more important than others are, such as "price or "taste." The following section analyzes the results of the second part of the survey.

3. Results and Discussion

The results of the survey are briefly summarized and presented as follows. The results from the data show that the number of students who typically eat yogurt was 491 and the number who do not typically eat yogurt was 253 in the sample. This indicates that approximately 66% of the students out of 744 valid responses consume yogurt. Respondents who habitually eat yogurt broke down as follows: 17 students who eat yogurt more than once a day, 94 students who eat yogurt once a day, 132 students who eat yogurt once every few days, 238 students who eat yogurt once a week, and 238 students who eat yogurt less often than that. The rate of university students who consume yogurt habitually (at least once every few days) was 51% of valid responses. Therefore, this tells us that there are a considerable number of students at Teikyo University who regularly consume yogurt.

The number of students who purchase yogurt broke down as follows: 245 students who purchase yogurt themselves, and 235 students whose parents or others purchase yogurt for them. This indicates that half of

the students buy yogurt on their own. The students who habitually eat yogurt and purchase it themselves were asked to fill in what they thought would be a reasonable price for yogurt (single serving). Results indicate that the average price considered to be reasonable was 145 JPY, the median was 120 JPY, and the mode was 100 JPY (28% answered, and there were 228 valid responses). We expect from the data that students feel that around 100 JPY is reasonable and that a range of prices between 100 JPY and 145 JPY is not too expensive. However, we need a more detailed study for pricing the yogurt when we sell it outside of the Teikyo University campus. We would like to leave this issue open for future research.

Regarding the determinant factors for buying yogurt, 59% of students consider taste to be the most important factor, while 68% consider the package design of the yogurt not to be an important factor. Price (second priority) was considered important by 64% of students and quantity (third priority) was considered important by 62% of students. It is hard to perform an analysis based only on these results and numerical values; however, it can still be ascertained that taste as well as price and quantity are important factors for university students. The number of students who have detailed knowledge of the ingredients of yogurt is limited. Furthermore, the tendency of Japanese people to prefer large-scale producers (brands) was not seen in students at Teikyo University.

This preliminary survey partially clarified the current status of yogurt consumption among students at Teikyo University. It reveals that the majority of our respondents consume yogurt on a regular basis and that the respondents' purpose in consuming yogurt is to enjoy it as a dessert rather than as a health food. We also found that the most significant determinant factor in buying yogurt is taste, followed by price and quantity. We also observed that respondents would be willing to pay between 100 JPY and 145 JPY for yogurt. These findings contributed in several ways to our understanding of consumer behavior among university students and provided a basis for an idea of a further survey as follows.

Firstly, an advanced survey design can be introduced. When people make purchasing decisions for goods, there is a trade-off among some attributes such as price, quality or brand. Therefore, we need to give respondents a hypothetical situation in the survey in which they are faced with a decision of whether to purchase a yogurt product. A choice experiment is one survey method. In a choice experiment, respondents are shown multiple choices and each choice has different characteristics. In such a situation, respondents are faced with a trade-off among different attributes and are required to select one of the choices according to their preferences. A related study used choice experiment questions and revealed that respondents are more likely to purchase yogurt that has labels indicating "Vitamin Enhanced", "Nutrition Info", or "Probiotic", while respondents are unlikely to purchase yogurt with a higher price and fat content (*3*). Our preliminary survey identified important factors for purchasing yogurt among university students, and they could be employed as attributes in choice experiment questions. Additionally, by using an advanced econometric model such as a latent class model, we can group respondents by preferences as well as quantitatively explain the difference in preferences by sociodemographic information or social beliefs. Since it is naturally expected that there is heterogeneity of preferences among university students, such an analysis could be useful in identifying the important factors for each specific group.

Secondly, packaging design could be important from a marketing standpoint. Some studies (*4,5*) showed that nutritional information did not affect the acceptability of yogurt. However, Bayarri *et al*. (2010) also suggested that different responses to information were observed among people with different individual characteristics, which suggests that university students could be affected by product information (*4*). Also, we know that corporate social responsibility has a positive impact on the image and sales of a product. For example, Managi *et al*. (2008) showed that the image of environmental friendliness in the production process positively affected consumers' purchasing decisions (*6*). In this regard, the possible economic impact on Fukushima and the Tama area could be positively correlated to the product value. In order to know exactly the extent to which this project affects their economies, input-output analysis can be employed. Another task would be to study how to advertise such information on packaging for effective marketing strategies.

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Brief Report

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Evaluation of the global action plan on antimicrobial resistance in Japan during its first eighteen months

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Summary **Antimicrobial resistance (AMR) has garnered the most attention among public health concerns worldwide. Japan formulated a national action plan for AMR in April 2016. The plan seeks to reduce the amount of antimicrobials used in 2020 to two-thirds of the use recorded in 2013. Prescription surveillance (PS) is being used to monitor trends in the amount of antimicrobials used. PS estimates the number of patients prescribed an antimicrobial each day. The number of patients who were prescribed an antimicrobial under the action plan was analyzed by including dummy variables with other control variables. Data from April 1, 2011 to 30 September 30, 2017 were analyzed. When the number of patients with an infectious disease (1 of 13 specified diseases) served as a dummy variable, estimates indicated that the coefficient of that dummy variable was not significant. If the number of patients with an infectious disease (1 of 13 specified diseases) was excluded as an explanatory variable, then the estimated coefficient was significant. The global action plan in Japan might not reduce the amount of antimicrobials used. The current results indicated that the number of patients who were prescribed an antimicrobial did not decrease significantly after initiation of the action plan. This finding does not exclude the possibility that the average amount of antimicrobials used per patient has decreased.**

Keywords: Action plan, antimicrobial resistance, evaluation, prescription surveillance, surveillance

1. Introduction

Antimicrobial resistance (AMR) has garnered the most attention among public health concerns since the World Health Organization (WHO) launched a global action plan in 2011 with the slogan "No action today, no cure tomorrow." According to the O'Neil Commission (*1*), 0.7 million persons were estimated to have died from AMR in 2013. The number of people dying due to AMR is estimated to increase to 10 million people if no countermeasures are taken. The rate of AMR will continue to increase by its current rate. Therefore, the nations of the world have started to take action. The WHO global action plan asks all member nations to formulate a national action plan. Following formulation of a national action plan in the UK and USA, Japan formulated such a plan in April 2016. The plan seeks to reduce the amount of used antimicrobials in 2020 to two-thirds of the use in 2013 and to reduce the rate of AMR.

A surveillance system, like the system started prior to 2013, is needed to monitor trends in the amount of antimicrobials used. Moreover, a timely and precise surveillance system is needed to evaluate measures intended to reduce antimicrobial use. If the surveillance system is not timely or precise, then measures cannot be modified to be maximally effective.

Fortunately, prescription surveillance (PS) is in operation in Japan. Since 2009, the PS system has been operated by the Japan Medical Association, the Japan Pharmaceutical Association, the School of Pharmacy of Nihon University, and EM Systems Co. Ltd. This nationwide syndromic surveillance system has reported the estimated number of patients with influenza and

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chicken pox and the number of patients prescribed certain types of drugs based on prescriptions filled at outside pharmacies (*2,3*). Under this system, the number of patients is estimated each day based on the number of prescriptions for neuraminidase inhibitors, anti-herpes virus drugs, antipyretic analgesics, and multi-ingredient cold medications as well as antimicrobials. The number of patients is estimated by prefecture and by age group. As of the end of April 2017, approximately 10,000 pharmacies were participating, accounting for about 20% of all pharmacies. The estimated number of patients is shown on a dedicated web page (*http://prescription.orca.med.or.jp/kanjyasuikei/*) the following morning.

2. Materials and Methods

2.1. *Data*

In fact, PS estimates the number of patients receiving a prescription each day. The number of prescriptions issued for certain medications by pharmacies participating in PS is tallied by prefecture. This number is multiplied by the reciprocal of the proportion of pharmacies participating in PS by prefecture and by the reciprocal of the percentage of off-site prescriptions by prefecture. Antimicrobials are classified into five types: penicillin, cephems, macrolides, new quinolones, and other antimicrobials (*2,3*). The current study focused on the total number of patients prescribed an antimicrobial while ignoring the specific type of antimicrobial.

2.2. *Analysis*

The number of patients who were prescribed an antimicrobial was included as a dummy variable with other control variables. An equation was calculated using the least-squares method

$$x_t = \alpha + \sum_{j=1}^{14} \beta_j d_{jt} + \sum_{k=2}^{52} \delta_k w_k + \sum_{l=2}^{7} \eta_l z_l + \zeta\ h_t + \psi\ n_t + \varepsilon_t \quad (1)$$

where x_t represents the number of patients prescribed an antimicrobial on day t, d_{jt} denotes the reported number of patients with an infectious disease j per sentinel site during a week with t days, and where w_k, z_l, h_t, and n_t are, respectively, dummy variables for the epidemiological week, day of the week, holidays, and the day after a holiday. In these variables, the subscripts k and l respectively indicate the number of the epidemiological week and days of the week in t days. Here, α_i is a constant term, and δ_{ik}, η_{il}, ζ_i, and ψ_i respectively represent coefficients for dependent variables. To assess the robustness of Eq. 1, the equation was also estimated while excluding the number of patients with an infectious disease (1 of 13 specified diseases).

The number of patients with certain specified infectious diseases at a sentinel site is reported officially each week. These infectious diseases are influenza, RS virus infection, pharyngoconjunctival fever, group A streptococcal pharyngitis, gastrointestinal infections, varicella, hand, foot and mouth disease, erythema infectiosum, exanthem subitum, pertussis, herpangina, mumps, and mycoplasma pneumonia. For RS, official surveillance figures only indicate the total number of patients per week. This information has been published officially and continually by the Ministry of Health, Labor, and Welfare.

Data from April 1, 2011 to September 30, 2017 were examined.

2.3. *Ethical considerations*

Data from PS were aggregated and not linked to personal information related to patients, medical institutions, and pharmacies, thereby yielding anonymous data. Therefore, there are no ethical concerns raised by the use of these data in this study.

3. Results and Discussion

Figure 1 shows variations in the number of antimicrobial prescriptions. The average number of antimicrobial prescriptions was 480,280 overall, 491,065 before initiation of the action plan, and 441,635 afterwards.

According to Eq. 1 where the number of patients with an infectious disease (1 of 13 specified diseases) served as a dummy variable, the coefficient of that dummy variable was –9876.085, and its p was 0.581. The adjusted R^2, which represents the overall goodness of fit, was 0.6607. Therefore, approximately 70% of the variation in the number of patients prescribed an antimicrobial was explained by Eq. 1. Moreover, if the number of patients with an infectious disease (1 of 13 specified diseases) is excluded as an explanatory

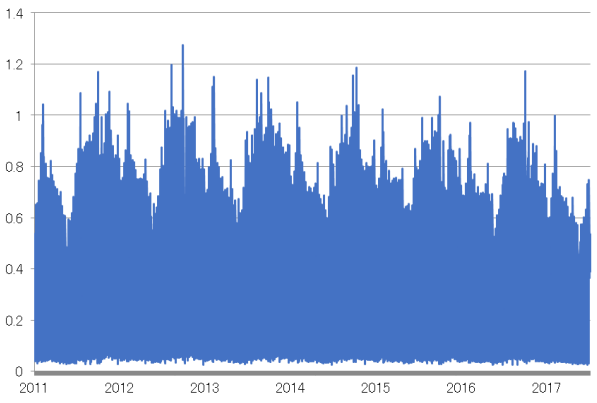

Figure 1. Variations in the number of patients prescribed an antimicrobial according to prescription surveillance (million patients). *Note*: Represents the daily estimate of the number of patients prescribed an antimicrobial according to prescription surveillance.

variable, then the estimated coefficient is –34109.6, and its p is less than 0.001. The adjusted R^2 was 0.6486.

Results indicated that the global action plan in Japan will significantly reduce the amount of antimicrobial use when the number of patients with an infectious disease (1 of 13 specified diseases) is excluded as an explanatory variable. However, including that number resulted in an insignificant reduction. The significant reduction in antimicrobials appears to reflect a lower incidence of the 13 specified infectious diseases after the plan was initiated than before it was initiated. Moreover, the estimated coefficient –34109.6 was only 7.1% of the average of the amount of antimicrobials used in all periods. Therefore, the global action plan in Japan might not reduce the amount of antimicrobials used.

However, the current finding does not necessarily mean that the global action plan in Japan has failed to reduce antimicrobial usage. Here, the amount of antimicrobials used was defined as the number of patients who were prescribed an antimicrobial each day. The amount of the antimicrobial is not specified. To be more accurate, the current results suggest that the number of patients prescribed an antimicrobial has not decreased markedly since initiation of the action plan. This finding does not exclude the possibility that the average amount of antimicrobials used per patient has decreased

4. Conclusion

This study examined the amount of all antimicrobials used without considering the type of antimicrobial. The action plan in Japan seeks to decrease all antimicrobials as well as certain type of antimicrobials. Therefore, the effects of the action plan must be assessed in terms of the type of antimicrobial. This is a topic for future study.

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