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### Editorial and Head Office:

Pearl City Koishikawa 603,
2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan
Tel.: +81-3-5840-9697
Fax: +81-3-5840-9698
E-mail: office@ddtjournal.com

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Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
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Original Article

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# Antioxidant activity and potential of *Caesalpinia sappan* aqueous extract on synthesis of silver nanoparticles

**Temsiri Suwan[1], Penpicha Wanachantararak[2], Sakornrat Khongkhunthian[3,4], Siriporn Okonogi[4,5,*]**

[1] *Interdisciplinary Program in Nanoscience and Nanotechnology, Chiang Mai University, Chiang Mai, Thailand;*
[2] *Dentistry Research, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand;*
[3] *Department of Restorative Dentistry and Periodontology, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand;*
[4] *Research Center of Pharmaceutical Nanotechnology, Chiang Mai University, Chiang Mai, Thailand;*
[5] *Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.*

**Summary** **The aim of this study was to investigate the antioxidant activity of *Caesalpinia sappan* aqueous extract (CE) and its potential on synthesis of silver nanoparticles (AgNPs). The antioxidant activity of CE was investigated using ferric reducing antioxidant power (FRAP) assay and two radical scavenging methods using 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) as free radicals. Silver nitrate ($AgNO_3$) was used as precursor for the synthesis of AgNPs. Effects of $AgNO_3$ concentration, reaction temperature, and duration of reaction were investigated. The obtained AgNPs was characterized using UV-Vis and photon correlation spectrophotometers. The antimicrobial activity of AgNPs was studied by means of diffusion method. The results from FRAP demonstrated that CE had high reducing property of 78.7 ± 2.4 mM $Fe^{2+}$/mg. The trolox equivalent antioxidant capacity of CE determined by ABTS was 64.8 ± 4.2 µM/mg. The concentration of CE that can inhibit 50% of DPPH radicals ($IC_{50}$) was 51.2 ± 3.2 µM. These results indicated that CE possesses strong antioxidant and reducing activities. The present study also showed that CE can act as reducing agent to produce AgNPs. The concentration of $AgNO_3$, reaction temperature, and reaction time play an important role on the particles size and zeta potential of the obtained AgNPs. The antimicrobial activity of the AgNPs against *Escherichia coli*, *Candida albicans*, and *Streptococcus mutants* was stronger than against *Staphylococcus aureus*.**

***Keywords: Caesalpinia sappan***, silver nanoparticles, reducing activity, antimicrobial activity

## 1. Introduction

Nowadays, silver nanoparticles (AgNPs) are of high interest due to their particular properties and wide applications. AgNPs are used to inhibit many pathogenic including bacteria such as *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, and *Pseudomonas aeruginosa* (*1-3*) and fungi like *Aspergillus* and *Candida* spp. (*4,5*). AgNPs can be synthesized by redox reaction of silver salt as a precursor and a reducing agent from synthetic chemicals. Recently, the eco-friendly process of AgNPs production was developed using reducing agents from natural sources like plants (*5*), algae (*6*), and microorganisms (*7*).

*Caesalpinia sappan* is a plant belongs to familily Leguminosae. It is wildly distributed and cultivated in Southeast Asia, Africa and the America (*8*). The wood of *C. sappan* contains several phytochemicals in alkaloids, phenolics, flavonoids, and glycosides (*9*). The major active compound of *C. sappan* is brazilin and brazilein, an oxidized from of brazilin (*10,11*). Many biological activities from different parts of *C. sappan* have been reported such as antioxidant activity from heart woods (*12*), antihelmintic property from leaves (*13*), and antimicrobial activity from barks (*14*).

The aim of this study was to synthesize AgNPs

*Address correspondence to:
Dr. Siriporn Okonogi, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail: siriporn.okonogi@cmu.ac.th

by using *C. Sappan* extract as a reducing agent. The plant extracts that can be used as reducing agents in AgNPs should dissolve well in water and its aqueous solution should have antioxidant or reducing property. However, all extracts of *C. Sappan* previously reported to have antioxidant activity were extracted from organic solvents and cannot dissolve well in water. Therefore, it is an essential to clarify whether the aqueous extract of *C. Sappan* has an antioxidant and reducing activities. In the present study, the reducing property of *C. sappan* aqueous extract was determined using ferric reducing antioxidant power (FRAP). The antioxidant activity of this extract was investigated by means of two standard radical scavenging methods using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,20-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as free radicals. The AgNPs were synthesized using silver nitrate ($AgNO_3$) as a precursor in the redox reaction. Effects of $AgNO_3$ concentrations, reaction temperature, and duration of reaction were studied. The obtained AgNPs were confirmed by UV-Vis absorption. The size, size distribution, and zeta potential of the obtained AgNPs were determined by photon correlation spectrophotometer (PCS).

## 2. Materials and Methods

### 2.1. *Materials*

All chemical reagent were analytical grade and purchased from commercial source without further purification. Two chemicals used as free radicals; DPPH and ABTS as well as 2,4,6-tripyridyl-s-triazine (TPTZ) were purchased from Sigma Adrich, Inc (St. Louis, MO, USA). $AgNO_3$ and sodium hydroxide (NaOH) 97% were supplied by RCI Lab-scan Co., Ltd. (Bangkok, Thailand). Hydrochloric acid (HCl) 37% was purchased from Carlo erba reagents (Rodano, Metropolitan City of Milan, Italy). Ferrous sulphate heptahydrate ($FeSO_4•7H_2O$) was purchased from RFCL limited (New Delhi, Delhi, India). Iron (III) chloride hexahydrate ($FeCl_3$) was purchased from Honeywell Riedel-de-Haën™ (Seelze GmbH Manufacturing Facility, Seelze, Hanover, Germany). Tryptic soy agar (TSA) and broth (TSB) were supplied by Difco™ (Balti-more, Maryland, USA). Brain heart infusion agar (BHI-A) and broth (BHI-B) were purchased from Becton, Dickinson and Company (Franklin Lakes, New Jersey, USA). Sabouraud dextrose agar (SDA) and broth (SDB) were purchased from BBL™ (Baltimore, Maryland, USA). All other chemicals and solvents were of analytical reagent grade or the highest grade available.

### 2.2. *Microbial strains*

Two aerobic bacterial strains, *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 (*E. coli*) which represent for Gram-positive and Gram-negative bacteria, respectively, and two strains facultative Gram-positive bacteria, *Streptococcus mutans* DMST 9567 and *S. mutans* DMST 41283 were used as pathogenic bacteria. *Candida albicans* ATCC 10231 was used as pathogenic fungi for the investigation of antimicrobial activity of the synthesized AgNPs.

### 2.3. *Collection of plant*

The fresh plant of *C. sappan* was collected from the local area in Chiang Mai province, Thailand. The heart wood of *C. sappan* was washed thoroughly using distilled water several times to remove dust and cut into small pieces before drying in the hot air oven at 50°C. The dried plant materials were ground into coarse powder.

### 2.4. *Preparation of plant extract*

Exact weight of 2 g of dried plant powder was macerated in 100 mL deionized water with constant stirring at room temperature for 24 h. The plant extract was filtered through Whatman No.1 filter paper and filtrate was subjected to a Freeze dry (Virtis®, Warminster, Pennsylvania, USA) to obtain an extract as a lyophilized form of *C. sappan* aqueous extract (CE). CE was stored in the refrigerator at 4°C for further studies.

### 2.5. *Antioxidant activity of CE*

#### 2.5.1. *FRAP assay*

The FRAP assay was done according to the method previously described (*15*) with some modification. Briefly, the FRAP reagent was freshly prepared by mixing 2.5 mL of 10 mM TPTZ solution in 40 mM HCl with 2.5 mL of 20 mM FeCl3 and 25 mL of 0.3 M acetate buffer pH 3.6. An exact amount of 20 µL of aqueous CE was mixed with 120 µL of FRAP reagent in 96 well plate. Blank samples were prepared by mixing acetate buffer and CE. The samples and blank were incubated for 10 min at room temperature and the absorbance of the samples was determined at 595 nm using microplate reader (Bio-Rad, Model 680, USA). The reducing power of the samples was evaluated by calculating the amount of $Fe^{2+}$ produced by CE aqueous solution using the calibration curve of $FeSO_4$. The experiment was done in triplicate.

#### 2.5.2. *ABTS assay*

This method was done by using ABTS free radical decolorization assay developed by Re *et al*. (*16*) with some modification. Briefly, the ABTS radicals was generated by reacting ABTS solution (7 mM) with

2.45 mM potassium persulfate ($K_2S_2O_8$). The mixture was allowed to stand for 15 h in the dark at room temperature. The solution was diluted with ethanol to obtain the absorbance of 0.7 ± 0.2 at 750 nm. CE aqueous solution was separately diluted with ethanol to reach a concentration of 0.1 mg/mL. An aliquot of 20 µL ethanolic test solution of CE was added to 180 µL ABTS free radical solution. The sample was incubated for 5 min and measured at 750 nm using a microtiter plate reader. All measurements were performed in triplicate. The free radical scavenging activity of each sample was expressed as trolox equivalent antioxidant capacity (TEAC), which was obtained by comparing the absorbance change at 750 nm in a reaction mixture containing CE with that containing trolox. This index is defined as the millimolar concentration of a trolox solution which antioxidant capacity is equivalent to 1.0 mg of CE.

2.5.3. *DPPH*

The scavenging activity on DPPH radical of CE was determined by modifying the methods of Gamez *et al*. (*17*). An aqueous solution of CE was diluted with ethanol to prepare an ethanolic test solution of different concentrations. DPPH was dissolved in ethanol and mixed with certain amount of the ethanolic CE solution. The solution was adjusted to a final DPPH concentration of 100 µM and the CE final concentrations of 0.1-1.0 mg/mL. The mixture was shaken vigorously and left to stand for 30 min in the dark place at room temperature (28°C). The amount of DPPH remaining in each period of standard was determined spectrophotometrically at 540 nm using a microtiter plate reader. All measurements were performed in triplicate. The radical scavenging activity was calculated as % inhibition.

2.6. *Synthesis of AgNPs*

In the synthesis of AgNPs, an aqueous solution of 1mg/mL CE was stirred and the pH was adjusted to 6 by HCl or NaOH. Then an aqueous solution of $AgNO_3$ was added dropwise to the CE solution until the volume ratio of CE to $AGNO_3$ was 100:1 and the final concentration of $AgNO_3$ was 0.01, 0.05, 0.1, 0.5, and 1 mM. The effect of reaction temperature was studied at 28, 50, 75, and 90°C and the reaction time studied was 15-300 min. The reaction mixture was kept under continuous stirring for all conditions.

2.7. *Characterization of AgNPs*

2.7.1. *UV-Vis absorption*

Characterization of the AgNPs was performed using UV–Vis spectrophotometer (Shimadzu UV 2450 double-beam spectrophotometer, Shimadzu-2450, Kyoto, Japan). The obtained AgNPs was diluted to 100 folds with deionized water before subjecting to this investigation. The optical property of the AgNPs solution was observed in the wavelength range of 300-800 nm. The UV-Vis absorbance spectra were recorded at room temperature.

2.7.2. *PCS*

The obtained AgNPs from different conditions were investigated for particles size, size distribution, and zeta potential by PCS (Malvern Zeta sizer Nano-Zs, Malvern Instruments, Worcestershire, UK). Each sample was diluted to 100 fold with deionized water before measurement. The size average of particles, size distribution expressed as polydispersity index (PdI), and zeta potential were recorded from three measurements taken of three independent AgNPs batches.

2.8. *Antimicrobial activity test of AgNPs*

The antimicrobial activity of the obtained AgNPs against the pathogenic microorganism; *S. aureus*, *E. coli*, *S. mutans*, and *C. albicans* was tested by means of Kirby-Bauer method (*18*). The aerobic and facultative bacteria were grown in TSA and BHI-A, respectively at 37°C for 24 h. Then they were diluted with TSB and BHI-B, respectively to a final density of $1.5 \times 10^6$ colony forming unit (CFU)/mL. *C. albicans* were cultured in SDA at 37°C for 36-48 h. The fungal suspension was diluted with SDB to a final concentration of $1\text{-}2 \times 10^5$ CFU/mL. The suspensions of the test microorganisms were swabbed on the surface of their corresponding agars. The wells of 6 mm were made on agar plates and each AgNPs sample of 40 µL was filled in the wells, whereas CE and $AgNO_3$ solutions were used as the negative controls. Ampicillin 100 µg/mL and Amphotericin B 100 µg/mL were used as the positive controls against bacterial and fungal strains, respectively. The plates were incubated at 37°C for 24 h and 48 h for bacterial and fungal strains, respectively. The antimicrobial activity of AgNPs was evaluated by determining the diameter of clear zone of inhibition (mm). All samples were done in triplicate for each pathogen.

2.9. *Statistical analysis*

Descriptive statistics for continuous variables were calculated and reported as a mean ± standard deviation. Data were analyzed using a One-way analysis of variance and Duncan's multiple range test using Statistic a software version 17 (SPSS Inc., Chicago, Illinois, USA). *P*-value less than 0.05 was considered as significant difference.

## 3. Results

### 3.1. *Antioxidant and reducing activities of CE*

The color of CE powder was dark orange-red and could dissolve well in water. The antioxidant and reducing activities of CE were performed when CE was in aqueous solution. The results from all tests demonstrated that CE aqueous solution possessed strong antioxidant and reducing capacities. The reducing activity of CE solution from FRAP was 78.7 ± 2.4 mM/mg whereas the free radical scavenging property of CE determined by ABTS showed the TEAC value of 64.8 ± 4.2 µM/mg. The minimum concentration to scavenge 50% of free radicals ($IC_{50}$) obtained from DPPH was 51.2 ± 3.2 µM

### 3.2. *Synthesis and antimicrobial activity of AgNPs*

#### 3.2.1. *Effect of $AgNO_3$ concentration*

In this experiment, the reaction temperature was fixed at 75°C and the reaction time was fixed at 60 min. The concentration of $AgNO_3$ was varied from 0.025 to 10 mM. The color of CE solution is orange whereas $AgNO_3$ solution is colorless. In the synthesis process, the color of mixture between CE and $AgNO_3$ was slowly changed from orange turned to brown-gray color which indicating the formation of AgNPs. The high concentration of $AgNO_3$ showed rapid change in color whereas the color change in low concentration of $AgNO_3$ was slow. Figure 1 show particles size and zeta potential value of obtained AgNPs obtained from different concentration of $AgNO_3$. It was found that the particles size of AgNPs that synthesized from 0.025, 0.5, 1, 5, and 10 mM $AgNO_3$ were 411.8 ± 20.4, 245.3 ± 34.2, 183.7 ± 15.8, 160.8 ± 4.0, and 233.3 ± 42.4 nm, respectively with the PdI values 0.28 ± 0.1, 0.24 ± 0.1, 0.23 ± 0.1, 0.18 ± 0.1, and 0.22 ± 0.1, respectively. The zeta potential of the AgNPs obtained from these concentrations were –24.6 ± 6.8, –27.4 ± 2.6, –28.0 ± 3.2, –32.6 ± 4.1, and –20.1 ± 8.1 mV, respectively.

For antimicrobial activity, it was found that AgNPs obtained from 5 mM $AgNO_3$ showed the highest activity against all microbial strains as they demonstrated the significantly widest inhibition zones. The inhibition zones of the AgNPs of this condition against *S. aureus* and *E. coli* were 13.4 ± 0.3 and 16.7 ± 0.7 mm, respectively whereas that against *S. mutans* DMST 9567 and *S. mutans* DMST 41283 were 14.1 ± 0.7 and 13.7 ± 0.3 mm, respectively. The inhibition zone of AgNPs against *C. albicans* was 14.3 ± 0.7 mm as shown in Table 1.

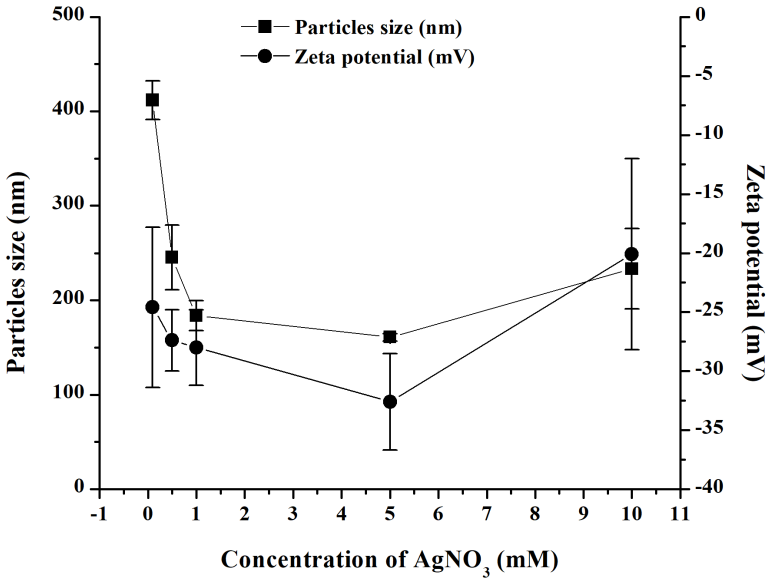


**Figure 1. Effect of concentration of AgNO3 on particles size and zeta potential of AgNPs.**

**Table 1. Effect of concentration of $AgNO_3$ on antimicrobial activity of AgNPs**

| Concentration of $AgNO_3$ (mM) | Diameter of inhibition zone (mm) | | | | |
|---|---|---|---|---|---|
| | *S. aureus* ATCC 25923 | *E. coli* ATCC 25922 | *S. mutans* DMST 9567 | *S. mutans* DMST 41283 | *C. albicans* ATCC 10231 |
| 0.1 | NZ | NZ | NZ | NZ | NZ |
| 0.5 | 9.2 ± 0.7 | 13.8 ± 0.7 | 9.0 ± 0.7 | 9.4 ± 0.8 | 9.2 ± 0.7 |
| 1 | 11.5 ± 0.7 | 15.2 ± 0.7 | 10.7 ± 0.3 | 11.2 ± 0.7 | 12.4 ± 0.6 |
| 5 | 13.4 ± 0.3 | 16.7 ± 0.7 | 14.1 ± 0.7 | 13.7 ± 0.3 | 14.3 ± 0.7 |
| 10 | 12.6 ± 0.5 | 14.8 ± 0.3 | 13.6 ± 0.7 | 12.8 ± 0.8 | 13.1 ± 0.7 |

NZ: No inhibition zone

3.2.2. *Effect of reaction temperature*

In the study of effect of temperature on the obtained AgNPs, the concentration of $AgNO_3$ was fixed at 5 mM and the reaction time was fixed at 60 min. The reaction temperature studied were at 28, 50, 75, and 90°C. The size of the AgNPs obtained from these conditions were 1310.1 ± 178.7, 222.2 ± 130.8, 137.5 ± 23.2, and 219.1 ± 81.2 nm, respectively with the PdI values of 0.48 ± 0.1, 0.32 ± 0.1, 0.18 ±0.1, 0.22 ± 0.1, respectively. The zeta potential of the AgNPs obtained from these concentrations were –5.1 ± 2.9, –19.6 ± 1.3, –29.3 ± 1.5, and –28.4 ± 2.6 mV, respectively as shown in Figure 2.

For antimicrobial activity, it was found that AgNPs obtained from the reaction temperature was 75°C showed the highest activity against all microbial strains as they demonstrated the significantly widest inhibition zones. The inhibition zones of the AgNPs of this condition against *S. aureus*, *E. coli*, *S. mutans* DMST 9567, *S. mutans* DMST 9567, and *C. albicans* were 12.4 ± 0.4, 16.8 ±0.6, 13.4 ± 0.7, 13.2 ± 0.3, and 14.5 ± 1.6 nm, respectively whereas AgNPs synthesized at 28°C showed no inhibition zone against all strains. The results show in Table 2.

3.2.3. *Effect of reaction time*

In the study of effect of reaction time, the concentration of $AgNO_3$ was fixed at 5 mM and the reaction temperature was fixed at 75°C. The reaction period studied was in the range of 15-300 min. The results of UV-Vis spectra showed that increase in reaction time caused significant increase in UV-Vis absorption. However, it was noted that the absorption of AgNPs obtained from the reaction tome of 60 and 90 min showed no significant difference. Moreover, the absorption intensity was decrease with the reaction time at 120, 180, and 300 min, respectively. The highest intensity of the prepared AgNPs spectra was obtained (data not shown) when the following condition was use; $AgNO_3$ at 5 mM, reaction temperature at 75°C and the reaction time of 60 min. The effect of duration of reaction on particles size and zeta potential was shown in Figure 3. The particles size of AgNPs obtained from the reaction times of 30, 60, 180, and 300 min was 128.7 ± 1.85, 120.9 ± 0.8, 235.3 ± 3.87, and 929.23 ± 68.9 nm, respectively whereas the PdI values of Effect of duration reaction of synthesis AgNPs at 30, 60, 180, and 300 min PDI 0.20 ± 0.1, 0.18 ±0.1, 0.24 ± 0.1, and 0.45 ± 0.1, respectively. The zeta potential of the AgNPs obtained from these concentrations were –31.1 ± 1.2, –30.6 ± 0.8, –24.2 ± 1.4, and –14.7 ± 3.2 mV, respectively.

For antimicrobial activity, it was found that AgNPs obtained from the reaction time of 60 min showed the highest activity against all microbial strains as they

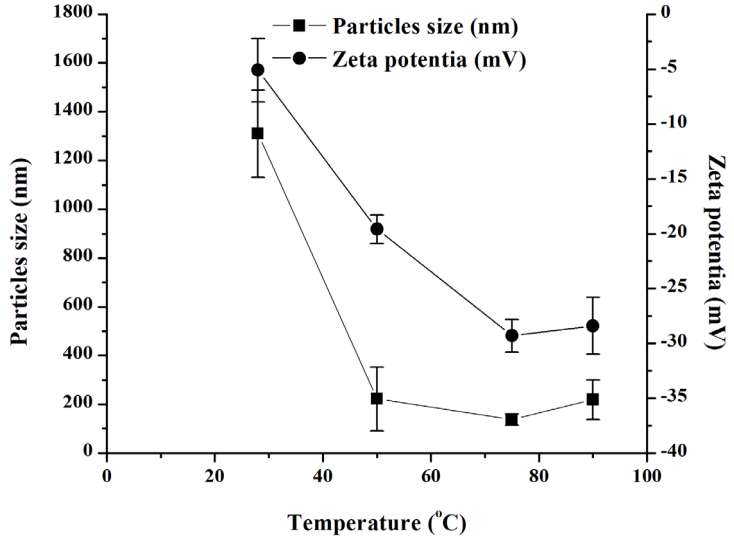


**Figure 2. Effect of reaction temperature on particles size and zeta potential of AgNPs.**

**Table 2. Effect of temperature on antimicrobial activity of AgNPs**

| Temperature (°C) | Diameter of inhibition zone (mm) | | | | |
|---|---|---|---|---|---|
| | *S. aureus* ATCC 25923 | *E. coli* ATCC 25922 | *S. mutans* DMST 9567 | *S. mutans* DMST 41283 | *C. albicans* ATCC 10231 |
| 28 | NZ | NZ | NZ | NZ | NZ |
| 50 | 9.5 ± 0.8 | 12.1 ± 0.7 | 10.1 ± 0.3 | 10.4 ± 0.7 | 11.6 ± 1.2 |
| 75 | 12.4 ± 0.4 | 16.8 ± 0.6 | 13.4 ± 0.7 | 13.2 ± 0.3 | 14.5 ± 1.6 |
| 90 | 12.4 ± 0.6 | 14.8 ± 0.4 | 11.6 ± 0.8 | 10.8 ± 0.7 | 10.5 ± 0.4 |

NZ: No inhibition zone

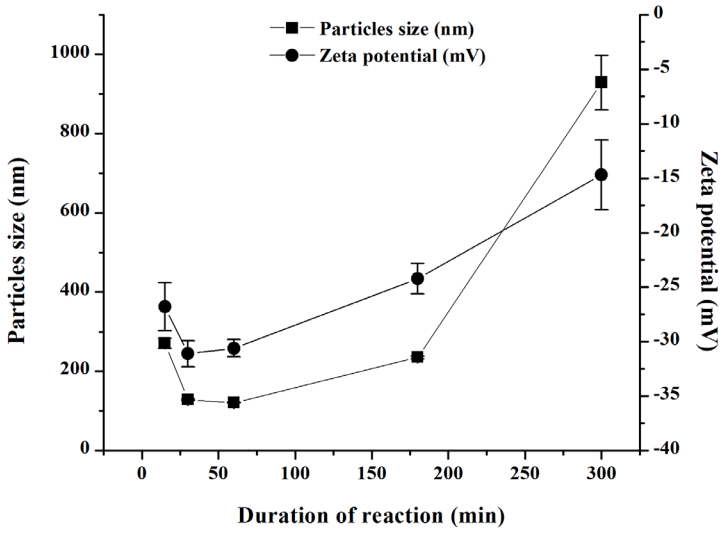


**Figure 3. Effect of reaction time on particles size and zeta potential of AgNPs.**

**Table 3. Effect of duration of reaction on antimicrobial activity of AgNPs**

| Duration of reaction (min) | Diameter of inhibition zone (mm) | | | | |
|---|---|---|---|---|---|
| | *S. aureus* ATCC 25923 | *E. coli* ATCC 25922 | *S. mutans* DMST 9567 | *S. mutans* DMST 41283 | *C. albicans* ATCC 10231 |
| 15 | 8.0 ± 0.5 | 9.0 ± 0.5 | NZ | NZ | NZ |
| 30 | 11.2 ± 0.5 | 12.0 ± 0.25 | 11.3 ± 0.3 | 10.5 ± 0.7 | 11.4 ± 0.7 |
| 60 | 12.0 ± 0.5 | 16.5 ± 0.5 | 13.6 ± 0.7 | 13.4 ± 0.7 | 14.5 ± 0.7 |
| 180 | 10.25 ± 0.5 | 11.5 ± 0.25 | 12.8 ± 0.7 | 12.4 ± 0.3 | 13.5 ± 0.7 |
| 300 | NZ | NZ | NZ | NZ | NZ |

NZ: No inhibition zone

demonstrated the significantly widest inhibition zones when compared with those obtained from the reaction time of 15, 30, 180 min, whereas those obtained from the reaction time of 300 min showed no inhibition zone against all microbial strain. The inhibition zones of the AgNPs obtained from the reaction time of 60 min against *S. aureus*, *E. coli*, *S. mutans* DMST 9567, *S. mutans* DMST 9567, and *C. albicans* were 12.0 ± 0.5, 16.5 ± 0.5, 13.6 ± 0.7, 13.4 ± 0.7, and 14.5 ± 0.7 mm, respectively as shown in Table 3.

## 4. Discussion

Previously, antioxidant property of *C.sappan* was investigated and reported that the activity was according to the phenolic compounds (*19*) such as alkaloid, tannins, steroids, and flavonoids (*14,20*), which most of flavonoid compounds in the extracts are flavone and flavonol (*21*). Most of these compounds are not soluble in water. The synthesis reaction of AgNPs needs to be in aqueous system. Therefore, it is essential to investigate whether there are some water soluble active compounds of this plant. The results in the present study reveal that the aqueous extract of *C. sappan* still possesses antioxidant and reducing properties. These results indicated that there are some bioactives in *C. sappan* that are water soluble and have antioxidant and reducing properties.

In the synthesis process, it has been reported that may factors including pH (*22*), reaction temperature (*23*), concentration of a precursor (*24*), and reaction time (*24*) that might affect the obtained AgNPs. Therefore, in the present study that the aqueous solution of *C. sappan* extract was used as a reducing agent, we investigated all of these factors and the results are in agreement with the previous reports that the concentration of the precursor ($AgNO_3$), the reaction temperature, and the reaction time play an important role particularly on the size and antimicrobial activity of the obtained AgNPs. The results in the present study show that the smallest size of AgNPs can be obtained from the suitable condition using 5 mM $AgNO_3$.

The obtained AgNPs synthesized from temperature 75°C showed smallest particle (137.5 ± 23.2 nm) and highly negative charge (–29.3 ± 1.5 mV). It was found that higher temperature gave smaller size of AgNPs. However, the temperature higher than 75°C was found to be not suitable condition for AgNPs preparation. Our results are in good agreement with the other research groups who have reported the AgNPs synthesized by using tea leaf extract that the particles size of AgNPs increased with the increased temperature

(*25*). The reaction time also affect the obtained AgNPs synthesized by CE. The obtained AgNPs synthesized at 60 min showed the smallest particles size, highest negative zeta potential, and low PdI value. The antimicrobial activity was according to the size of AgNPs. It was found that the smaller particle size gave the higher antimicrobial activity. It is considered that smaller size AgNPs having the large surface area which available for interaction microorganism cell than the larger size AgNPs (*26*). The mechanism of antimicrobial activity of AgNPs has been proposed that it is due to the adhesion of AgNPs on the surface of the microorganisms and change the properties of their membranes (*27*). Moreover, the effective of AgNPs on microbial is dependent on the microorganisms genus, species, strain and also isolates (*28*). The AgNPs obtained in the present study show less effective against *C. albicans* than bacteria. This is considered that *C. albicans* as a eukaryotic cell has thicker cell wall. The cell wall of *C. albicans* is composed of β1, 3-glucan that can form cross-linkages with other compounds and increase the strength and integrity of the cell wall. Therefore, it is more difficult for AgNPs to penetrate into fungal cell in comparison with the bacterial cells.

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Original Article

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# Effect of rice variety and reaction parameters on synthesis and antibacterial activity of silver nanoparticles

**Temsiri Suwan[1], Sakornrat Khongkhunthian[2,3], Jakkapan Sirithunyalug[3,4], Siriporn Okonogi[3,4,*]**

[1] *Interdisciplinary Program in Nanoscience and Nanotechnology, Chiang Mai University, Chiang Mai, Thailand;*
[2] *Department of Restorative Dentistry and Periodontology, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand;*
[3] *Research Center of Pharmaceutical Nanotechnology, Chiang Mai University, Chiang Mai, Thailand;*
[4] *Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.*

**Summary** **In the present study, three different rice varieties; Jasmine (JM), Niaw Koko-6 (NKK), and Saohai (SH) were determined for reducing power using ferric reducing antioxidant power (FRAP) assay. SH showed the highest reducing property followed by JM and NKK, respectively. All modified rice samples were used to fabricate silver nanoparticles (AgNPs) by reducing silver nitrate ($AgNO_3$) to metallic Ag. The obtained AgNPs from JM, NKK, and SH namely JM-AgNPs, NKK-AgNPs, and SH-AgNPs, respectively, showed maximum absorption at 410, 408, and 409 nm, respectively, which confirmed the spectra of AgNPs. Reaction parameters such as $AgNO_3$ and modified rice concentration as well as the reaction period were investigated. It was found that increasing of these parameters gave better AgNPs until the concentration of modified rice and $AgNO_3$ reached to 0.3% and 10 mM, respectively and the reaction period reached to 60 min, the most suitable AgNPs were obtained. Among the three rice varieties, SH showed the most potential for synthesis of AgNPs. SH-AgNPs showed the smallest size of 80.4 ± 2.8 nm and the highest zeta potential of – 45.9 ± 1.4 mV. The AgNPs obtained from all three rice varieties showed effective against *Escherichia coli* than *Staphylococcus aureus* and SH-AgNPs showed significantly higher antibacterial activity than JM- AgNPs and NKK-AgNPs.**

***Keywords:*** AgNPs, modified rice, rice variety, green synthesis, antibacterial activity

## 1. Introduction

Silver nanoparticles (AgNPs) have been increasing interested in medical applications because of their ability to inhibit many important pathogenic bacterial such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (*1*) owing to the effects of AgNPs on bacterial membrane (*2*). Moreover, AgNPs have shown anti-inflammatory (*3*) and anticancer (*4*) activities. Previously, AgNPs were prepared by using chemical reaction between silver nitrate ($AgNO_3$) and chemical reducing agents, such as hydrazine hydrate, sodium citrate, and tannic acid (*5,6*) to reduce $Ag^+$ to metallic silver ($Ag^0$). This may increase chemical waste to environment. Nowadays, there are many efforts to reduce generated hazardous waste by using active components from certain potential plants having reducing property, such as *Mentha piperita* (*7*), *Psidium guajava* (*8*), and *Cymbopogon citratus* (*9*).

Rice is the principle food for people in many countries. We previously reported that rice extract has antioxidant and reducing properties (*10*). However, the concentration of the modified rice used previously was too low to show significant reducing property. In the present higher rice concentration was used with the main aim of using modified rice as a reducing agent in the preparation of AgNPs (Rice-AgNPs). The effects of rice variety and rice concentration as well as other reaction parameters such as $AgNO_3$ concentration and time of reaction were investigated. The obtained rice-AgNPs were characterized by UV-vis, Fourier transmission infrared (FTIR) spectroscopy, photon correlation spectrophotometry (PCS). The inhibitory activity of

**Address correspondence to:*
Dr. Siriporn Okonogi, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail: siriporn.okonogi@cmu.ac.th

the rice-AgNPs to Gram positive and Gram negative bacteria was evaluated by measuring the inhibition zones under well diffusion method and determining minimum inhibition concentration (MIC) as well as minimum bactericidal concentration (MBC) using micro-dilution method.

## 2. Materials and Methods

### 2.1. *Materials*

Milled rice grains of three rice varieties including JM, NKK, and SH were obtained from a local market in Chiang Mai, Thailand. $AgNO_3$, methanol, and glacial acetic acid were supplied by RCI Lab-scan Co., Ltd. (Bangkok, Thailand). Potassium bromide (KBr) for FTIR was purchased from Fisher Scientific, (Loughborough, UK). Tryptic soy agar (TSA) and broth (TSB) were supplied by Difco™ (Baltimore, Maryland, USA). All other chemicals and solvents were of AR grade or the highest grade available.

### 2.2. *Bacterial strains*

The aerobic bacterial strains of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 represented for Gram-positive and Gram-negative bacteria, respectively, were used.

### 2.3. *FRAP assay*

Three rice grains of JM, NKK, and SH were chemical modified according to the method previously described by Okonogi *et al*. (*11*). The reducing power of the modified rice was determined using FRAP assay according to the method described previously (*12*) with some modification. Briefly, the FRAP reagent was freshly prepared by mixing 2.5 mL of 10 mM TPTZ solution in 40 mM HCl with 2.5 mL of 20 mM $FeCl_3$ and 25 mL of 0.3 M acetate buffer, pH 3.6. An amount of 20 µL dispersion of modified rice samples were mixed with 120 µL of FRAP reagent in 96 well plate. Blank samples were prepared by mixing acetate buffer and different concentration of JM, NKK, and SH. The samples and blank were incubated for 10 min at room temperature and the absorbance of the samples was determined at 595 nm using microplate reader (Bio-Rad, Model 680, Hercules, California, USA). The reducing power of the samples was evaluated by calculating the amount of $Fe^{2+}$ produced by modified rice starch samples using the calibration curve of $FeSO_4$. All data were run in triplicate.

### 2.4. *Synthesis of AgNPs*

#### 2.4.1. *Effects of reactant concentration*

Aqueous solutions containing 0.1, 0.2, 0.3, 0.6, and 0.9 % w/v of modified rice were prepared. In the same time, aqueous solutions of 2.5, 5, and 10 mM $AgNO_3$ were separately prepared. Then, add $AgNO_3$ solution dropwise to the rice solution at 75°C with continuous stirring until the volume ratio of the rice solution and $AgNO_3$ solution was 100:1. The reaction was kept at this temperature under continuous stirring for 60 min. The obtained rice-AgNPs were cooled down to room temperature for further studies.

#### 2.4.2. *Effects of reaction period*

An aqueous solution containing 0.3 % w/v of modified rice was prepared. Then, 10 mM $AgNO_3$ solution was added dropwise to the rice solution at 75°C with continuous stirring until the volume ratio of the rice solution and $AgNO_3$ solution was 100:1. The reaction was kept at this temperature under continuous stirring for 15, 30, 60, and 90 min. The obtained rice-AgNPs were cooled down to room temperature for further studies.

### 2.5. *Characterization of AgNPs*

#### 2.5.1. *UV-Vis*

The rice-AgNPs obtained from each preparation condition was diluted to 100 fold with deionized water. The outer color appearance of the rice-AgNPs was observed by visualization. The optical property of the rice-AgNPs solution was observed using UV-Vis spectrophotometer (Shimadzu-2450, Kyoto, Japan) in the wavelength range of 200-700 nm.

#### 2.5.2. *PCS*

The size, size distribution (PdI), and zeta potential of rice-AgNPs were investigated using PCS (Malvern Zetasizer Nano ZS, Malvern instrument, Worcestershire, UK) at 25°C. Each sample was diluted to 100 fold with deionized water before measuring.

#### 2.5.3. *FTIR*

The lyophilized rice-AgNPs and the modified JM, NKK, and SH in powder form were subjected to FTIR in order to investigated for functional group spectra. The samples were prepared in a KBr disc. The FTIR spectra of the samples were recorded in the range of 4,000-400 $cm^{-1}$ using a smart diffuse reflectance, Nicolet Nexus 470 FT-IR (Minneapolis, Minnesota, USA) in the diffuse reflectance mode at a resolution of 4 $cm^{-1}$ at room temperature. The spectra were collected against a KBr disc background at a controlled ambient temperature at 25°C.

### 2.6. *Evaluation of antimicrobial activity*

#### 2.6.1. *Well diffusion method*

A well diffusion method for evaluation of antibacterial activity against the test pathogenic bacteria of the obtained JM-AgNPs, NKK-AgNPs, and SH-AgNPs was based on Kirby-Bauer method (*13*). The test strains of *S. aureus* and *E. coli* were grown in TSA at 37°C for 24 h. Then, they were diluted in TSB to a final density of 1.5 × $10^6$ colony-forming units (CFU)/mL. The density of the microbial suspension was adjusted with 0.5 McFarland constant by observing the wavelength at 600 nm in a UV-vis spectrophotometer. The bacterial suspensions were swabbed on the agar surface by using sterile cotton swab. Aqueous mixtures (40 µL) of JM-AgNPs, NKK-AgNPs, and SH-AgNPs were added onto the wells in agar plates, the diameter of well was 6 mm. The plate were incubated at 37°C for 24 h. The antimicrobial activity was evaluated by determining the diameter of the clear zone of inhibition around the well expressed in millimeter (mm). All samples were done in triplicate.

2.6.2. *Broth dilution method*

Aqueous solutions containing 0.1 mg/mL of lyophilized rice-AgNPs were prepared in deionized water and diluted in TSB containing the test bacteria. The results were evaluated after 24 h of incubation at 37°C. The minimum rice-AgNPs concentration giving the clear solution in this step denoted the MIC. For MBC determination, the clear samples resulted from the MIC series were swabbed on the agar plates as indicated by Clinical and Laboratory Standards Institute (CLSI) guideline (*14*). The minimum rice-AgNPs concentration showing no bacterial growth in agar plate denoted the MBC. All samples were done in triplicate.

2.7. *Statistical analysis*

Data were analyzed using a One-way analysis of variance (ANOVA) and Duncan's multiple range test statistic a software version 17 (SPSS Inc., Chicago, United States). The values were presented as means ± standard deviation which a *p*-value less than 0.05 was considered as a significant difference.

## 3. Results

3.1. *Reducing property of the modified rice*

The reducing power of the samples analyzed by the FRAP assay was evaluated by calculating the amount of $Fe^{+2}$ produced by the test samples using the calibration curved of $FeSO_4$ (*15*). The modified rice of JM, NKK, and SH exhibited reducing power as a dose dependent manner as seen in Figure 1. Among the test concentrations, the highest reducing activity was found at the rice concentration of 0.9%. Among the modified rice samples from three rice varieties, SH showed the highest reducing capacity of 122.1 µmol $Fe^{2+}$/g sample whereas

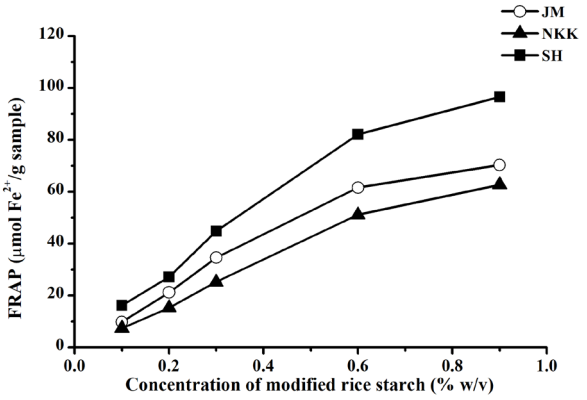


**Figure 1. Reducing property of modified rice starch.**

JM and NKK showed that of 78.3 and 72.5 µmol $Fe^{2+}$/g sample, respectively.

3.2. *Effects of reactant concentration*

The reactants in the chemical reaction of AgNPs preparation were the modified rice and $AgNO_3$ which both of them were clear and colorless solutions. However, it was noticed that the colorless solutions of the mixtures containing the modified rice and $AgNO_3$ changed to yellow color when the AgNPs were formed. After subjecting to UV-Vis spectroscopy, JM-AgNPs, NKK-AgNPs, and SH-AgNPs showed the maximum absorption at 410, 408, and 409 nm, respectively. We used these maximum wavelengths of AgNPs from each rice to determine the amount of the AgNPs obtained from each reactant concentration. The results shown in Figure 2 indicated that change of concentration of $AgNO_3$ significantly affected the obtained AgNPs. The higher the concentration of $AgNO_3$, the higher quantity of absorption obtained. The concentration of JM showed significant effect only when 5 mM $AgNO_3$ was used whereas that of NKK show no significant difference. Interestingly, the concentration of SH showed significant effect on the obtained AgNPs with all concentrations of $AgNO_3$. Among the test concentrations, the result demonstrated that the highest absorbance of rice-AgNPs was obtained from SH modified rice at 0.9% w/v with $AgNO_3$ at 10 mM.

3.3. *Effects of reaction period*

To investigate the role of reaction period on the formation of AgNPs, the gradual generation of AgNPs was monitored by visualization and UV-Vis spectroscopy. It was found that the reaction period affected the yield of AgNPs. During the synthesis process, the color of the mixture changed from colorless to light yellow indicating the beginning of AgNPs production. It was observed that the modified rice of different variety showed different manner of color changing. For example, at the reaction

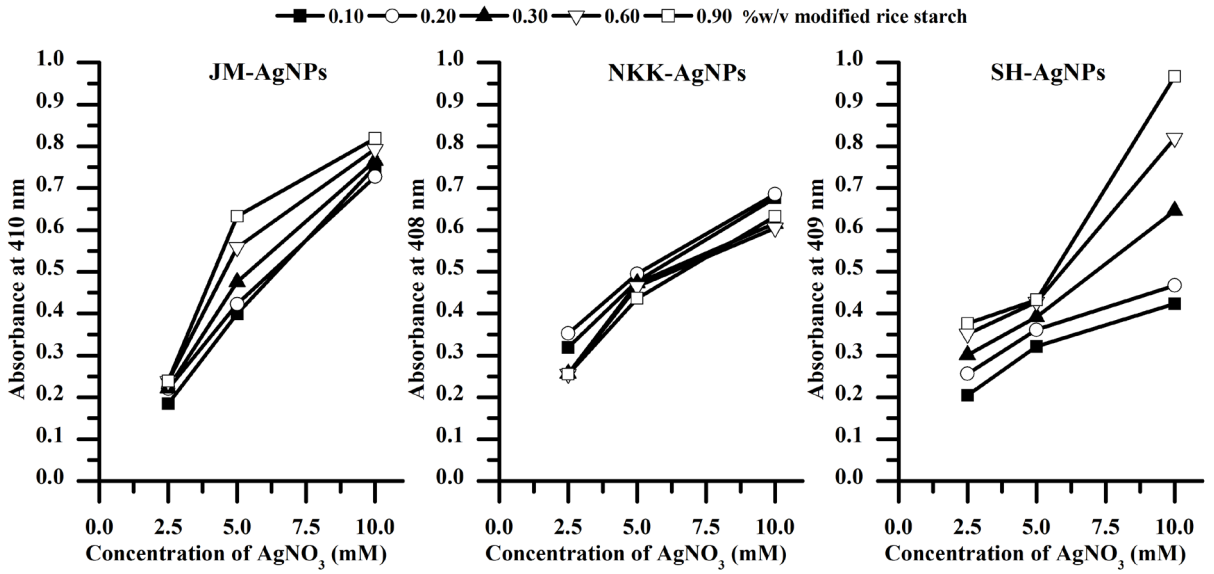


**Figure 2. Absorbance of the rice-AgNPs using different concentrations of modified JM, NKK, and SH and $AgNO_3$ investigated at wavelengths 410 408, and 409 nm, respectively.**

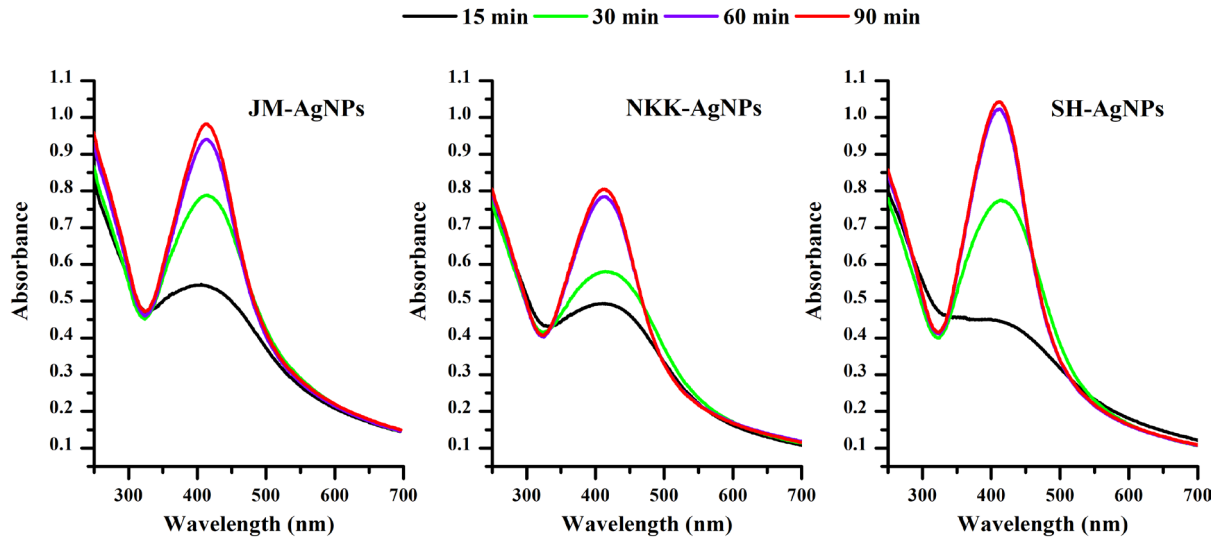


**Figure 3. UV-Vis spectra of JM-AgNPs, NKK-AgNPs, and SH-AgNPs synthesized using modified rice starch concentration 0.3%w/v in different reaction periods of 15, 30, 60, and 90 min.**

period of 15 min, the color of the mixtures using JM changed rapidly whereas the color of those using SH and NKK gradually changed. At 90 min, it was found that the yellow color of the mixtures using SH was the most intense compared to those using JM and NKK. The obtained of JM-AgNPs, NKK-AgNPs, and SH-AgNPs were confirmed by UV-Vis spectrophotometer at the scanning range of 200-700 nm and the quantity of their absorption was observed at their maximum absorption at 410, 408, and 409 nm, respectively. The UV-Vis absorption spectra of the colloidal dispersions of the rice-AgNPs prepared by using JM, NKK, and SH in different reaction period of 15, 30, 60, and 90 min are shown in Figure 3. It was found that with an increase of reaction time, a narrow Plasmon absorption peak around 400 nm remarkably increased due to the AgNPs formation. It was also noted that the absorption intensity at the reaction periods of 60 and 90 min showed no significant difference. Therefore, the optimal reaction period required for synthesized AgNPs from the modified rice was considered to be 60 min.

### 3.4. *PCS*

Size determined by PCS is the hydrodynamic size. The size of rice-AgNPs prepared by using 0.3% modified rice and 10 mM $AgNO_3$ at a reaction time of 60 min was compared. It was found that the size of AgNPs obtained from different rice variety was different. The size of SH-AgNPs was the smallest of 80.4 ± 2.8 nm whereas the size of JM-AgNPs and NKK-AgNPs was 113.6 ± 1.2 and 179.0 ± 5.6, respectively. The size distribution was also

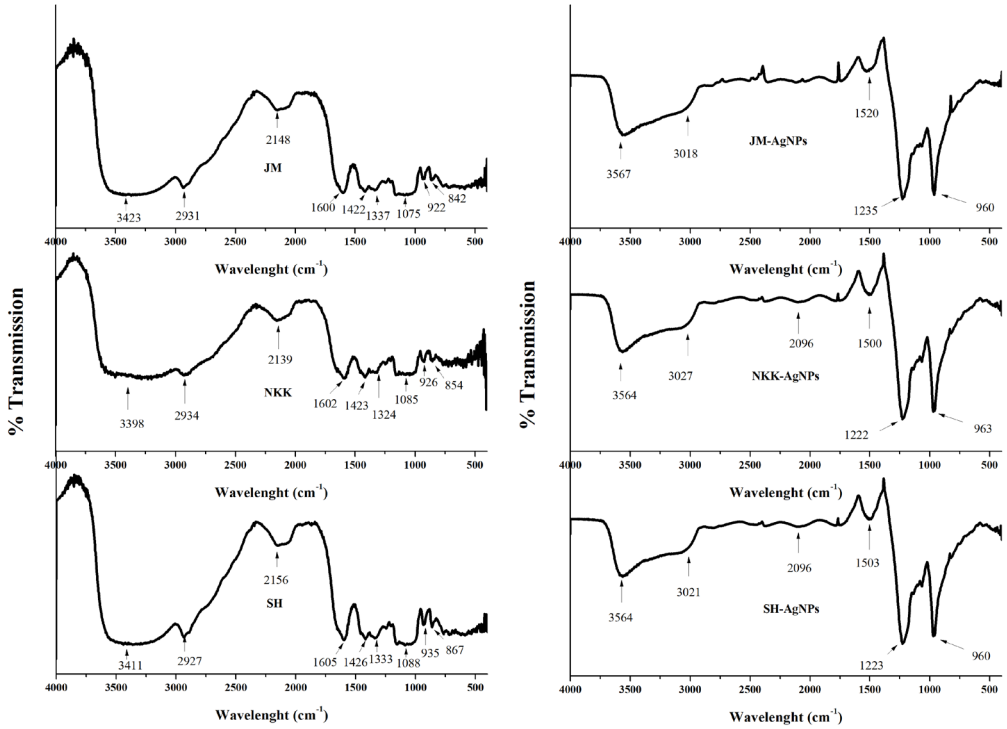


**Figure 4. FTIR spectra of the pure modified rice (left) and the rice-AgNPs (right) showing characteristic peaks at 4,000-400 $cm^{-1}$.**

different. It was found that the PdI values of JM-AgNPs, NKK-AgNPs, and SH- AgNPs were acceptable range of 0.214 ± 0.1, 0.248 ± 0.1, 0.184 ± 0.1, respectively. It was noted that the size distribution of SH-AgNPs was the narrowest. In addition, zeta potential values of these AgNPs were –36.3 ± 0.7, –36.1 ± 1.7, and –45.9 ± 1.4 mV, respectively. It was also noted that the zeta potential of the SH-AgNPs was the highest.

3.5. *FTIR*

The FTIR analysis was carried out to investigate whether the functional group of each modified rice was responsible for synthesis and stabilization of AgNPs. The results showed different stretches of bonds at different peak and the spectra are shown in Figure 4. FTIR spectrum of pure modified rice powder exhibited the typical absorption bands at 3,400, 2,930, 2,140, 1,600, 1,420, 1,337, 1,085, 930, and 850 $cm^{-1}$. After reacting with $AgNO_3$, the FTIR spectra of all synthesized rice-AgNPs showed the absorption bands at around 3,560, 3,020, 2,096, 1,500, 1,220, and 960 $cm^{-1}$.

3.6. *Antibacterial activity*

The rice-AgNPs was investigated for antibacterial activity against *S. aureus* and *E. coli*. The results showed that AgNPs from all rice varieties had inhibitory effects against both strains as shown in Table 1 and 2, respectively whereas the pure modified rice solution could not inhibit the tested strains. It was found that the rice-AgNPs obtained from 10 mM $AgNO_3$ and 0.3% of modified rice possessed the highest antibacterial activity. The MIC values of JM-AgNPs, NKK-AgNPs, and SH-AgNPs against *S. aureus* were 0.05, 0.1, and 0.025 mg/mL, respectively and against *E. coli* were 0.05, 0.05, and 0.025 mg/mL, respectively. The MBC value of rice-AgNPs from all three rice varieties against *S. aureus* was 0.1 mg/mL whereas that values of JM-AgNPs, NKK-AgNPs, and SH-AgNPs against *E. coli* were 0.1, 0.1, and 0.05 mg/mL, respectively as shown in Table 3.

## 4. Discussion

Rice is composed mainly of starch (> 85%) and other components (< 15%) which are protein, fat, and fiber. Rice starch comprises mainly two types of glucose polymers, amylose, and amylopectin. Okonogi *et al*. have investigated the amylose content in different rice varieties and reported that the amylose content of the non-glutinous SH was found to be the highest of 21% whereas that of the other rice varieties was significantly lower (*11*). They also reported that the raw rice grains from various rice varieties possess antioxidant activity (*10*). Moreover, some other research groups reported the difference in phenolic content in different rice varieties (*16,17*) confirming the antioxidant activity of rice. In the present study, we demonstrated that the rice grains after subjecting to chemical modification still possess antioxidant and reducing activity but in different levels. The reducing activity of the modified rice of non-glutinous rice (JM and SH) was found to be higher than

**Table 1. Inhibition zone of the rice-AgNPs against *S. aureus***

| AgNPs | $AgNO_3$ (mM) | Inhibition zone (mm) Concentration of modified rice (%w/v) | | | | |
|---|---|---|---|---|---|---|
| | | 0.1 | 0.2 | 0.3 | 0.6 | 0.9 |
| JM | 2.5 | NZ | NZ | NZ | NZ | NZ |
| | 5 | 7.4 ± 0.1 | 7.4 ± 0.4 | 7.6 ± 0.3 | 7.5 ± 0.2 | 7.2 ± 0.2 |
| | 10 | 7.8 ± 0.2 | 8.0 ± 0.2 | 10.6 ± 0.3 | 9.5 ± 0.5 | 8.4 ± 0.3 |
| NKK | 2.5 | NZ | NZ | NZ | NZ | NZ |
| | 5 | 7.2 ± 0.3 | 7.1 ± 0.4 | 7.4 ± 0.4 | 7.8 ± 0.2 | NZ |
| | 10 | 7.6 ± 0.2 | 7.8 ± 0.2 | 8.1 ± 0.6 | NZ | NZ |
| SH | 2.5 | NZ | 7.1 ± 0.3 | 7.3 ± 0.3 | 7.6 ± 2 | 7.8 ± 0.2 |
| | 5 | 7.4 ± 0.1 | 7.6 ± 0.2 | 7.8 ± 0.2 | 8.1 ± 0.1 | 8.0 ± 0.4 |
| | 10 | 8.2 ± 0.2 | 10.4 ± 0.2 | 14.2 ± 0.4 | 11.6 ± 0.2 | 8.1 ± 0.3 |

NZ: no inhibition zone. Data were represented as mean ± SD.

**Table 2. Inhibition zone of the rice-AgNPs against *E. coli***

| AgNPs | $AgNO_3$ (mM) | Inhibition zone (mm) Concentration of modified rice (%w/v) | | | | |
|---|---|---|---|---|---|---|
| | | 0.1 | 0.2 | 0.3 | 0.6 | 0.9 |
| JM | 2.5 | NZ | NZ | NZ | NZ | NZ |
| | 5 | 7.6 ± 0.4 | 8.1 ± 0.4 | 9.2 ± 0.2 | 8.8 ± 0.3 | 7.8 ± 0.3 |
| | 10 | 10.2 ± 0.2 | 11.8 ± 0.2 | 12.4 ± 0.3 | 12.0 ± 0.5 | 11.5 ± 0.2 |
| NKK | 2.5 | NZ | NZ | NZ | NZ | NZ |
| | 5 | 7.6 ± 0.2 | 7.8 ± 0.5 | 7.8 ± 0.2 | 7.3 ± 0.3 | 7.1 ± 0.3 |
| | 10 | 8.1 ± 0.3 | 8.6 ± 0.3 | 9.2 ± 0.5 | 8.8 ± 0.4 | 7.2 ± 0.2 |
| SH | 2.5 | 7.3 ± 0.2 | 7.5 ± 0.4 | 8.1 ± 0.4 | 7.8 ± 0.2 | 7.4 ± 0.6 |
| | 5 | 7.8 ± 0.2 | 8.2 ± 0.3 | 10.1 ± 0.2 | 9.6 ± 0.3 | 8.4 ± 0.4 |
| | 10 | 12.4 ± 0.4 | 14.8 ± 0.2 | 16.2 ± 0.4 | 14.1 ± 0.5 | 12.8 ± 0.3 |

NZ: no inhibition zone. Data were represented as mean ± SD.

**Table 3. MIC and MBC of the rice-AgNPs (mg/mL)**

| Rice-AgNPs | *S. aureus* | | *E. coli* | |
|---|---|---|---|---|
| | MIC | MBC | MIC | MBC |
| JM-AgNPs | 0.05 | 0.1 | 0.05 | 0.1 |
| NKK-AgNPs | 0.1 | 0.1 | 0.05 | 0.1 |
| SH-AgNPs | 0.025 | 0.1 | 0.025 | 0.05 |

the glutinous rice (NKK). This difference in reducing power leads to the different effect on AgNPs synthesis. As the amylose content in the non-glutinous rice was significantly higher than in the glutinous rice and the results from the FRAP assay indicated that the reducing property of the non-glutinous rice was higher than of the glutinous rice, it is considered that the amylose may also play an important role in reducing property of rice besides the other phenolic components in rice grains. These quantity differences of rice compositions mutually influence the characteristics of their respective modified rice (*18*). The chemical modification of rice starch yielded different degree of substitution values depending on the amylose content and time of reaction (*19*). Amylose adopts a left-handed helical form in aqueous solution and can entrap hydrophobic molecules in the helix, which acts as a one-dimensional supramolecular host molecule (*20*). The end reducing group at C6 of the starch molecules is looked upon as the reducing agent for fabrication of AgNPs (*21*). In the same time, soluble starch chains act as capping agent due to their hydroxyl groups to prevent aggregation of the synthesized AgNPs (*22*). The results in the present study suggest that the modified rice of JM, NKK, and SH still have these functions to reduced $AgNO_3$ and stabilize AgNPs in the fabrication process.

It has been reported that the concentration of reducing agent is an affecting factor in the intensity of the extinction peak of the synthesized AgNPs spectra. Thus, it denotes that increase concentration causes increase absorption peak height (*23*). It has also been reported that increasing concentration of $AgNO_3$, the maximum absorbance of the obtained AgNPs is increase (*24*). The result of the present study was according to the previous report for the increasing concentration of $AgNO_3$. However, for the reducing agent that the modified rice was used, the result was different. Among the three different rice varieties, only modified SH showed significant effect as increase SH concentration caused increase absorption peak height.

The shape of the UV-Vis spectra gives preliminary information about the size and the size distribution of the AgNPs (*25*). The results of the present study show that the shape of spectra depended on the reaction period and rice variety. The absorption peaks of AgNPs obtained

from non-glutinous rice (JM and SH) are sharper than that obtained from glutinous rice (NKK). The intense band in the 380-400 nm range indicates much smaller colloidal particles than the another wavelength (*26*). The rice-AgNPs prepared in various reaction periods of 15-90 min showed the absorption peaks around 400 nm indicating that the fabrication parameters used are suitable to give the AgNPs in a small size. This result also implies that the AgNPs prepared by this synthesis method is very stable without aggregation. This can be confirmed by size measurement using PCS. The particle size of the rice-AgNPs was found to be less than 200 nm depended on the type of rice used in the synthesis process. The AgNPs from non-glutinous rice was smaller than that from the glutinous rice. High zeta potential, either positive or negative, is generally required to ensure stability. In general, systems with zeta potential more than ± 30 mV are considered to be kinetically stable (*27*). Our results showed that the rice-AgNPs were negative charge with high value of zeta potential of higher than 30 mV particularly SH-AgNPs which supports long term stability of AgNPs. This result implies that SH-AgNPs are very stable without aggregation.

FTIR spectra provide functional groups like OH or C=O interacted to the surface of AgNPs (*28*). The IR peaks of the obtained AgNPs in the current study indicate that many functional groups are involved. The peak at 850 $cm^{-1}$ is considered to be the stretching vibration of CC-CHO (*29*). The peaks at 960 $cm^{-1}$ is considered to be the stretching vibrations of C–OCH3, C–H stretching of alkenes and C–O stretching aromatic side chain of proteins (*30*). The absorption band at 1,085 $cm^{-1}$ could be attributed to stretching vibrations of the C–O bond in either group (*31*). The peak at 1,420 and 2,930 $cm^{-1}$ are ascribed to the –C-H stretch of the alkyl group. The peak at 1,600 $cm^{-1}$ the characteristic asymmetrical and symmetrical stretching vibrations of the C=O group (*29*). The broad peak at 3,400 $cm^{-1}$ are assigned as OH stretching that could possibly emanate from carbohydrates. The IR spectra of the modified rice of all three test varieties suggest that they contain various active molecules rich in hydroxyl group and carboxyl group that are responsible for reduction of the metallic ion. The presence of these groups on the surface of AgNPs leads to the negative charge and high zeta potential causing the increase stability of the obtained AgNPs.

The antibacterial activity of SH-AgNPs is significantly higher than JM-AgNPs and NKK-AgNPs, respectively. It has been reported that the AgNPs are found to accumulate in the bacterial membrane (*32,33*). The results in the present study indicate that the rice-AgNPs has slightly higher effective to Gram negative bacteria than Gram positive strains. This result is due to certain difference between Gram positive and Gram negative bacteria. Gram-positive and Gram-negative cells differ markedly in their cell walls which Gram-positive cells is much thicker with higher amount of peptidoglycan in the cell walls than Gram-negative. The thicker cell wall is therefore extensive practical importance in protecting the cell from penetration of silver ions into the cytoplasm (*34-37*).

It can be concluded that the modified rice can be used as reducing agent in the synthesis of AgNPs. Among three rice varieties, SH has the highest potential on the synthesis followed by JM and NKK, respectively. The reaction period and concentration of $AgNO_3$ play an important role in the preparation of AgNPs. The increase absorbance intensity of the rice-AgNPs was dependent on amount of modified SH and $AgNO_3$ as a reducing agent and a precursor, respectively. The size of AgNPs synthesized from non-glutinous modified rice is smaller than from glutinous rice. SH-AgNPs are the most stable with the highest antibacterial activity to both Gram-positive and Gram-negative bacteria.

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# Preparation and characterization of rice gels containing tooth bleaching agent

**Adchareeya Kaewpinta[1], Sakornrat Khongkhunthian[2,3], Pisaisit Chaijareenont[2,4], Siriporn Okonogi[2,5,*]**

[1] *Interdisciplinary Program in Nanoscience and Nanotechnology, Chiang Mai University, Chiang Mai, Thailand;*
[2] *Research Center of Pharmaceutical Nanotechnology, Chiang Mai University, Chiang Mai, Thailand;*
[3] *Department of Restorative Dentistry and Periodontology, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand;*
[4] *Department of Prosthodontics, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand;*
[5] *Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai, Thailand.*

**Summary** **In the present study, the modified white rice of Jasmine (JM) and Saohai (SH) were used to prepare the rice gels. Carbamide peroxide (CP) containing rice gels (CP rice gels) of JM (CP-JM) and SH (CP-SH) were prepared. The rice gels and CP rice gels show homogenous texture. Rice variety influences the characteristics and properties of the rice gels. Amylose content of JM was lower than SH. Rheological behavior of JM and CP-JM was pseudoplastic without thixotropy whereas that of SH and CP-SH was pseudoplastic with thixotropy. CP-SH showed higher adhesive property and viscosity than CP-JM whereas CP-JM showed faster *in vitro* drug release than CP-SH. For *ex vivo* efficacy evaluation, 55 normal human teeth were subjected to the CP rice gels. Samples were applied on tooth surface according to the dental bleaching techniques. For at-home bleaching technique, the CP rice gels with 10% and 20% CP were used with bleaching time of 8h and 4h, respectively. For in-office bleaching technique, the CP rice gels with 35% CP was used with bleaching time of 1 h. The developed CP rice gels showed significantly higher efficacy than the positive and negative controls. For at-home bleaching technique, CP-SH was the most effective gels whereas for in-office bleaching technique, CP-JM was the most effective gels.**

***Keywords:*** Jasmine rice, Saohai rice, modified rice, carbamide peroxide, tooth bleaching

## 1. Introduction

Tooth color is an important issue in aesthetic dentistry because the abnormal tooth color can affect the quality of life (*1*), physical appearance, beauty, and self-confidence (*2,3*). Abnormal tooth color is caused by aging, high levels of fluoride, and other drugs. It is also caused by chromogenic agents such as dental plaque, food, colored beverage, and smoking (*4*). Dissatisfaction with tooth color is widely reported (*5*). An oral health related quality of life questionnaire for use among young adults reported that tooth color was the most important concern (*6*). This concern has been shown to be associated with increased desire for treatments that improve color of the human teeth.

Tooth color can be improved by many methods including toothpastes (*7*), scaling and polishing (*8*), bleaching, enamel microabrasion (*9*), placement of crowns and veneers (*10*). Among those methods, bleaching is the most convenient treatment for tooth color improvement (*11*). Carbamide peroxide (CP), hydrogen peroxide, sodium percarbonate, and calcium peroxide have been widely used as bleaching agent for the teeth (*12*). Peroxide will diffuse into the teeth and bleach the color or darker shades called chromogens that are accumulated in the tooth to give brighter teeth (*13*). CP is more commonly used than others because of its effectiveness and safety for oral use with a controlled concentration (*14*).

The tooth bleaching techniques are divided into two categories; at-home bleaching and in-office bleaching. For at-home bleaching technique, CP at low

**Address correspondence to:*
Dr. Siriporn Okonogi, Department of Pharmaceutical Sciences, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand.
E-mail: okng2000@gmail.com

concentrations, usually between 10 to 20% is used. In general, 10% CP was used for 8 h per day, and 15 to 20% CP was used for 3 to 4 h per day. For in-office bleaching technique, CP at high concentration like 35% was used for short time of 30 min to 1 h per day (*9*). Tooth bleaching products generally help to improve the overall color. Gels are the formulations that enhanced ability of bleaching agents and have become widespread worldwide as an effective tooth bleaching treatment (*15*). Moreover, gels was a preferable formulation in terms of patient compliance, comfortable, and easy to apply on the tray that used for bleaching the teeth (*12*).

Most of gelling agents using nowadays are usually made from synthetic polymers which mostly produced from chemical polymerization and may cause serious problems to the environment. Therefore using natural polymer particularly from the edible raw material is increase because of environmental benefits (*16*).

Rice (*Oryza sativa* L.) is a plant in family Gramineae. The main element of rice is carbohydrates or starch. Fat and protein can be found in rice but very small amount. Starch is polysaccharides which are long chains of monosaccharide linked by glycosidic bonds. The main components of starch are amylose and amylopectin. Amylose is a linear polymer chain and amylopectin is branch polymer chain. Many researches have been reported that the structure of rice starch can be modified and change the physicochemical properties of starch. Structural modification of rice starch can be made by heat, chemicals, or enzymes. The modified rice is widely used in pharmaceutical field. It can be used as gelling agents, flocculants, thickeners, stabilizers, fillers, binders, and disintegrate (*16*). Many researches have studied about rice structural modification, and it is able to make a good natural gelling agents (*17*). We have reported that the gels developed from rice powder of some rice varieties showed suitable properties of buccal drug delivery (*18,19*). Recently, we reported that colored rice gels could be feasible to be the good gelling agents for CP (*20*) however, colored rice gels may decrease patient compliance due to their color which were red-brown or yellow-brown (*21*), whereas rice gels obtained from white rice are normally white or colorless. Furthermore, there is limited information available about using different rice varieties of white rice as gelling agent for CP. The present study emphasized on using white rice as a main component in gel formulation as drug delivery system.

## 2. Materials and Methods

### 2.1. *Materials*

Two rice varieties; Jasmine (JM) and Saohai (SH) were from a local supermarket in Chiang Mai province, Thailand. CP, triphenylphosphine (TPP), silver nitrate, glacial acetic acid, sodium hydroxide, monochloroacetic acid and amylose standards were from Sigma chemical Co. (St. Louis, MO, USA). Dichloromethane, methanol, and glacial acetic acid were obtained from RCI Labscan Co., Ltd. (Bangkok, Thailand). CP commercial gels with 10%, 20%, and 35% CP concentration were from manufacturer (Ultradent Product Inc, Salt Lake City, UT, USA). All other chemicals and solvents were analytical grade or the highest grade available.

### 2.2. *Preparation of modified rice powder*

#### 2.2.1. *Preparation of rice powder*

Raw rice powder was prepared from JM and SH rice grains by wet milling method previously described by Okonogi *et al.* (*17*) with some modification. Briefly, the clean water-soaked rice grains were blended and filtered through 80 mesh sieve. The water of the filtrates was removed and the solid mass obtained was dried in the hot air oven. The dried solid mass was pulverized and kept in a desiccator for further study.

#### 2.2.2. *Determination of amylose*

The analysis of amylose content in the rice was done according to the iodine colorimetric method described by Juliano (*22*) with some modification. Briefly, 0.1 g of rice powders was mixed with 1 mL of 95% ethanol and 9 mL of 1 N sodium hydroxide. The mixed solution was heated to 100°C for 10 min. Then, distilled water was added to make 100 mL solution. A 5 mL of the obtained solution was added to a volumetric flask, 1 mL of 1 M acetic acid and 2 mL iodine-potassium iodide were added into the flask and the volume was adjusted to 100 mL by using distilled water. The solution was protected from light and incubated for 20 min at room temperature. Spectrophotometer measurements were made at 620 nm by using UV-Spectrophotometer (UV 2450 Spectrophotometer, Shimadzu Corporation, Kyoto, Japan). Standard curve was generated using the amylose standards.

#### 2.2.3. *Modification of rice*

The raw SH and JM powder was subjected to chemical modification according to the previous method described by Okonogi *et al*. (*17*) with some modification. Briefly, a solvent of methanol-water was mixed with sodium hydroxide solution. The raw rice powder was added to the mixture followed by monochloroacetic acid solution. The mixture was refluxed at 50 ± 1°C for 3 h. The mixture was adjusted to neutral pH. The solid mass was collected and washed by using methanol until the chloride of the filtrate was negative to silver nitrate testing. The dried solid mass was pulverized and filtered through the 80 mesh sieve.

The fine powder of the modified rice was kept in a desiccator for further study.

2.3. *Morphology of rice particles*

The morphology of rice particles was characterized using scanning electron microscope (SEM) (JEOL JSM-5910LV, JEOL Ltd., Tokyo, Japan). The samples were placed on the surface of the stub and coated with gold before examination. The excitation voltage of 10-20 kV under low vacuum mode (0.7 - 0.8 torr) and 10,000 magnifications were used.

2.4. *Preparation CP rice gels*

Rice gel base was prepared by hydration method previously described (*19*) with some modification. Briefly, exact weight of each modified rice powder was dispersed in distilled water to obtain 10% w/w, and then heated to 90°C and stirred for 1 h to obtain homogenous gels. CP powder was incorporated in the prepared gel base to obtain JM gels containing CP (CP-JM) with concentrations of 10% (10CP-JM), 20% (20CP-JM) and 35% (35CP-JM) and SH gels containing CP (CP-SH) with concentrations of 10% (10CP-SH), 20% (20CP-SH) and 35% (35CP-SH). The gels were kept in 4°C until further study.

2.5. *Outer appearance of CP rice gels*

In order to detect miscibility, rice gels containing CP were visually observed for drug precipitation and gel separation over the whole study period of 5 months.

2.6. *Rheological behavior and viscosity of the gels*

Rheological characterization and viscosity of the rice gels and rice gels containing CP were determined using Rheometer (Rheometer R/S-CPS, plate&plate, Brookfield engineering laboratories, Middleboro, MA, USA) with P25 DIN plate. Shear rates from 1 to 360 $s^1$ and back from 360 to 1 $s^1$ were used to determine the rheological properties of the gels under shear stress. The temperature was maintained at 25 ± 0.2°C.

2.7. *Adhesive property of the gels*

The adhesive property of the gels was investigated by an *in vitro* adhesive test previously described (*23*) with some modification. Briefly, the exact amount of gels was applied on the smooth surface plate with a width of 20 mm and a length of 100 mm. This surface plate was set next to the angle 30° inclined plate. A 15 mm diameter glass ball was released from the top of the inclined plate with a ball running length of 200 mm and further run on the surface plate until it stopped by gel adhesion. The length of the ball running from the beginning of the surface plate to the stop point was recorded.

2.8. *In vitro drug release*

*In vitro* drug release was studied by using an activated dialysis bags with a molecular weight cut-off at 12,000 daltons (Cellu Sep® T4 regenerated cellulose tubular membrane, Membrane filtration products Inc., Seguin, TX, USA) were used. The 50-mL artificial saliva was used as a release medium. The amount of 1 g of tested gels was filled in the dialysis bag. The bag was sealed and added to the medium with the controlled temperature at 37 ± 1°C and stirring speed of 100 rpm. After 5, 10, 15, 20, 30, 40, 50, and 60 min, the samples were collected and the fresh medium with the same volume was replaced. High-performance liquid chromatography (HPLC) (Hewlett Packard series 1,100, Agilent technologies, Santa Clara, CA, USA) was used for drug determination. Briefly, 1,000 µL of samples was mixed with 1,000 µL of 0.1M TPP and stirred for 2 h and protected from light. A reversed phase column 4.6 × 250 mm Hypersil ODS Agilent technologies with UV detection at 225 nm was used, the injection volume was 10 µL and the flow rate was adjusted to 1.0 mL/min. Determination was carried out at 25 ± 0.2°C with a mobile phase containing acetonitrile-water at a ratio of 50:50. At 6.5 min of running time, the composition of mobile phase was changed to a ratio of 100:0. After that, at 10 min, the composition of mobile phase was changed back to a ratio of 50:50 until the retention time of 25 min was reached. The calibration curve was prepared using solution of CP at 50-200 µg/mL and a linear response were obtained with correlation coefficient ($r^2$ = 0.9997).

2.9. *Ex vivo bleaching efficacy*

2.9.1. *Preparation of the teeth*

This investigation was approved by the Human Experimentation Committee, Faculty of Dentistry, Chiang Mai University (No. 58/2016). Fifty-five human teeth from normal volunteers of Chiang Mai University were collected by dentists. Cleaned teeth without caries or structural enamel defects were selected and kept in saturated 0.1% thymol solution at 4°C until further test.

2.9.2. *Tooth color measurement*

The teeth were randomly allocated into 11 experimental groups according to gel formulations; the treatment groups were JM gels and SH gels with 10%, 20%, and 35% CP. Commercial gels containing 10% CP (10CP-PC), 20% CP (20CP-PC) and 35% CP (35CP-PC) were used as positive controls. Rice gel bases; SH gels and JM gels were used as negative controls. The bleaching

protocol used in the present study was according to the dental bleaching techniques (*12,24*). At-home bleaching technique, 10% CP was applied for a long period (8 h per day) and 20% CP was applied for a medium period (4 h per day). In-office bleaching technique, 35% CP was applied for a short period (1 h per day). Duration of the bleaching was 14 days.

To demonstrate bleaching efficacy, tooth color values were measured using colorimeter (Fru WR10 portable precision colorimeter, Shenzhen wave optoelectronics technology Co.,Ltd, Shenzhen, China). The tooth color values were determined based on the Commission international de l'Eclairage (International commission on illumination: CIE) b* (yellow–blue) scales (*25*). After initial tooth color measurement, each day, the 0.1 mL samples were placed on tooth surface surrounded with 0.05 mL artificial saliva and kept in close container. The relative humidity was controlled at 100% and the temperature was maintained at 25 ± 1°C. After that, the gels were removed by using deionized water and the teeth were measured for color changing and stored in artificial saliva until the next bleaching session. The collected data was calculated to measure the bleaching efficacy.

### 2.10. *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 followed by Duncan's multiple range test ($p < 0.05$) using SPSS statistic a software version 22.

## 3. Results

### 3.1. *Raw rice and modified rice*

Raw JM powder was white powder whereas raw SH powder was yellowish powder. After both raw rice powders were chemically modified by etherification, the obtained modified rice powders from both rice varieties were white. The outer appearances of raw rice powder and modified rice powder were shown in Figure 1.

### 3.2. *Amylose content*

Different amylose levels were found in the rice samples. Amylose content of JM was found to be 15.24 ± 0.07% whereas that of SH was 22.75 ± 0.06%. These values were used to classify the rice based on their amylose content. Generally, amylose content was categorized into five classes: waxy (0-2%), very low amylose (3-9%), low amylose (10-20%), intermediate amylose (20-25%) and high amylose (above 25%) (*26*). JM were classified as low amylose and SH were classified as intermediate amylose.

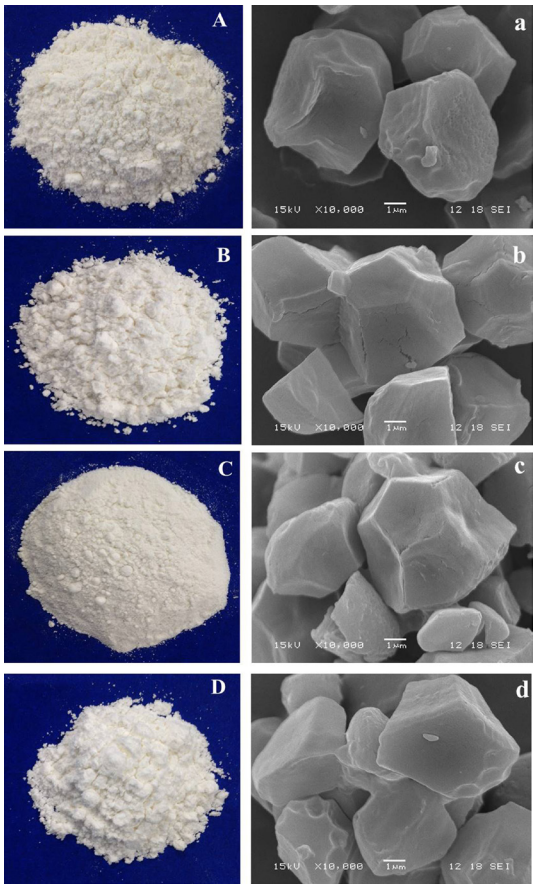


**Figure 1. Outer appearance of raw JM (A), raw SH (B), modified JM (C), and modified SH (D) powders and SEM morphology of raw JM (a), raw SH (b), modified JM (c), and modified SH (d) powders.**

### 3.3. *Morphology of rice particles*

The SEM results demonstrated different morphology between raw and modified rice of each rice varieties as presented in Figure 1. The raw rice showed polygonal shapes with heterogeneous sizes. Size of raw rice particles was approximately 5-7 µm. The modification of rice caused a change to the rice particles. The modified rice particles were swollen and merged together. Some small particles were attached to the surface of the large. Some of surface edges were slightly unsharpened.

### 3.4. *Outer appearance of CP rice gels*

The obtained rice gels and CP rice gels showed transparent semisolids and homogenous textures. After incorporating CP into the rice gel bases, both gels showed good compatibility to the drug. The CP rice gels showed no phase separation and drug precipitation after keeping at room temperature for 5 months.

### 3.5. *Rheological behavior*

The rheological behavior of the rice gel bases, CP rice gels, and CP-PC was shown in Figure 2. The stress-

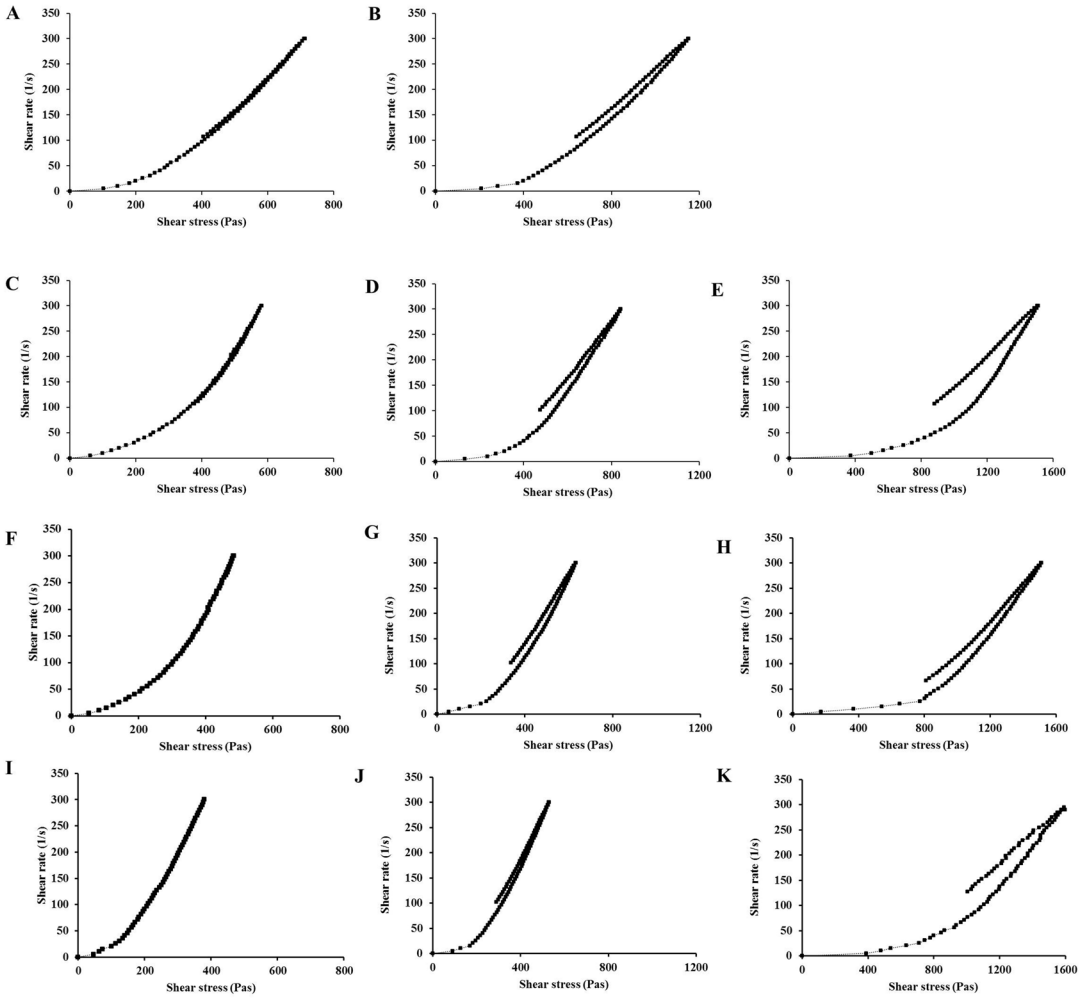


**Figure 2. Rheograms of JM gels (A), SH gels (B), 10CP-JM (C), 10CP-SH (D), 10CP-PC (E), 20CP-JM (F), 20CP-SH (G), 20CP-PC(H), 35CP-JM (I), 35CP-SH (J), and 35CP-PC(K).**

strain relationship was non-linear for all samples. The rheological behavior of the rice gel bases, CP rice gels and CP-PC was non-Newtonian flow. JM gel base and CP-JM gel showed pseudoplastic flow without thixotropy. SH gel and CP-SH gel showed pseudoplastic flow with thixotropy.

The viscosity of the rice gel bases, CP rice gels, and CP-PC was presented in Table 1. CP-PC showed the highest viscosity. It was found that the viscosity of SH gels was higher than that of JM gels. The viscosity of the CP rice gels was slightly lower than their corresponding gel bases.

**Table 1. Viscosity of the gels and the distance of running ball**

| Formulations | Viscosity (Pas) | Distance (cm) |
|---|---|---|
| JM | 4.17 ± 0.27 | 5.40 ± 0.11 |
| SH | 8.98 ± 0.18 | 4.78 ± 0.10 |
| 10CP-JM | 3.52 ± 0.24 | 6.12 ± 0.12 |
| 10CP-SH | 7.75 ± 0.17 | 5.23 ± 0.10 |
| 10CP-PC | 13.20 ± 0.26 | 1.60 ± 0.18 |
| 20CP-JM | 2.16 ± 0.35 | 8.50 ± 0.21 |
| 20CP-SH | 6.84 ± 0.50 | 6.10 ± 0.14 |
| 20CP-PC | 12.50 ± 0.31 | 1.88 ± 0.28 |
| 35CP-JM | 1.75 ± 0.12 | 11.43 ± 0.10 |
| 35CP-SH | 4.88 ± 0.20 | 8.72 ± 0.12 |
| 35CP-PC | 14.01 ± 0.22 | 1.90 ± 0.15 |

### 3.6. *Adhesive property*

The adhesive property of the gels as shown in Table 1 was expressed as the distance of the running ball on the surface covered with the test gels. The shorter the distance indicated the higher the adhesive property of the gel samples. Among CP rice gel formulations, 10CP-SH showed the shortest distance. The results indicated that SH gels possessed higher adhesive property than JM gels.

### 3.7. *In vitro drug release property*

Drug release profile of CP rice gels and CP-PC was presented in Figure 3. The determination of CP was based on an oxidation of TPP into triphenylphosphine oxide (TPPO) (*27*). TPP and TPPO are the agents that can be detected by HPLC. TPP was oxidized by peroxide and formed TPPO. Determination of drug was

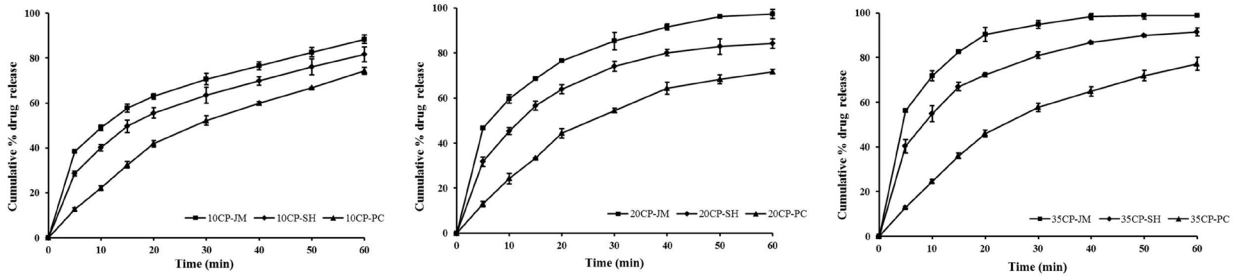


**Figure 3.** ***In vitro*** **cumulative drug release profiles from the gels.**

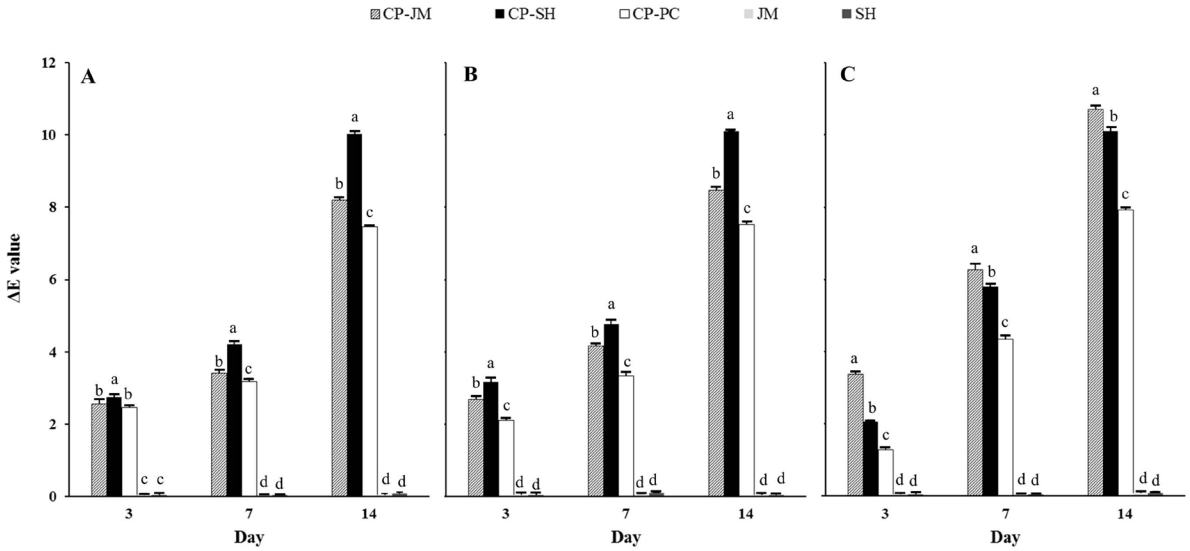


**Figure 4.** ***Ex vivo*** **tooth bleaching efficacy of CP-JM, CP-SH, and CP-PC, at 3 different CP concentrations, 10% CP (A), 20% CP (B), and 35% CP (C).** Data are mean ± SD. Different lowercase letter in same day for each group implied the statistically different ($p < 0.05$).

made by external quantification using TPPO peak area. All CP rice gels demonstrated faster drug release than CP-PC. Among the CP rice gels, 35CP-JM showed the highest amount of drug release.

### 3.8. *Ex vivo bleaching efficacy*

Visual observation revealed that tooth color of CP rice gels groups and positive groups after treatment was highly whiter than the initial day. The values of color changing were collected by colorimeter. The result was expressed by the mean value of measurements of the tridimensional coordinates of the CIELab system. In this system, the color was determined by the relationship between three axes. The L* axis represents the lightness with values ranging from 0 (black) to 100 (white). The a* axis represents the amount of red (positive a* value) or green (negative a* value). The b* axis represents the amount of yellow (positive b* value) or blue (negative b* value). The values of L*a*b* were calculated for color changing (ΔE) by using an equation following (*25*); $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$. After calculations, the results indicated that the groups of CP rice gels and CP-PC were obviously effective. The mean ΔE values of these groups were significantly higher than the negative control group. Moreover, the mean ΔE values of day 14 were the highest when compared to those of day 3 and day 7. The results of 10% CP gel groups were presented in Figure 4A. At day 14, 10CP-SH showed the significant highest mean ΔE value of 10.04 ± 0.07 ($p < 0.05$). Even ΔE value of 10CP-JM was lower than 10CP-JM, but it was significantly higher than 10CP-PC. The mean ΔE values of 10CP-JM and 10CP-PC were 8.20 ± 0.08 and 7.46 ± 0.04, respectively. The results of 20% CP gel groups were presented in Figure 4B. At day 14, it was also found that ΔE value of 20CP-SH was the significantly highest of 10.10 ± 0.03. Similar to the 10% CP gels, the ΔE value of 20CP-JM (8.47 ± 0.09) was significantly higher than that of 20CP-PC. However, the results of 35%CP gel groups were different from 10% and 20% CP gel groups as presented in Figure 4C. At day 14, 35CP-JM showed significant tooth bleaching effective with the highest ΔE value of 10.71 ± 0.11 followed by 35CP-SH and 35CP-PC with mean ΔE values of 10.11 ± 0.12 and 7.92 ± 0.07, respectively. These values indicated that 35CP-SH had significantly higher ΔE values than 35CP-PC ($p < 0.05$).

## 4. Discussion

Chemical modification of rice base on carboxymethylated etherification can improve the properties of rice (*16*). This modification method can enhance water soluble property of the rice starch. The particle morphology of the obtained modified rice is obviously changed from the raw rice. The modified rice of both JM and SH shows good property for forming hydrogels by using simple hydration method. As CP is a water soluble active compound. Therefore, this drug can miscible well with the rice hydrogels after incorporation to the gel bases leading to obtain the good texture CP rice gels.

The suitable topical gel formulations should have suitable rheological behavior. This property can provide the flow behavior information and microstructural environment of the gels which are responsible for drug diffusion and also possible drug compatibility (*28*). The rheological property of the developed JM and SH gels is non-Newtonian behavior. Both gels can immediately flow after stress application, and their viscosity changes during the process of shearing. Rice variety plays important role on rheological behavior of the rice gels. JM gels possess pseudoplastic flow without thixotropy whereas SH gels possess pseudoplastic flow with thixotropy. Thixotropy indicates the reformation of gel structure after shear stress is applied. Higher thixotropy indicates the lower gel structure reformation. It has been reported that a synthetic polymer, carboxypolymethylene, is commonly used as gelling agent in many tooth bleaching commercial gels (*24*) and gives non-Newtonian behavior, pseudoplastic flow with thixotropy (*29*). The obtained CP-SH rice gels developed in our present study possess pseudoplastic flow with thixotropy, the same rheological behavior as the commercial gels used as positive controls.

High adhesive property helps the topical gel formulations to retain at the application area for the desired duration of action whereas the formulations with low adhesive property are easily removed. For tooth bleaching gels, high adhesive property is important for efficacy and safety (*30,31*). Considering rice gel formulations, the viscosity and adhesive property of SH gels are higher than JM gels. Recent research has reported that the amylose content effects to rice swelling after modification and properties of gels (*32*). The results from our present study indicate that JM can be classified as a low amylose content group whereas and SH can be classified as an intermediate amylose content group. The different properties of rice gels are considered to be due to the amylose content in different rice variety. Moreover, CP also affects to the rice gels, increasing CP concentration decreases viscosity and adhesive property of the gels.

The release study demonstrates that 35CP-JM has the highest drug release property. It is considered that the release property of the gel is influenced by the viscosity of the gel base. Drug molecules can diffuse easily through the low viscosity gel and pass the definite MW cut-off dialysis membrane to the medium. High viscosity of gels may prolong releasing of the drug. This effect causes 35CP-JM with low viscosity possesses the fastest drug release. The *ex vivo* study in human teeth was done in order to compare the bleaching efficacy. The treatment sessions of 10%, 20%, and 35% CP used in this study are according to the common bleaching technique. All CP gel formulations possess high effective for tooth bleaching. CP rice gels and CP-PC have the similar tooth bleaching efficacy but in different level, which the developed CP rice gels are higher efficacy than the CP-PC and the negative controls.

Comparing ΔE values of CP rice gels and CP-PC, it is indicated that for at-home bleaching technique, the efficacy of 10CP-SH and 20CP-SH is higher than the others. For in-official bleaching technique, the efficacy of 35CP-JM is the highest which is considered to be due to the fastest drug release of this formulation. Considering other factors that can affect bleaching efficacy, it is noted that not only the release property of the gels but also the adhesive property. SH gels had the suitable adhesive property and good ability of drug release. Therefore, at the medium and long period, 10CP-SH and 20CP-SH showed the highest tooth bleaching efficacy.

In conclusion, the developed rice gels containing tooth bleaching agent, CP-JM and CP-SH are homogeneous texture with tooth bleaching effectiveness. Rice variety plays a role on the characteristics and properties of the derived gels CP rice gels showed higher efficacy than the positive controls. Moreover, CP-SH is the most effective gels for at-home bleaching technique whereas CP-JM is the most effective gels for in-official bleaching technique.

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# The process of surplus medicine accumulation by elderly Japanese patients with chronic disease: A qualitative study

**Natsuki Kimura[1], Akiko Miki[2], Hiroki Satoh[2], Hiroshi Yamazaki[3], Yasufumi Sawada[2,*]**

[1] *Faculty of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan;*
[2] *Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan;*
[3] *School of Health Sciences, Shinshu University, Matsumoto, Nagano, Japan.*

**Summary** **The Japanese government actively urges pharmacists to support efforts to reduce surplus medicines. However, these activities currently serve only to dispose of surplus medicines; no measures are being taken to fundamentally prevent the accumulation of surplus medicines from the outset. A deep understanding of patients' views about storing medicines at home and how they might be accumulating surplus medicines would contribute to the prevention of surplus accumulation. This study aimed to characterize the process by which elderly chronic disease patients in Japan accumulate surplus medicines. Semi-structured interviews were conducted with 18 elderly patients, and the interview data were analyzed using a modified grounded theory approach (M-GTA) to present the process by which surplus medicines were accumulated at patients' homes. The results suggest that elderly patients with chronic diseases often wish to avoid unnecessary medications because of anxiety about medicines, and that these patients seek to maximize medicine suppression. In this context, patients use their own judgment to decide whether to use medicines as needed. Additionally, when patients accumulate surplus medicines, they hesitate to throw them away because they feel that to do so is *mottainai* (wasteful), or because they accumulate surplus medicines as emergency household medicines. These findings reveal when and how surplus medicine accumulation occurs and the points at which pharmacists can easily intervene to promote a close relationship with patients.**

***Keywords:*** Modified grounded theory approach (M-GTA), medicine suppression, as-needed medications, family pharmacist

## 1. Introduction

It is estimated that the overall monetary value of surplus medicines in patients' homes exceeds tens of billions of yen annually (*1-2*). Such accumulation factors into serious problems, including the improper use of this surplus medicines, the burden it places on the medical economy, and associated environmental issues. In particular, cancer recurrence has been confirmed in patients who had taken doxifluridine for approximately one year following rectal cancer surgery and after these patients were switched to treatment using tegafur/gimeracil/oteracil potassium (S-1); a case report indicated that severe side effects arise from improper concomitant use of the pretreatment medicine doxifluridine, a contraindicated medicine that was stored in patients' homes prior to receiving S-1 (*3*). This incident drew attention to the issue of medicine interactions caused by the concomitant use of surplus medicines and newly prescribed medicines. In addition, accumulation of surplus medicines resulting from noncompliance is also at issue. Even when evaluating the outcomes of medicine treatment and evaluating the value of medicines themselves, there is a danger that medicines will be erroneously evaluated because of patients using medicines in ways other than their prescribed uses.

In light of this, the Japanese government actively urges pharmacists to support efforts to reduce surplus medicines. Following the revision of regulations for

**Address correspondence to:*
Dr. Yasufumi Sawada, Laboratory of Drug Lifetime Management, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan.
E-mail: sawada@mol.f.u-tokyo.ac.jp

compensating compounding in 2012, "confirmation of surplus medicines" was specifically designated as an obligation of pharmacists (*4*). As a result of the revisions to dispensing fees made in 2014, pharmacists must "confirm patients' surplus medicines status following receipt of prescriptions before preparing their medicines" (*5*). Accordingly, excess-reduction bags and "brown bag" activities are now being implemented at pharmacies based on patients' declarations (*1*). However, these are merely interventions by pharmacists who are focused on adjusting the amount of medicines dispensed and are not fundamental measures to prevent the accumulation of surplus medicines from the outset. Specifically, for pharmacists to be able to intervene and resolve situations involving surplus medicines it is necessary that they understand the factors causing the accumulation of surplus medicines, such as how patients manage their medication and their reasons for not taking them, and take measures accordingly. Only several reports to date have addressed actual conditions, such as the amount and value of surplus medicines at patients' residences, as well as efforts to reduce surplus medicines and the effects of these efforts on reducing medicine costs (*2*). However, although some efforts have been made to identify the reasons that patients accumulate surplus medicines, there has been no discussion on the various processes and factors that lead to such accumulation.

It is believed that by developing a deep understanding of the kinds of ideas that patients have regarding medicines in their homes – *e.g.* how they are accumulating surplus medicines, how they perceive the situation, and so on – it will be possible to provide guidance on medicine in line with patients' perspectives, leading to improvements in medicine adherence and progress toward preventing the accumulation of surplus medicines.

Accordingly, this study aims to identify the process by which surplus medicines are accumulated in the homes of elderly Japanese chronic disease patients based on qualitative data, such as results of interviews on medication use and surplus medicines that were conducted with patients using a modified grounded theory approach (M-GTA) (*6*).

## 2. Materials and Methods

### 2.1. *Definition of surplus medicines*

Although the definition of "surplus" differs among medical professionals and patients (*2,7,8*), all surplus medicines existing in patients' homes have been considered to be used inappropriately or misused from the viewpoint that there is an inherent danger in surplus medicines. The term "surplus medicines" is uniquely defined in this study as "medicines taken by patients that were prescribed in the past that are not meant to be used at present, but were kept without being discarded." However, cases of patients planning to keep a certain amount of medicines on hand for situations in which seeking medical attention is difficult, because of sudden disaster or unforeseen circumstances, were not considered surplus medicines.

### 2.2. *Setting and participants*

Through several pharmacies that employ cooperating pharmacists, the researchers visited patients' houses and conducted interviews, after acquiring patients' consent. The subjects were elderly patients aged 60 years or older who were receiving ongoing medicine therapy for chronic conditions. The reasons for this selection of participants were as follows: multiple chronic diseases develop as people age; with aging, the number of medicines taken increases, patients' cognitive function declines, and patients leave their homes less frequently; and, in contrast with younger patients, the process by which elderly patients accumulate surplus medicines is believed to have procedural characteristics. This survey was conducted between January 2013 and October 2015.

Interviews were conducted following a semi-structured format. Subjects were asked semi-structured questions such as "How do you feel about acknowledging your illness or symptoms?"; "How do you feel about having leftover prescription medicine (if there is any remaining prescription medicine after use)?"; "How do you feel when you leave leftover prescription medicine? Why do you think you leave extra medicine?"; and "Do you reuse leftover prescription medicine? If so, what kind of medicines do you reuse and in what situations do you do this?". Subjects responded freely to these questions. Subjects' responses were recorded using an IC recorder only when their consent to do so had been obtained. Interview lengths differed for each subject but were generally approximately 1-2 hours long. The data gathered during the interviews were recorded verbatim. A total of 18 subjects were eligible. Table 1 shows characteristics of the participants.

### 2.3. *Data analysis*

A qualitative research method, M-GTA, proposed by Kinoshita (*6*), was used in the present study. M-GTA is the revised version of the grounded theory approach (*9*) that permits the application of the original method to real research data.

The reasons for selecting M-GTA for analysis of the interview data are given below. Kinoshita highlighted each of the following as study types suitable for the M-GTA (*10*): 1) Research related to social interaction, in which people interact with other people. 2) Research concerning human service areas. (Research cycles that return theories accumulated as research results back to practice sites and there enter active application and validation are the most natural.) 3) Research concerning

**Table 1. Characteristics of the participants**

| No. | Gender | Age | Diagnoses | Family situation | Individual(s) responsible for administering medication |
|---|---|---|---|---|---|
| A | M | 60s | asthma, depression | living alone | self, home-visiting nurse |
| B | F | 70s | cardiac disorder (The patient herself is unaware of the specific diagnosis) | living alone | self |
| C | F | 80s | arrhythmia, hypertension, fibromyalgia syndrome | living alone | self |
| D | M | 80s | diabetes mellitus, digestive disorder | living alone | self |
| E | F | 70s | cardiomyopathy, cardiomegaly, atrial fibrillation | living alone | self |
| F | F | 70s | hypertension | living alone | self |
| G | M | 80s | diabetes mellitus, hypertension | living alone | self |
| H | F | 70s | diabetes mellitus, hypertension | living alone | self |
| I | M | 60s | bradycardiac atrial fibrillation, implanted pacemaker, cerebral infarction | living with family | self, spouse |
| J | M | 70s | diabetes mellitus | living alone | self |
| K | M | 80s | implanted pacemaker, hemifacial spasm | living alone | self |
| L | M | 70s | intracerebral bleeding, dementia | living alone | self |
| M | M | 60s | atrial fibrillation, diabetes mellitus | living with family | self, spouse |
| N | M | 70s | cerebral infarction, arrhythmia | living with family | self |
| O | M | 70s | cerebrovascular/vascular stenosis, diabetes mellitus, hypertension | living with family | self |
| P | F | 90s | Implanted pacemaker, diabetes mellitus | living with family | self |
| Q | M | 80s | esophageal cancer, urinary bladder cancer, prostate cancer, esophagostenosis, reflux esophagitis | living with family | self |
| R | F | 60s | colorectal cancer, diabetes mellitus | living with family | self |

Gender – M: male / F: female

phenomena exhibiting procedural characteristics.

The current study meets all these criteria, as shown below. 1) This research clarifies changes in the patient's medication situation and the state of medicine management at their home according to direct and indirect exchanges (interactions) with the patient's pharmacist, physician, and acquaintances. 2) This research examines the human-service components of pharmacists' work by considering patients' prescriptions, medications, and methods of home medicine management leading to proper medicine use. In addition, based on the circumstances in patients' homes, this research can offer recommendations regarding how pharmacist guidance on medication and intervention can stop the accumulation of surplus medicines (and the improper use thereof) at patients' homes and to promote proper medicine use. 3) This research aims to characterize the underlying processes that result in accumulations of surplus medicines at patients' homes through interviews with patients.

2.4. *Method of analysis*

The focus of this analysis was the "process by which elderly chronic disease patients accumulate surplus medicines in their homes." A series of underlying concepts guided this analysis using analysis worksheets (hereinafter "worksheets"). An example of an analysis worksheet is shown in Table 2. Next, the verbatim interview transcripts were read repeatedly, and portions of the data considered to be related to the surplus medicine accumulation process analysis theme were extracted and inserted in the "Variations" row. This portion of the data then helped the researchers to interpret the meaning of the participants' own viewpoints on the process of accumulation of surplus medicines and was provisionally described in the "Definitions" row. Next, a brief way to express the definition was created while paying attention to theoretical applicability and then described as a provisional concept in the "Concept" row. Other similar portions of the data were then searched for; findings were individually added to the sheet and then compared against the provisional definition and the concept name, with corrections and refinements made as necessary. Ideas and doubts during concept creation were described in the "Theoretical notes" row. When concept generation reached a certain point, the researchers began investigating the relationship between the concepts. Directional concepts were

**Table 2. Example analysis worksheet**

| Concept | Medicine, in excess, can also be poison |
|---|---|
| Definition | Medicines are considered to be "unnatural" and "bad for you," and patients' dislike of medicines and anxiety regarding side effects are strengthened. |
| Variations | 1) *I try not to take too much, you know? I would be in trouble if I took the same medicine twice or made some other mistake. I think they are, so I take as little as I can. (Patient E)*<br>2) *This medicine, Adoair, it is a steroid medicine, is it not? I wonder if it has side effects. I have been taking it for a long time. (Patient A)*<br>3) *I take medicines in the morning, noon, and at night. I have been taking this medicine for many years. I have been taking those small ones from before for decades too. I wonder if these medicines have any kind of surplus effects on my body. (Patient J)*<br>4) *Because medicines are scary. I do not know what kinds of effects they might have. (Patient P)* |
| Theoretical notes | • Taking medicine is considered to be treatment for a disease; however, patients have a vague sense of anxiety and dislike of medicines (medicine, in excess, can also be poison). Patients feel ambivalent towards medicines.<br>• Why do you feel anxious?<br>Because you experienced side effects?<br>Because you heard that taking too many medicines was bad? |

illustrated using arrows. Additionally, in the course of studying relationships between concepts, when it was possible to determine that a relationship between multiple concepts could be more briefly expressed using one label, these concepts were aggregated into a category. There were also cases in which a concept with a high abstract explanatory capacity was adopted as a category as well. Lastly, the researchers examined the categories and concepts to create a results diagram that would ultimately explain the process of surplus accumulation and the story line (*11*).

During the analysis, after the first author performed data analysis, the other two researchers checked the content of the analysis worksheets, concepts, results diagram, and the story line. Moreover, conceptions of the analytical focus and targets were shared among the three researchers; data were extracted from the interview recordings; concepts and categories were created; analytical results were prepared; and validity was maintained as far as possible through repeated discussion and analytical operations.

### 2.5. *Ethical concerns*

This study was conducted with the approval of the "Research Ethics Committee for Human Studies" of the Faculty of Pharmaceutical Sciences, Graduate School of Pharmaceutical Sciences at The University of Tokyo. Written informed consent was obtained prior to conducting interviews. The survey subjects were briefed regarding the purpose of this study and they agreed to its purpose and methodology. In addition, the researchers explained to the subjects that their personal information would be protected, and that any information that could be used to identify individuals would not be made public when the research results were published.

## 3. Results

In the following section, the process by which surplus medicines were accumulated at patients' homes is described using the central categories, concepts, and narratives obtained during interviews (indicated by lenticular brackets [], angle brackets <>, and *italics* respectively). The central categories and concepts are given in Table 3. Supplementary explanations are provided in parentheses (), when the subject's narrative is unclear from a direct quotation alone. Finally, results in the story line and diagram were proposed using four categories as stated below.

**Table 3. Central categories and concepts**

| Central categories | Concepts |
|---|---|
| Maximal medicine suppression | underestimate their conditions<br>in excess, can also be poison<br>doubts about prescriptions |
| As-needed medications | using body sensory as the base<br>alternative practice<br>grading medicine efficacy by pill count<br>decision-making based on accessible information |
| Loss of yakushiki | stay away from unreadable information<br>judging by medicine look<br>just have to take the medicines |
| Emergency household medicines | in case of an emergency<br>mottainai (wasteful) |

### 3.1. *Central categories*

#### 3.1.1. *Maximal medicine suppression*

The elderly patients all adhere to the socially accepted notion that medicine <in excess, can also be poison>. These patients believe that medicines are inherently "unnatural" or "bad for you" and feel anxious about medications and their potential side effects. These feelings become stronger as the number of medications they take increases, or if their doctor keeps them on a medication for a long time, and eventually they begin to want to minimize medicine use as much as possible.

*Patient E: I try not to take too much, you know? I*

*would be in trouble if I took the same medicine twice or made some other mistake. Aren't medicines scary? I think they are, so I take as little as I can.*

In addition, because many chronic diseases progress slowly and subjective symptoms are minimal, patients are less likely to realize the importance of taking their medications and tend to <underestimate their conditions>.

*Patient O: Sometimes, about once per week, I will go golfing. I go play without taking my medicine. I know that I did not take it. I will be fine even if I do not take it; I am confident that nothing will happen.*

Elderly patients with chronic diseases justify their accumulation of surplus medicines using [maximal medicine suppression] as a behavioral standard, alongside their socially accepted notion that medicine <in excess, can also be poison> and a trend to <underestimate their conditions>. Patients have <doubts about prescriptions> and their preference for [maximal medicine suppression] is reinforced, particularly when they do not fully understand the intent of their physician's prescription and begin to believe that their medications are unnecessary.

*Patient Q: I do not understand it. Diuretics are a good example – I do not have any swelling or pain, no fever (but I received medicine from my doctor)…why do I have to take it?*

3.1.2. *As-needed medications*

Elderly patients with chronic diseases, although given prescriptions that must be taken on a specific schedule for pharmacological reasons, will consider these medicines as [as-needed medications] based on their preference for [maximal medicine suppression]. These patients place great trust in their own judgment. They often decide that medicines have no effect and are unnecessary if they do not feel that their symptoms are being relieved or if they feel that they are healthy without the medicines.

*Patient O: I will not use my psoriasis medication if my skin condition improves. I use it when it gets really bad, but when I think I feel better…I come off.*

In this way, patient self-adjustment or interruption of a course of medication is done without consulting a physician or a pharmacist, based on how patients feel about their physical conditions. Such ideas of <decision-making based on accessible information> are reinforced by mass media and friends.

*Patient J: The plaster, something in it makes me cough, so I stopped using it. I am allergic to it. Aspirin medicines. It is happening even now. So, I stopped using that medicine because I heard that you could put a medicine in it that will not make me cough. I tried looking it up on the Internet. It might start again.*

These patients consider the instructions of a physician or pharmacist to be only one of various sources of medicine information. Patients who feel repulsed by or anxious about their medicines may seek out <alternative practices> as much as possible, such as diet restriction, exercise, and massage rather than medication.

*Patient O: Just now I'm dieting. Hmm, (the size of the meal) have portion control. I only take this medicine in the morning. So, I just changed how I eat. Doing this was great.*

In this way, when patients' physical conditions improve, and especially if the improvement is seen as a result of <alternative practices>, patients begin to believe that they can improve their condition without using medicine. Thus, they interrupt their medication regimen using their own judgment, leading to accumulation of the remaining medicine.

Furthermore, because many patients take many medications in the morning, it is believed that morning periods are the most important. The result is that patients sometimes neglect their medication schedules for the remainder of the day or for other medications.

*Patient P: In the morning, I take a lot of pills, so I think that my (most important) medicines must be taken then.*

Patients' whose own <grading medicine efficacy by pill count> results in medicines being considered low priority are considered to be in non-compliance. Medicines that seem to patients to be important are taken regularly, and the rest are taken only when the patient believes it to be necessary, further establishing their idea of [as-needed medications].

3.1.3. *Loss of yakushiki (medicational self-understanding)*

*Yakushiki* is defined as patients having not only medical knowledge regarding their prescriptions but also an understanding and acceptance of the importance of taking prescribed medications (*12*). Patients often do not know the exact names of their medications or what kind of effects they will experience when taking medicines, and they may experience [loss of *yakushiki*]. These patients leave or discard medicine information sheets, often without reading them. For elderly people with chronic diseases, this information can be quite a challenge to understand as the letters are small, it uses complicated medical terms, and the names of medicines that are foreign words are written in katakana, and they

may <stay away from unreadable information>.

*Patient J: I cannot read a thing on those (medicine bags or medicine labels) without my glasses. I did not really look at it. It is just too much of a pain to read that tiny text.*

Patients thus distinguish different types of medicines and how to take them on the basis of their colors or the appearance of their containers.

*Patient D: Usually, I…uh, the colored one…the one that was light pink…it was multicolored, right? I wonder what kind of medicine that was.*

As these patients cannot easily access knowledge about the action and efficacy of different medicines, when they consider different medicines using their own judgment they tend to believe that they <just have to take the medicines> prescribed by their physician.

*Patient E: I have not really thought too seriously about my medicine until now. I just felt like I was given them, so I have to take them. I know that these medicines have various side effects, but I have not really thought much about what each of them are.*

Patients <stay away from unreadable information> and identify which medicines to take <judging by medicine look>. Also, they come to think that they <just have to take the medicines> at some point but not on the prescribed schedule. This mindset causes patients to experience an increasing sense of [loss of *yakushiki*], reinforces reliance on [as-needed medications], and leads to the accumulation of surplus medication.

3.1.4. *Emergency household medicines*

Elderly patients with chronic diseases identify surplus medicines not only as if they are [as-needed medications] but also as if they may be [emergency household medicines] that can be stocked. While possibly a product of their upbringing, some of these patients dislike throwing away medicines because they find it <*mottainai* (wasteful)>.

*Patient J: I basically never throw away medicine. It is a psychological thing. I almost never throw them away.*

These patients also believe that surplus medicines should be stored away <in case of an emergency>.

*Patient H: I keep any leftovers over there. I think I might need them again someday.*

The patient quoted above believed that it is <*mottainai* (wasteful)> to throw away surplus medicines, believing that they may be useful <in case of an emergency>. Such surpluses are often viewed as [emergency household medicines]; patients do not have awareness of them being "surpluses" and do not consider them as such.

3.2. *Summary of the process by which elderly chronic disease patients accumulate surplus medicines in their homes*

Elderly Japanese with chronic diseases routinely seek [maximal medicine suppression]. This is caused by their belief that medicine, <in excess, can also be poison>, while their tendency to <underestimate their conditions> is due to a lack of subjective symptoms. [Maximal medicine suppression] is especially reinforced when patients have <doubts about prescriptions>: that is, they suspect that their doctors are overprescribing.

Because of these beliefs, tendencies, and doubts, patients begin to perceive prescribed medicines they need to take regularly as [as-needed medications]. Patients' regard for how they feel about their physical conditions serves as a reliable criterion in deciding whether or not to take medicines. This belief is supported by their lay knowledge of medication, which is based on information from mass media and friends. When patients feel well and do not think taking medicines is necessary, they resort to <alternative practices> such as exercise.

Patients' perceptions and practices about [as-needed medications] are reinforced when [loss of *yakushiki*] occurs. Medicine information sheets often contain too much and too technical information written in small letters, discouraging elderly patients from reading them. Patients <stay away from unreadable information>, choosing to identify which medicines to take <judging by medicine look>. Also, they come to think that they <just have to take the medicines>. Finally, they come to believe that the self-reduced dosage is good enough so long as they are not completely off medications.

[Maximal medicine suppression], [as-needed medications], and [loss of *yakushiki*] cause medicine surplus. Elderly Japanese with chronic diseases do not regard keeping surplus medicines as problematic. They feel that discarding surplus medicines is <*mottainai* (wasteful)> and that these medicines will be useful on future occasions. Ultimately, patients positively identify surplus medicines as [emergency household medicines] to be stocked.

Figure 1 summarizes the result diagram identified above.

## 4. Discussion

The process revealed by the results of this study, as identified above, makes it clear that it is difficult

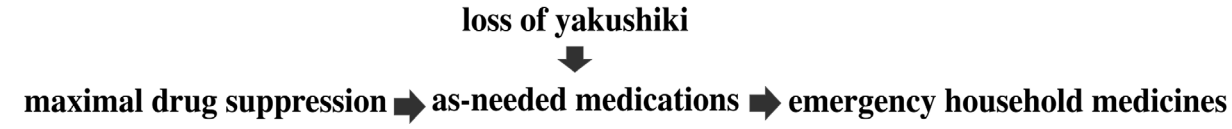


**Figure 1. Process by which elderly chronic disease patients accumulate surplus medicines in their homes.**

to identify the factors leading to surplus medicine accumulation while relying solely on the information reported at pharmacies by patients themselves. The concepts and categories offered by this study reveal why and when surplus medicine accumulation occurs as well as the points at which pharmacists can easily intervene. In addition, regarding patients' avoidance of declaring or consulting on surpluses with healthcare professionals, the interview data obtained suggests that patients believe that it is impossible to talk with physicians and pharmacists on an equal footing because they lack medical knowledge, and therefore patients tend to simply defer to the professional.

It is clear that communication between pharmacists and patients is important in solving these problems. There have also been reports that patients who were given brief explanations about their medications had lower levels of satisfaction (*13*), even when there was no voluntary consultation from the patient who had no changes in prescriptions. Pharmacists should assume that their customers' situations are changing and make efforts to determine whether a patient is wondering about taking medicines and whether they have any concerns.

As shown by the anxieties reported by participants during this study, they believe that medicine, <in excess, can also be poison>. Elderly patients with chronic diseases also have feelings of concern, fear, and disgust toward medicines. In the past, most patients have stated that they want information regarding possible side effects when prescribed a new medication; 60 percent want their doctors to explain these effects (*14,15*). Pharmacists must advise patients taking medicine while recognizing that patients' resistance to their medication may be stronger than any professional's direction to "take medicine." However, there have been reports that pharmacists tend to overestimate patients' cognitive abilities more than physicians in terms of a patient's understanding of medical terminology (*16*); pharmacists must accurately convey medicine information to patients, but if they cannot accurately assess patients' level of literacy they may be unable to provide sufficient information.

The results of this study also revealed that many of the patients interviewed had not taken advantage of offers for medicine information sheets, medicine bags, medicine diaries, or similar explanations and tools. Pharmacists often provide a one-sided explanation, promoting patients' <*loss of yakushiki*>. In elderly patients, visual acuity, hearing ability, and cognitive function are often declining, suggesting that more innovation is needed in provision of information. In the interviews conducted during this study, many patients stated that they could not read the fine print on medicine information sheets or could not remember details when they were provided a large amount of information. For such patients, when receiving directions regarding medications, for example, it is believed that by providing information that incorporates photographs, illustrations, and large print, and confirming what information the patient wishes to know in advance, patients are provided with easy-to-use guides on how to take their medications. It is also important to establish systems (*e.g.* 24-hour correspondence, home visits, making regular phone calls, *etc.*) that allow patients to talk to a pharmacist even between consultations.

This study has some limitations. The data necessary for theoretical sampling were insufficient, and in one case an idea that was considered important was ultimately deleted from the results diagram because of lack of variation. For example, in the research process, it was believed that the presence or absence of the involvement of patients' families, such as spouses in medicine management, was primarily related to the process of surplus accumulation, but only three of the 18 subjects had spouses involved in medicine management; therefore, variations were insufficient in number to establish this as a concept. However, one prior report has suggested that family member cooperation can affect medicine adherence (*17*).

Also, there were several cases cited in this study in which patients' wives had to consult their husband's PTP sheets on a daily basis to prevent them forgetting to take medications, and in other cases spouses reminded patients when they found that they had forgotten to take their medications; family cooperation and engagement are considered important. In another instance, it was believed that "forgetting to take medicines" is an important factor in surplus medicine accumulation, but sufficient variations were not established. This factor requires further research.

Differences in diseases also greatly affected the way patients understood their conditions. Two of the 18 patients interviewed were cancer patients, and there was a strong tendency for these two patients to have a more substantial understanding of their conditions, but this number of samples is insufficient. In order to obtain findings that enable deeper insight, it is necessary to conduct a theoretical sampling on a specific population, such as patients with a specific disease, patients who

rely on spousal support, solitary patients, and so on.

Finally, this study analyzed elderly patients with chronic diseases, and these findings cannot be generalized to all patients with conditions such as acute diseases or to younger patients.

Previously, a qualitative interview study with five patients analyzed various factors in the prevalence of surplus medicines among senior patients in Japan (*18*). However, this study was the first attempt to characterize the process by which surplus medicines accumulate in the homes of elderly chronic disease patients using M-GTA for analysis of interview data.

Accurately grasping patients' medication situations, assessing their actual condition and therapeutic effects pharmacologically, and proposing optimal treatment for each patient are duties of pharmacists. It is necessary for pharmacists to build trust between patients and their physicians by fulfilling the role of "family pharmacist," who connects patients with physicians through actions such as listening to patients' dissatisfaction with their conditions and prescription medicines, alleviating unnecessary anxiety, suggesting lifestyle changes, and describing medication rules for individual patients and proposing prescription plans to physicians.

Some of the concepts that emerged from analysis of interview data may include reasons for the accumulation of surplus medicines that had previously been regarded as tacit knowledge. However, the results of this study made it possible to provide new perspectives on methods to check surplus medicines that had been previously left to be addressed by pharmacists' own experiences and skills.

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Brief Report

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# Establishment of a gnotobiotic silkworm model

**Hiroto Nakajima[1], Yasuhiko Matsumoto[2], Kazuhisa Sekimizu[1,2,*]**

[1] *Genome Pharmaceuticals Institute Co., Ltd., Tokyo, Japan;*
[2] *Teikyo University Institute of Medical Mycology, Tokyo, Japan.*

**Summary** **Gnotobiotic animals are useful for investigation of the effects of specific lactic acid bacteria on individual animals. Here we report that lactic acid bacteria colonize and proliferate in the intestinal tract of germ-free silkworms. When silkworms hatching from formalin-treated eggs were reared to fifth-instar larvae with an artificial diet containing antibiotics, bacteria and fungi were not observed in their intestines. An antibiotic-free diet supplemented with viable lactic acid bacteria, such as *Enterococcus faecalis* 0831-07, *Lactococcus lactis* 11/19-B1, or *Leuconostoc carnosum* #7-2, was fed to the germ-free silkworms for 1 day. After feeding the larvae on a diet without lactic acid bacteria for 5 days, each type of lactic acid bacterium was found in the intestine. Moreover, an increase in the number of *Enterococcus faecalis* 0831-07 was observed in the intestine 2-5 days after ingestion. These findings suggest that we successfully established a method to construct a gnotobiotic silkworm model.**

***Keywords:*** Gnotobiotic animal, lactic acid bacteria, silkworm

## 1. Introduction

Lactic acid bacteria used for various fermented foods, such as yogurt and pickles, are considered to contribute to human health (*1-3*). Some lactic acid bacteria are reported to colonize in the mammalian intestinal tract to form intestinal microbiota (*4,5*).

In general, mammals are used to evaluate the effects of lactic acid bacteria for promoting health and preventing disease (*4,5*). The influence of lactic acid bacteria and intestinal bacteria on the host is often studied using gnotobiotic animals in which specific bacterial species are maintained as viable bacteria in the intestinal tract (*6-11*). The use of gnotobiotic animals allows for investigation of the effects of specific bifidobacterium and lactic acid bacteria on individual animals without confounding by other intestinal microbiota (*12*). Gnotobiotic mammalian animals are expensive, however, and special equipment and facilities are required to rear them. Furthermore, utilizing a large number of mammals raises ethical problems from the viewpoint of animal welfare. To overcome the problems associated with the use of mammals, insects such as fruit flies, honey bees, and cockroaches have been proposed as gnotobiotic animals (*8,10,11*). These animals, however, are too small for injecting sample solutions with syringes and collecting their blood for biochemical analysis. We established silkworm infection models and diabetes models for exploring candidate functional foods and pharmaceuticals (*13-17*). Silkworms are associated with lower costs and fewer ethical problems compared with mice, yet their size is sufficiently large for injection experiments (*13,14,16-21*). We successfully obtained useful lactic acid bacteria using the silkworm infection and diabetes models (*22-25*), although a method for constructing gnotobiotic silkworms has not been established.

In this study, we report that lactic acid bacteria colonize and proliferate in the silkworm intestinal tract. The gnotobiotic silkworms we developed are expected to be useful for evaluating the effects of lactic acid bacteria.

## 2. Materials and Methods

### 2.1. *Silkworm rearing conditions*

Silkworms were reared according to the previously reported method (*24*). Fertilized silkworm eggs (*Bombyx mori*, Hu · Yo x Tukuba · Ne) were purchased from

**Address correspondence to:*
Dr. Kazuhisa Sekimizu, Teikyo University Institute of Medical Mycology, 359 Otsuka, Hachioji, Tokyo, 192-0395, Japan.
E-mail: sekimizu@main.teikyo-u.ac.jp

**Table 1. Isolation of lactic acid bacteria from silkworm intestine**

| Ingested bacteria | Recovered bacteria from silkworm intestine | |
|---|---|---|
| Bacteria species | Viable cell number (CFU / intestine of silkworm) | Bacterial species |
| *Enterococcus faecalis* 0831-07 | 1.4-3.8 × $10^8$ | *Enterococcus faecalis* |
| *Lactococcus lactis* 11/19-B1 | 0.9-5.0 × $10^6$ | *Lactococcus lactis* |
| *Leuconostoc carnosum* #7-2 | 0.5-1.5 × $10^5$ | *Leuconostoc carnosum* |
| Saline (control) | < 100 | None |

Silkworm larvae on the first day of the fifth-instar stage were fed for 1 day with a diet with saline or one of the following lactic acid bacteria: *Enterococcus faecalis* 0831-07 (2.3 × $10^7$ cfu/larva), *Lactococcus lactis* 11/19-B1 (2.6 × $10^7$ cfu/larva), or *Leuconostoc carnosum* #7-2 (7.3 × $10^6$ cfu/larva). The silkworms were then reared on a diet without lactic acid bacteria for 5 days, and extracts of the intestinal tracts were prepared to measure the number of viable cells ($n$ = 2 /group). Bacterial species of two independent colonies on each agar plate were determined by sequence analysis of the amplified DNA encoding 16S rRNA.

Ehime sericulture incorporated company (Ehime, Japan). The eggs were treated with 4% formalin. Larvae hatched from the eggs were fed an artificial diet containing antibiotics (Silkmate 2S, Nihon Nosan Corporation, Tokyo, Japan) up to the fifth-instar stage at 25°C. Square dishes (type 2, Eiken Instruments) were used as rearing cages for the first and second-instar larvae, and plastic food packs (192 × 120 × 46 mm, Chuo Kagaku, Saitama) were used for rearing the larvae in the later stages. Larvae on the first day of the fifth-instar stage that were fasted since the fourth molt were used for the experiments. A diet containing lactic acid bacteria was prepared by mixing 15 μL of lactic acid bacteria culture and 1 g of antibiotic-free artificial food, Silkmate 2S (Katakura Industries Co., Ltd., Tokyo).

### 2.2. *Culture of lactic acid bacteria*

*Enterococcus faecalis* 0831-07 (*26*), *Lactococcus lactis* 11/19-B1 (*22*), and *Leuconostoc carnosum* #7-2 (*25*) were streaked on deMan, Rogosa, and Sharpe (MRS) agar (Becton, Dickinson and Company, MD, USA) plates, and incubated at 30°C under anaerobic conditions. Bacterial colonies were isolated and further cultured to full growth in MRS broth (Becton, Dickinson and Company) at 30°C for 2-3 days under anaerobic conditions.

### 2.3. *Measurement of viable cell number in the silkworm intestinal tract*

The entire intestinal tract was aseptically isolated, chopped with scissors in 10 mL saline, homogenized, and the supernatant obtained. The supernatant was diluted in saline, and a 100-μL aliquot was spread on Brain Heart Infusion (BHI; Becton, Dickinson and Company, MD, USA) agar plates. After incubation at 30°C for 2 days, the number of colonies was counted and the number of viable cells in the sample was calculated.

### 2.4. *Identification of bacteria*

Bacterial species were identified by sequencing genes encoding 16S rRNA according to a previously reported method (*24*). Colonies that formed on BHI agar plates were picked up, and the DNA sequence encoding bacterial 16S rRNA was amplified by colony polymerase chain reaction using universal primers. Species of the bacteria were determined by sequencing analysis of the amplified DNA.

## 3. Results and Discussion

### 3.1. *Viability of orally administered lactic acid bacteria in the silkworm intestinal tract*

In our laboratory, silkworm eggs are treated with formalin and the larvae are fed an artificial diet containing antibiotics. Under such rearing conditions, viable bacteria and fungi are not detected on agar plates when the materials in the silkworm intestinal tract are spread and incubated (*27*). In the present study, we examined whether lactic acid bacteria can be recovered from the intestinal tract when silkworms are fed a diet supplemented with lactic acid bacteria (Table 1). Fifth-instar silkworms were fed with *Enterococcus faecalis* 0831-07, *Lactococcus lactis* 11/19-B1, or *Leuconostoc carnosum* #7-2 for 1 day. The silkworms were then fed a diet without antibiotics for 5 days, and the materials in the intestinal tracts were spread on BHI agar plates and incubated. A number of colonies formed on the agar plate. In the control experiments, silkworms were fed a diet supplemented with physiologic saline, and no colonies formed. Two colonies that formed on the plates were picked up, and the DNA region encoding 16S ribosomal RNA was sequenced. The results demonstrated that the two independent colonies were the lactic acid bacterial species added to the diet. The result suggests that lactic acid bacteria supplemented in the diet can survive in the silkworm intestinal tract for at least 5 days.

### 3.2. *Growth of lactic acid bacteria in the silkworm intestinal tract*

We then examined whether orally administered lactic

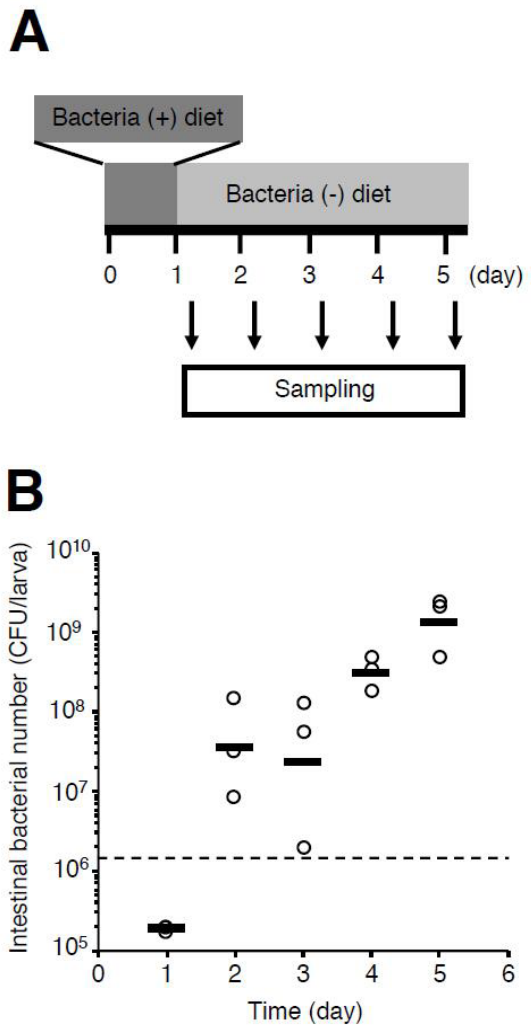


**Figure 1. Growth of *Enterococcus faecalis* 0831-07 in intestinal tracts of silkworms. (A)** Schematic of the experiment schedule. **(B)** Silkworm larvae on the first day of the fifth-instar stage were fed a diet with saline or *Enterococcus faecalis* 0831-07 ($1.3 \times 10^6$ cfu/larva, shown by dashed line in the figure) for 1 day. Then, the silkworms were reared on a diet without lactic acid bacteria or antibiotics for 5 days, and the viable cell numbers in the intestinal tract were measured over time. Bars in the figure show the mean ($n$ = 3/group).

acid bacteria grow in the silkworm intestinal tract. Silkworms were fed a diet with *Enterococcus faecalis* 0831-07 ($1.3 \times 10^6$ cfu/larva) for 1 day, then further fed a diet without lactic acid bacteria, and the numbers of viable bacteria in the intestinal tract were monitored over 5 days (Figure 1). The bacterial number decreased from $1.3 \times 10^6$ cfu/larva on the day of ingestion to 1.7-$2.0 \times 10^5$ cfu/larva on the subsequent day. Thereafter, the number of bacteria increased and reached 0.5-2.4 $\times 10^9$ cfu/intestine on day 5. This value was 100 times higher than that of the bacteria fed to the silkworms, indicating that *Enterococcus faecalis* 0831-07 proliferated in the silkworm intestinal tract. This finding confirms that our experimental system to establish a single bacterial species in the silkworm intestinal tract was successful, thereby providing a gnotobiotic silkworm model.

## 4. Conclusion

In this study, we produced a gnotobiotic animal using silkworms. Silkworms have several advantages as animal models, such as lower costs and fewer ethical problems as experimental animals. Silkworm infection and diabetes models are established, and the gnotobiotic silkworm model might be useful for screening for lactic acid bacteria with the potential to prevent or cure infectious diseases and diabetes.

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Brief Report

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# Is there a need for shifting patients on long term nevirapine based regimens to efavirenz based regimens: a cross-sectional study?

**Nitin Gupta, Ankit Mittal, Kutty Sharada Vinod, Farhan Fazal, Wasim Khot, Sanjay Ranjan, Neeraj Nischal*, Manish Soneja, Ashutosh Biswas, Naveet Wig, Rita Sood**

*Department of Medicine, All India Institute of Medical Sciences, New Delhi, India.*

**Summary** **Non-nucleoside reverse transcriptase inhibitors (NNRTIs) are the backbone of effective anti-retroviral therapy in the developing world. Efavirenz is the current NNRTI of choice due to reports of higher incidence of serious adverse events with nevirapine. Majority of patients with Human immunodeficiency virus (HIV) infection in India are still on nevirapine based therapy. The aim of the study was to evaluate the need of shifting these patients to efavirenz based therapy. A cross-sectional study was conducted on adult patients, who were on NNRTI based regimen for more than one year with good adherence. The patients were divided into efavirenz or nevirapine groups based on the treatments they were receiving at the time of study. The different arms were compared based on their clinical and laboratory profile, adverse events and immunological response. A total of 244 patients were recruited. A total of 125 patients were receiving nevirapine based regimen while 119 patients were receiving efavirenz based regimen. There was no significant difference in the frequency of hematological and biochemical derangements between the two groups. There was no difference in the median highest CD4 count achieved during therapy between the two groups. Clinically observed side effects were more common in the efavirenz group. These results suggest that there isn't enough evidence to shift patients tolerating long term nevirapine based therapy to efavirenz based therapy.**

***Keywords:*** Adverse events, efficacy, non-nucleoside reverse transcriptase inhibitor

## 1. Introduction

Non-nucleoside reverse transcriptase inhibitors (NNRTI) form the backbone of the three-drug first line regimen against human immunodeficiency virus (HIV) infection in most parts of the world (*1*). It has been effectively used in the developing world for a long time owing to their low cost and high potency. In the World Health Organization (WHO) guidelines (antiretroviral therapy for HIV infection in adults and adolescents) published in 2010, both nevirapine and efavirenz were mentioned as preferred NNRTIs for the first line therapy (*2*). However, nevirapine slowly fell out of favor because of higher incidence of serious adverse events in early part of therapy, especially in individuals with higher CD4 counts (*3*). Therefore, in the WHO consolidated guidelines (Use of antiretroviral drugs for treating and preventing HIV infections) of 2013, efavirenz containing regimens were preferred as the first line therapy while nevirapine based regimens were moved to the alternative first line list (*4*). However, the question of shifting those patients, who were already on long term nevirapine based therapy to efavirenz containing regimens was still unanswered. The primary objective of this study was to therefore, compare the long-term tolerability of patients receiving nevirapine based regimen vs those receiving efavirenz based regimens.

## 2. Methods

A cross-sectional study was conducted after taking approval from the Institute's Ethics committee. Adult patients (> 18 years) on NNRTI based regimens for

**Address correspondence to:*
Dr. Neeraj Nischal, Department of Medicine, Teaching block, 3rd floor, All India Institute of Medical Sciences, New Delhi-110029, India.
E-mail: neerajnischal@gmail.com

more than one year with good adherence (> 95% adherence over last one year by pill count method) attending the anti-retroviral therapy (ART) center were recruited after taking informed consent.

A one-time interview was done based on a structured questionnaire. The patients were divided into efavirenz or nevirapine groups based on the NNRTI they were receiving. Patients were interviewed and examined for any adverse effects (clinician and patient reported) to ART. The following laboratory parameters were collected: complete blood count, liver/kidney function tests, and electrolyte levels. Their records were reviewed for treatment details and immunological parameters. CD4 count at baseline, highest CD4 count achieved during treatment and the most recent CD4 count were recorded. The highest change from the baseline was calculated by subtracting baseline CD4 count from the highest CD4 count. Immunological failure was defined as persistently low CD4 count (< 100/μL)/decrease in CD4 count below the base line or half of the highest CD4 attained during the treatment (4). The two arms were compared based on their clinical and laboratory profile, clinical adverse events and immunological response.

*Statistical analysis* Data was collected on a pre-designed pro forma. All data was presented as mean ± SD or median and interquartile range. Frequency of each of the outcomes were expressed in percentage with 95% confidence interval (CI) determined for each of the percentage. Appropriate parametric/non-parametric tests were used based on the type of variables and their distribution.

## 3. Results and Discussion

A total of 244 patients were recruited in the study, 68% (165) of whom were male. A total of 125 (51%) patients were receiving nevirapine based regimen while 119 (49%) were receiving efavirenz based regimen. Out of the 125 patients receiving nevirapine, zidovudine and lamivudine formed the rest of the regimen in 117 (94%) patients. Out of the 119 patients receiving efavirenz, 108 (91%) patients were also receiving tenofovir plus lamivudine. The patients were also classified into the following groups based on the year of initiation (2005-2008-40, 2009-2012-63, 2013-2017-141). Median baseline CD4 count at the time of initiation of treatment was 214.5/μL (121-320.5). Median highest CD4 count achieved during treatment was 591.5/μL (413.5-789.5). Median highest increase in CD4 count from the baseline was 362.5/μL (207.25-546.75). Immunological failure was noted in 12 patients. A total of 19 patients had a history of concurrent tuberculosis with HIV. A total of 33 (13.5%) patients reported some clinical side effect during the interview. None of them were severe enough to require discontinuation. The following clinical side effects were reported by the patients: neuropsychiatric symptoms ($n$ = 13), gastro-intestinal symptoms ($n$ = 8), lipodystrophy ($n$ = 5), peripheral neuropathy ($n$ = 3), rash ($n$ = 2) and gynecomastia ($n$ = 2).

The following hematological abnormalities were noted: anemia (< 11g/dL) -41, leucopenia (< 4,000/mm3)-13, leukocytosis (> 11,000/mm3)-7, thrombocytopenia (< 1,000,000/mm3)-34, and macrocytosis (> 100fl)-132. The following biochemical abnormalities were noted: transaminitis-69, deranged kidney function-4, hypophosphatemia (< 2.5mg/dL)-53 and hypocalcaemia (< 8.8mg/dL)-74.

There was no significant difference between the nevirapine and efavirenz group in the following demographic features: locality, distance of home from the ART center, literacy, employment status, addictions and baseline CD4 count (Table 1). However, there was significant difference between the two groups in terms of mean age at the time of analysis and year of initiation. There was no significant difference in the frequency of hematological and biochemical derangements except for that of macrocytosis which was more common in the nevirapine group. There was no difference in the median highest CD4 count achieved during therapy but the median of highest change in CD4 count was significantly higher in the nevirapine group. Clinically observed side effects were more common with efavirenz (Table 1).

The treatment options for AIDS has drastically changed since 1987, when the first drug, zidovudine was approved (*5*). Although zidovudine was efficacious, it had serious side effects like bone marrow suppression. Also, monotherapy was eventually leading to resistance and failure. Since then, the world has moved on to effective combination therapy, commonly consisting of three drugs (*6*). Two of these drugs are Nucleoside Reverse Transcriptase Inhibitor (NRTI), while the third drug can be either an Integrase inhibitor (INSTI) or Protease inhibitor (PI) or Non-nucleoside reverse transcriptase inhibitor (*6*). The Department of Human and Health Services (DHHS) favors an integrase inhibitor based regimen because of its high barrier to resistance, high potency and less side effects (*7*). However, WHO and national guidelines in India still recommend a NNRTI based regimen owing to their lower cost. Although, NNRTIs have been associated with some side effects (Nevirapine- severe hypersensitivity, Efavirenz- central nervous system (CNS) side effects), most of them are observed in the initial few weeks to months after the initiation of treatment (*8*). Both the drugs have shown to have lower frequency of side effects in patients who have been on these drugs for long durations. In our study, a total of only 13.5% patients had clinically observed side effects at the time of interview. This included side effects related to both NRTIs and NNRTIs. None of the side effects were severe enough to require discontinuation. However, clinical observed side effects, predominantly persistent neuropsychiatric symptoms were observed

**Table 1. Comparison of demographic characteristics, clinical and laboratory parameters between patients on nevirapine vs efavirenz based regimens**

| Characteristics | Nevirapine ($n$ = 125) | Efavirenz ($n$ = 119) | $p$ value |
|---|---|---|---|
| Age | 37.9 ± 10 | 35.3 ± 9.1 | 0.03 |
| Year of initiation | | | < 0.01 |
| 2005-08 | 32 | 8 | |
| 2009-12 | 48 | 15 | |
| 2013-16 | 45 | 96 | < 0.01 |
| Mean duration of therapy in years | 6.2 | 3.1 | 0.6 |
| Locality | | | |
| Urban | 110 | 107 | 0.8 |
| Rural | 15 | 112 | |
| Distance | | | 0.1 |
| < 100 km | 97 | 94 | |
| > 100 km | 28 | 25 | |
| Literacy | | | |
| Primary | 43 | 38 | |
| Secondary | 49 | 36 | |
| College | 17 | 16 | |
| Illiterate | 16 | 29 | |
| Employed | 83 | 75 | 0.6 |
| Addiction (smoker or alcoholic) | 24 | 18 | 0.4 |
| CD4 baseline | 192 (102-310) | 232 (157.5-324.5) | 0.2 |
| Highest CD4 | 645 (427-852) | 568 (406-735) | 0.3 |
| Change in CD4 | 423 (237-600) | 288 (199.5-447) | 0.005 |
| Immunological failure | 6 | 6 | 0.6 |
| Clinically observed side effects | | | 0.03 |
| Overall (33) | 11 | 22 | |
| Gastro-intestinal symptoms ($n$ = 8) | 3 | 5 | |
| Neuropsychiatric symptoms ($n$ = 13) | 1 | 12 | |
| Peripheral neuropathy ($n$ = 3) | 1 | 2 | |
| Rash ($n$ = 2) | 1 | 1 | |
| Lipodystrophy ($n$ = 5) | 5 | 0 | |
| Gynaecomastia ($n$ = 2) | 0 | 2 | |
| Macrocytosis | 105 | 27 | < 0.01 |
| Anaemia | 17 | 24 | 0.2 |
| Leucocytosis | 2 | 5 | 0.2 |
| Leucopenia | 8 | 5 | 0.4 |
| Thrombocytopenia | 18 | 16 | 0.8 |
| Transaminitis | 36 | 33 | 0.8 |
| Deranged KFT | 1 | 3 | 0.3 |
| Hypocalcemia | 36 | 38 | 0.6 |
| Hypophosphatemia | 32 | 32 | 0.1 |

$^{*}p < 0.05$; $^{**}p < 0.01$ compared with control group.

more in the efavirenz group.

No significant difference in hematological and biochemical derangements were observed between the two groups except for that of higher frequency of macrocytosis in the nevirapine group. This was because nevirapine was co-administered with zidovudine in 94% of cases, which competes with deoxy nucleoside triphosphates and results in impaired synthesis of the erythrocyte precursor in the bone marrow (*9*).

In our study, although we did not find any difference between the highest CD4 count attained during treatment between the two groups, there was a higher median CD4 count change (between baseline and highest) in the nevirapine group. This was probably due to the longer duration of treatment in the nevirapine group. In a meta-analysis by Cochrane infectious diseases group, no significant difference was observed between nevirapine and efavirenz in terms of virological success and progression to AIDS (*10*). Studies from India have shown good tolerability with both nevirapine and efavirenz (*11-14*).

In conclusion, since most serious adverse events associated with nevirapine are seen during the first few weeks/months of the therapy, nevirapine might be as good as efavirenz, if not better, in those patients who are tolerating it well for years. There isn't enough evidence for shifting the patients on long term nevirapine based therapy to efavirenz containing regimen.

Limitations of the study: This was a cross-sectional study without any follow up and the data was collected from a single interview.

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Brief Report

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# Bio-guided fractionation and iron chelating activity of agricultural residues

**Farid A. Badria[1,*], Sara N. Suliman[1], Marwa Elsbaey[1], Mai H. El-Naggar[2]**

[1] *Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt;*
[2] *Department of Pharmacognosy, Faculty of Pharmacy, Sohag University, Sohag, Egypt.*

**Summary** **Iron overload is implicated in many disorders in the body such as heart failure, liver cirrhosis and fibrosis, gallbladder disorders, diabetes, arthritis, depression, infertility, and cancer. Even though synthetic chelating agents are available, they have several limitations such as poor oral bioavailability, short plasma half-life, high cost and numerous side effects. Therefore, the aim of this study is using agricultural residues as sources for alternative efficient, benign, and economic iron chelators of natural origin. Eighteen agricultural residues were screened for iron chelating activity using 2, 2'-bipyridyl assay. The results showed that the extract of *Mangifera indica* leaves had the highest iron chelation activity (69.7%), in comparison to ethylenediaminetetraacetic acid (EDTA) (70.3%) (standard iron chelator). The *M. indica* leaves extract was further investigated for its flavonoidcontent, phenolic content and antioxidant activity. The high concentration of phenolic (405.5µg/g expressed as gallic acid equivalent) and flavonoid (336.9 µg/g expressed as quercetin equivalent) phytochemicals in the extract, as well as its significant antioxidant capacity (96.95%) compared to ascorbic acid (91.90%) (standard antioxidant agent), suggested that the *M. indica* leaves could represent a good source for new iron chelating agents in iron overload disorders.**

***Keywords:*** Iron overload, *Mangifera indica*, antioxidant activity, flavonoids, phenolics

## 1. Introduction

Iron is avital trace element of the body (*1*). It is essential for oxygen and electrons transport within cells and as an integrated part of important enzyme systems in various tissues (*2*). Since iron deficiency and iron overload are both harmful and are associatedwith several disorders, it is very important to maintain iron homeostasis in the body.

Iron overload (hemochromatosis) is caused by genetic disorder involved in a protein regulating iron absorption, or due tomultiple transfusions of iron in chronic anemia or thalassemia, and liver cirrhosis patients (*3*). Accumulation of iron in the body results in initiation and propagation of reactive oxygen species, which start to attack the cell vital macromolecules such as proteins, lipids, RNA and DNA causing DNA mutation, cell damage and ultimately cell death. The high oxidative stress status is associated with many health complications such as heart failure, liver cirrhosis and fibrosis, gallbladder disorders, diabetes, arthritis, infertility, and cancer (*2*). About 71% mortality are recorded in beta thalassemia patients who suffer from cardiac diseases due to accumulation of iron in myocardium (*4*). Iron chelators can remove the accumulated iron from body before causing irreversible tissue damage by forming soluble, stable complexes that can be excreted in feces and/or urine. Although, available iron chelators reduce iron-related complications, their severe side effects, poor oral bioavailability or short plasma half-life make them suboptimal (*5*). Deferoxamine (Desferal®) is the most common drug used for this purpose for many years (*6*). Besides being an expensive drug, its use is painful due to its administration by intravenous or subcutaneous routes. Moreover, it had a negative effect on patient's life through chronic treatment such as neurotoxicity (*4*).

**Address correspondence to:*
Dr. Farid A. Badria, Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.
E-mail: faridbadria@gmail.com

Other available drugs such as deferasirox (Exjade®) and deferiprone (Ferriprox®) are also expensive and their use is associated with side effects such as gastrointestinal disorder (*4*). Several researchers have been interested in studying iron overload problems in the body and finding natural iron chelators from different sources. Catechins and curcumin were represented as natural iron chelators. They were proven to have a significant ability to reduce the level of iron overload in the body (*7-9*). Agricultural residues are considered as a potential source for economic biologically active compounds (*10,11*). Therefore, this study was designed to search for natural and efficient iron chelators with high safety margin and economic cost from agricultural residues.

## 2. Materials and Methods

### 2.1. *Plant materials*

Eighteen agricultural residues (Table 1) were collected from Mansoura city and its vicinity in August 2017 and authenticated by Prof. Ibrahim Mashaly at Ecology and Botany Department, Faculty of Science, Mansoura University. *Citrus sinesis* L. peel and leaves of all other agricultural residueswere used all over this study.

### 2.2. *Chemicals*

Methanol, petroleum ether, methylene chloride, ethyl acetate (EtOAc), n-butanol, sodium hydroxide, Ferric chloride, sulfuric acid, hydroxyl amine hydrochloride, Potassium acetate, and sodium carbonate were purchased from EL-Nasr Company, Cairo, Egypt. Other chemicals and reagents obtained from different sources as follow; alcoholic α-naphthol (BiochemPharma Limited, Cairo, Egypt), mercuric chloride (Noreshark, Cairo, Egypt), ferrous sulfate (Nobel Company, India), trisHCl (PrevestDenpro Limited, Digiana, Jammu, India), 2,2'-bipyridyl solution (Hopkin and Williams Essex, London, England), ethylenediamine tetra acetic acid (EDTA) (BDH Laboratory, England), aluminum chloride (Laboratory Rasayan S.D Fine, Chem. Limited, Mumbai, India), folin-ciocalteu reagent (Sigma, Louis, USA), azino-bis-(3-ethyl benzthiazolin-6-sulfonic acid), (Fluka, Germany), manganese dioxide (Oxford Laboratory, Mumbai, Maharashtra, India), ascorbic acid (Cevarol tablet, Memphis pharmaceutical Co, Cairo, Egypt). Quercetin and Gallic acid were previously isolated and identified in the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University.

### 2.3. *Preparation of the crude plant extracts*

The plant material was collected and washed under running tap water to remove the dust. The plant samples were then air dried and crushed into powder and stored in polythene bags for further use (*12*). Powdered plant material (5 g) was exhausted by 70% methanol (3 × 50 mL). The extract was evaporated under reduced pressure, kept in dark at about 4°C (*13*).

### 2.4. *Large scale preparation and fractionation of the crude extracts of Mangifera indica leaves*

The plant powder (3 kg) was extracted by the same method which used before with plant samples and yielded 200 g dry extract. The extract was dissolved in the least amount of 50% methanol. Using liquid-liquid partitioning method; it was fractionated successively till exhaustion with petroleum ether, methylene chloride, ethyl acetate and n-butanol. The fractions, in each case, were evaporated to dryness under reduced pressure, and

**Table 1. The studied agricultural residues with their iron chelation activity at concentration of 1 mg/mL**

| Plant | Common name | Family | Iron chelation activity % |
|---|---|---|---|
| *Brassica oleracea* Var.capitata | Cebbage (green) | Brassicaceae | 28.93 ± 4.46 |
| ***Caricapapay* L.** | **Papya** | **Caricaceae** | **34.88 ± 9.44** |
| *Ceratonia siliqua* | Carob | Fabaceae | 27.25 ± 0.21 |
| ***Citrus lemon* L.** | **Lemon** | **Rutaceae** | **36.11 ± 1.30** |
| *Citrus sinesis* L. | Orange | Rutaceae | 24.39 ± 0.29 |
| *Cymbopogoncitratus* | Lemon grass | Poaceae | 12.80 ± 0.91 |
| ***Ficuscarica*** | **Figs** | **Moraceae** | **34.50 ± 3.78** |
| ***Mangiferaindica* L.** | **Mango** | **Anacardiaceae** | **69.71 ± 0.27** |
| *Morus alba* | Berry | Moraceae | 24.32 ± 0.93 |
| ***Populus alba.* L.** | **White poplar** | **Solanaceae** | **34.11 ± 13.13** |
| *Prunus armeniaca* | Apricot | Rosaceae | 28.98 ± 0.41 |
| *Prunus persica* | Peach | Rosaceae | 22.60 ± 6.06 |
| ***Psidium guajava*** | **Guava** | **Myrtaceae** | **58.38 ± 3.37** |
| *Punicagranatum* | Pomegranate | Lythraceae | 19.79 ± 0.20 |
| *Pyrus communis* | Pear | Rosaceae | 27.54 ± 4.43 |
| *Solanum melongena* | Aubergine | Solanaceae | 14.0 ± 6.77 |
| *Vitis vinifera* | Grape | Vitaceae | 23.27 ± 3.76 |
| ***Ziziphus spinacristi*** | **Buskthorn** | **Rhamnaceae** | **40.19 ± 2.14** |

Results are expressed as mean ± SD.

kept in refrigerator for further investigation.

2.5. Determination of iron chelation activity

The metal chelating activity was assessed by bipyridyl assay method (*14*). Briefly, 250 µL of 3 Mm $FeSO_4$, 1 mL of 0.2 M 2,2'-bipyridyl solution, 1 mL of 0.2 M Tris-HCl, 400 µL of 10% hydroxyl amine, 2.5 mL of methanol and 100 µL of distillated water were added to each extract sample (250 µL, 1 mg/mL). The absorbance was determined at $\lambda_{max}$ 522 nm and used to evaluate $Fe^{2+}$ chelating activity using ethylenediaminetetraacetate (EDTA) as a standard, the results were expressed as the percentage of iron chelation activity = $(A_{control} - A_{test})/(A_{control} - A_{blank}) \times 100$.

$IC_{50}$ of EDTA, total extract of *M. indica* leaves as well as its EtOAc fraction were determined at serial dilutions ranging from 1,000-7.8 µg/mL. The iron chelation activity was recorded at each concentration and a calibration curve was established.

2.6. *Preliminary phytochemical screening*

The plant extracts were tested for the presence of saponins, alkaloids, carbohydrates, flavonoids, and tannins using Foam test, Mayer's test, Molish's test, NaOH test, and Ferric chloride test respectively. The phytochemical screening was carried out using 0.5 mg of each extract (*15,16*).

2.7. *Determination of total flavonoid content*

The assay was performed according to the method described by Ebrahimzadeh *et al*. (2008) (*5*). To 250 µL of the extract (1 mg/mL stock solution), 750 µL of methanol, 50 µL of aluminum chloride, 50 µL of potassium acetate solution, and 1,400 µL of distilled water were added and kept for 30 min. The absorbance was measured at $\lambda_{max}$ 415 nm. Quercetin was used as a positive control at serial dilutions (1,000-7.8 µg/mL). The calibration curve was established. The total flavonoid content was expressed as µg/g quercetin equivalent using standard curve equation y = 0.0048 x + 0.0012, $R^2 = 0.09995$.

2.8. *Determination of total phenolic content*

To 40 µL of the extract (1 mg/mL stock solution), 1,800 µL of Folinciocalteu reagent were added then 1,200 µL of sodium carbonate were added after 5 min. and kept for one hour in dark area. The absorbance of the solution was measured at $\lambda_{max}$ 750 nm (*17*). Gallic acid was used as a standard at serial dilutions (1,000-12.5 µg/mL). The calibration curve was established. The total phenolic content was expressed as µg/g gallic acid equivalent using standard curve equation y = 0.0008 x + 0.0143, $R^2 = 0.9982$.

2.9. *Screening of the antioxidant activity*

This method was carried out according to Lissi *et al*. (1999) (*18*). The reaction mixture consisted of 2 mL of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution, and 3 mL of manganese dioxide solution prepared in phosphate buffer (pH 7). The mixture was shaken, centrifuged and decanted. The absorbance of $ABTS^+$ radical solution was recorded at $\lambda_{max}$ 734 nm. The test absorbance was measured upon the addition of 20 µL of the test sample solution in spectroscopic grade MeOH/buffer (1:1, v/v) to the ABTS solution. The decrease in absorbance was expressed as % inhibition which is calculated from the equation: %inhibition = $(A_{control} - A_{test})/A_{control} \times 100$. Ascorbic acid (20 µL) was used as a standard antioxidant at serial dilutions (1,000-1.56 µg/mL). $IC_{50}$ was determined by preparing a serial dilution of ascorbic acid, total extract as well as the EtOAc fraction ranging from 1,000-0.78 µg/mL. The % ABTS inhibition was recorded at each concentration and a calibration curve was established.

## 3. Results and Discussion

3.1. *Determination of iron chelation activity*

The results of iron chelation assay (Table 1) indicated that the leaves of *M. Indica* had had the highest iron chelation activity (69.71 ± 0.27%) that was comparable to the standard iron chelator, EDTA (70.30 ± 0.08%). In addition, *P. guajava*, *Z. spinacristi*, *C. lemon*, *C. papay*, *F. carica* and *P. alba* leave extracts showed high activities (58.38 ± 3.37%, 40.19 ± 2.14, 36.11 ± 1.30, 34.88 ± 9.44, 34.50 ± 3.78 and 34.11 ± 13.13, respectively). Meanwhile, *S. melongena* and *C. citratus* leave extracts showed the lowest iron chelation activities 14.0 ± 6.77% and 2.80 ± 0.91%, respectively. These outcomesencouraged further investigations of the different fractions of *M. indica* leaves extract to determine the most active iron chelationfraction. The obtained results showed that the EtOAc fraction had the highest activity among the tested fractions (29.28 ± 2.52%). Other fractions; petroleum ether, methylene chloride, and *n*-butanol showed 5.87 ± 0.38 %, 8.13 ± 1.65%, and 24.05 ± 2.49% iron chelation activities, respectively. In addition, the $IC_{50}$ value of *M. indica* and the EtOAc fraction were found to be 362.7 ± 37.19 µg/mL and 150 ± 2.05 µg/mL respectively in comparison to EDTA (745.2 ± 12.72 µg/mL).

3.2. *Preliminary phytochemical screening*

Preliminary phytochemical screening of the used plant extracts was carried out to investigate the nature of the compounds responsible for iron chelation activity. Results showed presence of flavonoids and tannins in

**Table 2. Preliminary phytochemical screening**

| Plant name | Alkaloids | Tannins | Saponins | Carbohydrate | Flavonoids & Anthraquinones |
|---|---|---|---|---|---|
| *Brassica oleracea Var.capitata* | + | - | - | + | + |
| *Caricapapay* L. | + | - | - | ++ | ++ |
| *Ceratonia siliqua* | - | + | - | + | ++ |
| *Citrus lemon* L. | + | +++ | - | + | ++ |
| *Citrus sinesis* L. | + | +++ | - | + | ++ |
| *Cymbopogoncitratus* | + | +++ | - | + | ++ |
| *Ficuscarica* | - | - | - | + | ++ |
| *Mangiferaindica* L. | + | +++ | - | + | +++ |
| *Morus alba* | - | + | - | +++ | + |
| *Populus alba.* L. | + | +++ | - | + | + |
| *Prunus armeniaca* | + | +++ | - | + | + |
| *Prunus persica* | +++ | +++ | - | + | - |
| *Psidium guajava* | +++ | +++ | - | + | + |
| *Punicagranatum* | +++ | +++ | - | + | + |
| *Pyrus communis* | +++ | +++ | - | + | + |
| *Solanum melongena* | + | +++ | - | +++ | +++ |
| *Vitis vinifera* | + | +++ | - | ++ | ++ |
| *Ziziphusspinacristi* | +++ | +++ | - | + | ++ |

**Table 3. Total phenolic (TPC), total flavonoid contents (TFC), and antioxidant activity of the M. indica leaves total extract and EtOAc fraction**

| Plant | TPC (µg/g) | TFC (µg/g) | % $ABTS^+$ inhibition |
|---|---|---|---|
| Total extract | 403.59 ± 1.25 | 336.982 ± 3.56 | 96.95 ± 0.98% |
| EtOAc fraction | 631.82 ± 1.26 | 616.126 ± 1.97 | 99.90 ± 0.29% |
| Ascorbic acid | — | — | 91.90 ± 0.29% |

Results are expressed as mean ± SD.

the active plant extracts (Table 2).

### 3.3. *Determination of total phenolic and flavonoid content*

Since both the total extract of the leaves of *M. indica* as well as the EtOAc fraction showed the highest iron chelation activities, they were further investigated to determine their phenolic and flavonoids contents. EtOAc fraction showed higher total phenolic and total flavonoid contents than the total plant extract (Table 3).

### 3.4. *Determination of antioxidant activity*

The percentage ABTS inhibition against different concentrations (µg/mL) was determined for *M. indica* leavestotal extract and the EtOAc fraction. The results showed that EtOAc fraction had higher inhibitory activity than the total extract and both had higher inhibitory activities than the positive control, ascorbic acid (Table 3). Furthermore, the $IC_{50}$ values of the *M. indica* leaves extract and the EtOAc fraction were determined and found to be 85.29 ± 16.28 µg/mL, and 24.18 ± 1.83 µg/mL, respectively, in comparison to ascorbic acid (37.18 ± 1.83 µg/mL).

Analyzing the previous results, collectively, it could be concluded that, there is a direct relation between the iron chelating activity of the plant extracts and their content of active compounds. The activity of *M. indica* leave extract is attributed to its content of phenols, and flavonoids as represented by the phytochemical screening and studying its total flavonoid and total phenolic contents, while the chelating activity of *P. guajava*, *Z. spinacristi*, *C. lemon*, and *P. alba* was largely attributed to their tannins content only. Moreover, the good chelating activity showed by each of *C. papay* and *F. carica* was relatively attributed to their flavonoid content. In contrast, the leave extracts for each of *S. melongena* and *C. citratushave* a rich content of polyphenolic compounds and showed the weakest chelating activity. In addition, there was a direct correlation between the antioxidant activity and the polyphenolic compounds content observed by *M. indica* and its EtOAc fraction, both showed a potential antioxidant activity higher than ascorbic acid (standard antioxidant). The antioxidant activity of the EtOAc fraction was stronger than *M. indica* total extract since its total phenolic and total flavonoid contents were higher than that of *M. indica*. This indicated a direct correlation between the antioxidant activity and the polyphenolic content. In contrast, the EtOAc fraction had lower iron chelating activity than the total extract indicating no correlation between the iron chelation activity and the polyphenolic content.

The iron chelating and the antioxidant activities may not be correlated to the polyphenolic content of some plant extracts. This was also presented in other published researches (*5,19*). Overall, the leaves extract of *M. indica* that contained high polyphenolic content exhibited the best iron chelating activity and high antioxidant activity. It is worth to be mentioned that the previous literature proved the potential use of *M. indica* extract as antioxidant (*20*) and as iron chelator (*21*). We need to conduct a bio-guided study to pin point the activity of the extract, even though some literature cited that Mangiferin is the compound responsible for the activity (*22,23*).

In conclusion, the vital use of agricultural residues should be recognized, especially *Mangifera indica* leaves as potential therapeutic agents in iron overload health complications. Therefore, future strategies are in progress to determine the mechanism of action through *in vivo* and bioavailability studies. This may pose a hope for utilizing the mango leaves and/or the pure compound(s) as a valuable natural, safe, and efficient alternative for the currently used iron chelation therapies.

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Case Report

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# Ultrasonographic evaluation of changes over time in one defecation cycle in adults with functional constipation: A report of two cases

**Masaru Matsumoto[1,2], Shiho Tanaka[3], Koichi Yabunaka[1,2,*], Mikako Yoshida[1,2], Yuka Miura[4], Takuya Tsutaoka[1,5], Mayumi Handa[1,6], Gojiro Nakagami[2,3], Junko Sugama[4], Shingo Okada[7], Hiromi Sanada[2,3]**

[1] *Department of Imaging Nursing Science, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[2] *Global Nursing Research Center, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[3] *Department of Gerontological Nursing/Wound Care Management, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan;*
[4] *Institute for Frontier Science Initiative, Kanazawa University, Kanazawa, Ishikawa, Japan;*
[5] *Imaging Technology Center, Research & Development Management Headquarters, Fujifilm Corporation, Tokyo, Japan;*
[6] *Marketing Planning Group, Ultrasound Promotion Department, Fujifilm Medical Corporation, Tokyo, Japan;*
[7] *Department of Surgery, Kitamihara Clinic, Hakodate, Hokkaido, Japan.*

**Summary** **We described fecal retention during the defecation cycles of adults with functional constipation *via* ultrasonography (US) of the large intestine. US was performed continuously after the last defecation until the next defecation. We defined the fecal finding level on US as follows: weak fecal retention, a marginally high echo in the colonic lumen; or strong fecal retention, a strongly echoic colon lumen with showing a crescent-shaped acoustic shadow on transverse images and haustrations on longitudinal images. The findings confirmed weak fecal retention in the colon throughout the defecation cycle and a pattern of strong fecal retention in the descending and sigmoid colon and over the colon, including the transverse colon and ascending colon, in patients with functional constipation.**

***Keywords:*** Ultrasonography, functional constipation, slow transit, fecal retention, adults

## 1. Introduction

Ultrasonography (US) can be used to observe the position of fecal storage and the state of the feces, and is therefore considered useful for the evaluation of constipation. Previously, objective constipation evaluations have confirmed the time of colon transit as a measure of defecation function (*1,2*). However, although these evaluations demonstrated the areas of fecal stagnation, they could not provide real-time information about where and what type of feces were stored in the colon. Therefore, we demonstrated through a comparison with computed tomography findings that fecal properties, such as the existence of haustration and crescent-shaped, highly echoic area with acoustic shadows, could be evaluated on US images (*3*). In addition, we demonstrated that US could be used to assess fecal retention patterns that did and did not involve the rectum in patients with chronic constipation (*4*). Functional constipation types can be classified as normal transit, slow transit, and anorectal dysfunction (AD) (*5-7*), and we demonstrated the ability to evaluate the latter type by confirming fecal retention in the rectum using US.

However, the previous study was a cross-sectional investigation, and no study has investigated changes in the fecal retention state over time in the large intestine up to the point of fecal discharge. To clarify the characteristics of colonic fecal retention patterns in elderly patients with constipation, it is first necessary to investigate the colonic fecal retention patterns in adult patients with constipation. In this case report, we observed the fecal retention status and subjectively

*Address correspondence to:
Dr. Koichi Yabunaka, Department of Imaging Nursing Science, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan.
E-mail: kyabunaka-tky@umin.ac.jp

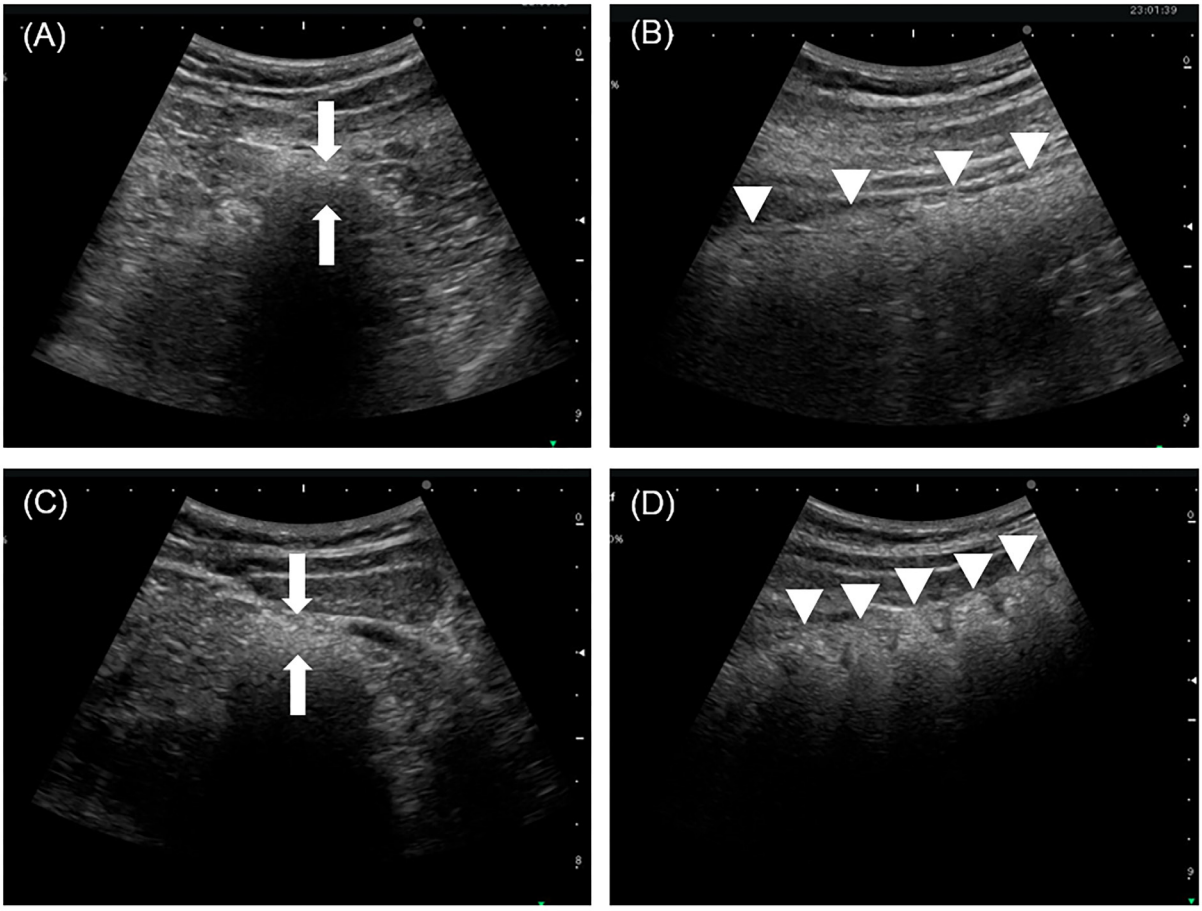


**Figure 1. Standards for ultrasonographic imaging evaluations. (A, B)** Ultrasonography (US) images of weak fecal retention in a 32-year-old male patient. **(A)** A transverse US image showing a marginally highly echoic colonic lumen (arrows) and posterior echo behind the descending colon. **(B)** A longitudinal US image showing a flattened outer boundary wall and highly echoic descending colon wall (arrowheads). **(C, D)** US images of strong fecal retention in a 25-year-old male patient. **(C)** A transverse US image showing a strongly echoic colonic lumen (arrows) and a strong acoustic shadow behind the descending colon (arrowheads). **(D)** A longitudinal US image showing a crescent-shaped acoustic shadow with haustrations and strong echoes on the descending colon wall (arrowheads).

evaluated one defecation cycle using US with a longitudinal view in adult patients with functional constipation, and compared the findings with those from a case of non-constipation.

## 2. Case Report

### 2.1. *Ultrasound technique*

All subjects were adults without disease during treatment and without morphological abnormalities of the large intestine. US of the ascending, transverse, descending and sigmoid colon and rectum was performed continuously from after the most recent defecation until after the subsequent defecation in our laboratory, using an US system (FUJIFILM FC1-X, FUJIFILM SonoSite, Bothell, WA, USA) with a curved-array probe (2-5 MHz). The resulting images were supplemented by transverse and longitudinal sonographic scans. According to a previous study (*3*), we defined the US levels of fecal finding as follows: a weak fecal retention finding was indicated by a marginally highly echoic colonic lumen and posterior echo behind the colon on transverse images, and a flattened outer boundary wall and highly echoic colon wall on longitudinal images; a strong fecal retention finding was indicated by a strongly echoic colonic lumen and strong acoustic shadow behind the colon on transverse image, and a crescent-shaped acoustic shadow with haustrations and a strongly echoic colon wall on longitudinal images (Figure 1). US was performed by a trained researcher. Static images were interpreted by a certified sonographer with 30 years of experience.

The Rome IV criteria were used to determine the presence or absence of functional constipation (*3*). Two items were selected from the Constipation Assessment Scales and used to evaluate subjective discomfort due to constipation (*8*). Fecal properties were evaluated using the Bristol stool form scale (*9,10*), and fecal amounts were evaluated using King's stool chart (*11-13*). This study was approved by the Ethical Committee of the Graduate School of Medicine, The University of Tokyo, Japan (No. 11521).

**Table 1. Ultrasonographic findings and subjective evaluation during observation period**

| | Evaluation | Site/item | Immidiately after last defecation | After 8 hours | After 16 hours | After 24 hours | After defecation |
|---|---|---|---|---|---|---|---|
| Non-constipation | Ultrasonographic evaluation | Ascending colon | + | + | + | + | + |
| | | Transverse colon | – | + | + | + | + |
| | | Descending colon | – | + | + | + | – |
| | | Sigmoid colon | – | – | + | ++ | + |
| | | Rectum | – | – | – | – | – |
| | Subjective evaluation | Abdominal distention or bloating | 0 | 0 | 1 | 1 | 0 |
| | | Sensation of rectal pressure or fullness | 0 | 0 | 0 | 0 | 0 |
| | | | | After 1 day | After 2 days | After 3 days | After defecation |
| Case1 | Ultrasonographic evaluation | Ascending colon | | – | + | ++ | + |
| | | Transverse colon | | ++ | ++ | ++ | ++ |
| | | Descending colon | | ++ | ++ | ++ | + |
| | | Sigmoid colon | | ++ | ++ | ++ | ++ |
| | | Rectum | | – | – | – | – |
| | Subjective evaluation | Abdominal distention or bloating | | 1 | 1 | 1 | 0 |
| | | Sensation of rectal pressure or fullness | | 0 | 0 | 0 | 0 |
| Case2 | Ultrasonographic evaluation | Ascending colon | | + | + | + | + |
| | | Transverse colon | | + | + | + | + |
| | | Descending colon | | + | ++ | ++ | ++ |
| | | Sigmoid colon | | + | ++ | ++ | ++ |
| | | Rectum | | – | – | – | – |
| | Subjective evaluation | Abdominal distention or bloating | | 0 | 0 | 1 | 0 |
| | | Sensation of rectal pressure or fullness | | 0 | 0 | 0 | 0 |

After 1 day indicates the day after the last defecation day. +: Weak fecal loading (+), ++: Strong fecal loading (++), –: No fecal loading findings. Number: the score of subjective evaluation: 0, none; 1, some; 2, severe.

2.2. *Non-constipation*

A 32-year-old healthy man with a history of daily defecation was followed up. US follow-up was performed for 8 hours from the last defecation until the next defecation during a 2-day period. The US findings and subjective evaluations during the observation period are shown in Table 1. Weak fecal retention was observed only in the ascending colon at baseline, whereas strong fecal retention was also confirmed in the distal intestine over time. Findings indicative of strong fecal retention were observed in the sigmoid colon after 24 hours (Figure 2). The strong fecal retention in the sigmoid colon and 'abdominal distention or bloating' were resolved after defecation. The defecated stools were assigned a 4-point score (normal stool) on the Bristol stool form scale and a F-type score (> 200 g) on King's stool chart.

2.3. *Functional constipation*

2.3.1. *Case 1*

A 25-year-old man with a defecation cycle of 3-4 days was followed up. His diet comprised regular meals. US follow-up was performed for approximately 24 hours from the last defecation until the next defecation over a 4-day period. The US findings and subjective evaluations during the observation period are shown in Table 1. Fecal retention was almost always observed in the colon, excepting the rectum. After 3 days, fecal retention was found in the ascending colon, as well as the transverse, descending and sigmoid colon. Strong fecal retention finding was observed in the transverse and sigmoid colon throughout the observation period. As shown in Figure 2, findings of strong fecal retention finding were observed in the transverse and sigmoid colon, despite defecation. The patient received a score of 1 point for 'abdominal distention or bloating' after 1-3 days, which decreased to 0 points after defecation. During the observation period, the 'sensation of rectal pressure or fullness' was 0-point. The defecated stools were classified as 2 points (hard stool) on the Bristol stool form scale and as B-type (100-200 g) on King's stool chart.

2.3.2. *Case 2*

A 28-year-old female with a defecation cycle of 3-4 days was followed up. Her diet comprised regular meals. The US follow-up was performed for approximately 24 hours from the last defecation to the

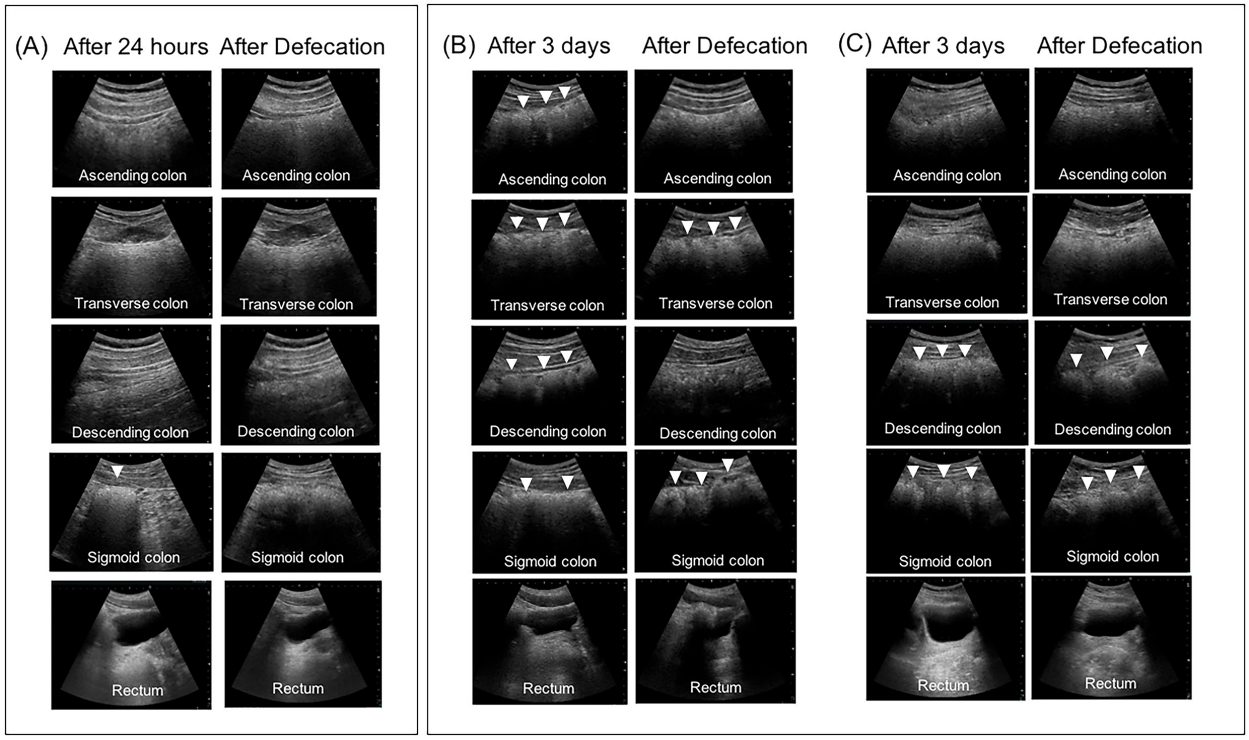


**Figure 2. Fecal retention observed by ultrasonography in cases of non-constipation and functional constipation. (A)** A post-defecation longitudinal ultrasonography (US) image of a 28-year-old female subject with no constipation after 24 hours. **(B)** A post-defecation longitudinal US image of a 25-year-old male patient with functional constipation after 3 days. **(C)** A post-defecation longitudinal US image of a 28-year-old female patient with functional constipation after 3 days. Arrowheads indicate fecal retention, as depicted by a crescent-shaped acoustic shadow with haustrations and strong echoes on the longitudinal US image.

next defecation over a 4-day period. The US findings and subjective evaluations during the observation period are shown in Table 1. Fecal retention was always observed in the colon, except for the rectum, although findings of strong fecal retention were only observed in the descending and sigmoid colon throughout the observation period. In addition, as shown in Figure 2, these findings of strong fecal retention in the descending and sigmoid colon persisted despite defecation. The score of 'abdominal distention or bloating' was only 1 point after 3 days, but decreased to 0 points after defecation. During the observation period, the 'sensation of rectal pressure or fullness' score remained at 0 points. The defecated stools were classified as 2 points (hard stool) on the Bristol stool form scale, and as A-type (< 100 g) on King's stool chart.

## 3. Discussion

In this study, we observed the fecal retention status on longitudinal US images and subjective evaluations during one bowel movement cycle in adult patients with functional constipation; additionally, we compared these findings with those from a non-constipated subject for the first time. This study targeted adults with a perception of normal bowel movements, which may explain the lack of fecal retention in the rectum. Therefore, the two cases of functional constipation in this study appear to meet the criteria of the slow transit type.

Our evaluation of functional constipation revealed that feces are always stored in the colon (excluding the rectum) throughout the defecation cycle. By contrast, the subject without constipation did not exhibit fecal storage in the colon after defecation, and the feces had gradually moved to the distal colon. We note that the post-defecation US findings from the non-constipation case may not have coincided with the findings observed after the previous defecation because of variations in the time of US examination. As defecation occurred 24 hours after the last defecation, we considered that fecal retention findings were observed in the transverse and sigmoid colon after defecation because stool moved distally from the proximal colon.

This study demonstrates the ability of US to evaluate two types of slow transit in patients with functional constipation. Previous studies based on colon scintigraphy have reported that slow transit type is characterised by a pattern exhibiting a transport delay throughout the colon, as well as a pattern of transport delay in the descending and sigmoid colon (*2*). In our study, the evaluation of fecal retention findings at two levels also revealed two slow transit patterns associated with functional constipation; the first exhibited persistent strong fecal retention, especially in the descending and sigmoid colon, whereas the second exhibited persistent strong fecal retention in

descending and sigmoid colon, as well as the ascending and transverse colon. The results of US were consistent with those of colon scintigraphy. Our findings indicate that US is more useful than previous bowel function tests for the non-invasive evaluation of constipation (*1,2*), as this tool can evaluate not only the site of fecal retention, but also the properties of feces.

In conclusion, adults with functional constipation always exhibit fecal accumulation in the colon (excluding the rectum) throughout the defecation cycle. Moreover, US could be used to confirm a pattern of fecal retention findings characterised by strong echoes and acoustic shadows in the descending and sigmoid colon, as well as a pattern of echogenic findings throughout the colon (including the transverse and ascending colon) in adults with functional constipation.

*Conflict of interest*

This was a joint research program with FUJIFILM Corporation, and the study was conducted under the sponsorship of this organization. Masaru Matsumoto, Koichi Yabunaka, and Mikako Yoshida belong to a social collaboration department which receives funding from FUJIFILM Corporation.

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Case Report

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# Successful treatment of repeated hematemesis secondary to post-sclerotherapy esophageal ulcer in a cirrhotic patient: A case report

**Zhaohui Bai[1,2,§], Xiaozhong Guo[1,§], Xiaodong Shao[1], Yingying Li[1,3], Qianqian Li[1,4], Xiangbo Xu[1,2], Zhendong Liang[1], Jiao Deng[5], Xia Zhang[6], Hongyu Li[1,*], Xingshun Qi[1,*]**

[1] *Department of Gastroenterology, General Hospital of Shenyang Military Area, Shenyang, Liaoning, China;*
[2] *Postgraduate College, Shenyang Pharmaceutical University, Shenyang, Liaoning, China;*
[3] *Postgraduate College, Jinzhou Medical University, Jinzhou, Liaoning, China;*
[4] *Postgraduate College, Dalian Medical University, Dalian, Liaoning, China;*
[5] *Department of Pharmacology, General Hospital of Shenyang Military Area, Shenyang, Liaoning, China;*
[6] *No. 4 People Hospital of Shenyang City, Shenyang, Liaoning, China.*

**Summary** **Esophageal variceal bleeding is a common lethal complication of cirrhosis. Endoscopic injection sclerotherapy (EIS) is one of the major endoscopic approaches for treating esophageal variceal bleeding. However, complications may occur after EIS, which mainly include retrosternal discomfort/pain, dysphagia, re-bleeding, esophageal ulcer, esophageal strictures, and esophageal perforation, etc. In this article, we reported a 36-year-old male who developed esophageal ulcer related bleeding after EIS. Currently, there is no consensus on the treatment strategy for esophageal ulcer-related bleeding after EIS. In the present case, the following treatment strategy may be effective for ulcer related bleeding. The first step is to inhibit gastric acid secretion and reduce portal pressure by intravenous infusion of esomeprazole and somatostatin, respectively. The second is local hemostasis by oral norepinephrine and lyophilizing thrombin powder. The third is to protect digestive tract mucosa by oral Kangfuxin Ye and aluminum phosphate.**

***Keywords:*** Esophageal varices, endoscopic injection sclerotherapy, endoscopic band ligation, esophageal ulcer, portal hypertension

## 1. Introduction

Esophageal and gastric varices are common complications of chronic liver diseases. On the other hand, esophageal varices are one of the most common causes of acute upper gastrointestinal bleeding (*1,2*). The 6-week mortality rate of each variceal bleeding episode is 15-20%, ranging from 0% among patients with Child class A to approximately 30% among patients with Child class C (*3-5*). Before the 1970s, the major treatment options of variceal bleeding included vasoconstrictors and surgical intervention. Since the mid-1970s, endoscopic injection sclerotherapy (EIS) has been gradually employed for the treatment of esophageal variceal bleeding (*6*). EIS is superior to vasoconstrictors or balloon tamponade in controlling acute esophageal variceal bleeding (*7,8*). However, EIS is associated with a number of complications, such as esophageal ulcer, stenosis, and perforation (*9*). Among them, the incidence of ulcer related bleeding after EIS is 4.3-12.8% (*10-19*). At present, there is no consensus on the treatment strategy for esophageal ulcer-related bleeding after EIS. In this article, we reported a case of esophageal ulcer related bleeding after EIS and discussed the management of this complication.

## 2. Case presentation

On June 17, 2018, a 36-year-old male with a 19-month history of hepatitis C virus related liver cirrhosis was admitted to the Department of Emergency of our hospital due to intermittent hematemesis for 11 hours.

[§]These authors contributed equally to this work.
******Address correspondence to:*
Drs. Xingshun Qi and Hongyu Li, Department of Gastroenterology, General Hospital of Shenyang Military Area, No. 83 Wenhua Road, Shenyang 110840, Liaoning Province, China.
E-mail: xingshunqi@126.com (Qi X); 13309887041@163.com (Li H)

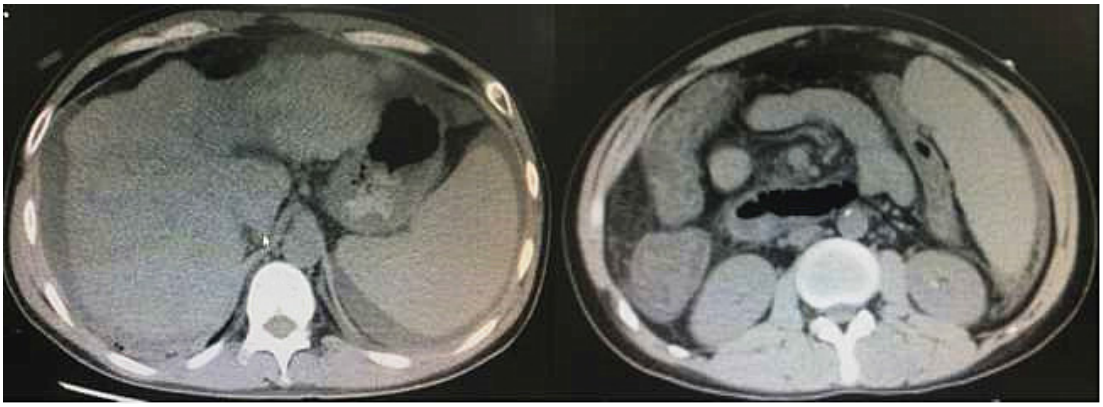

**Figure 1. Abdominal CT scans showed cirrhosis, splenomegaly, ascites, and left renal calculus.**

The volume of fresh blood vomited was about 300 mL. Immediately, infusion of terlipressin 2 mg, esomeprazole 80 mg, somatostatin 6 mg, hemocoagulase injection 2 u, and hydroxyethyl starch sodium chloride injection 500 mL was given at the Department of Emergency. He developed hematemesis again. The volume of fresh blood vomited was about 300ml. On June 18, 2018, he was transferred to our department. He had undergone endoscopic band ligation (EBL) with and without gastric variceal tissue adhesive injection for the treatment of acute variceal bleeding three times (on March 1, 2017, August 1, 2017, and March 27, 2018). He had a 10-year history of smoking and drinking.

After his admission, the patient did not have hematemesis or melena. Heart rate was 78 b.p.m. and blood pressure was 132/80 mmHg. Physical examinations demonstrated that his skin and sclera were yellow. On laboratory tests, red blood cell (RBC) was $4.05 \times 10^{12}$/L (reference range: 4.0-5.5 $\times 10^{12}$/L), hemoglobin (Hb) was 125 g/L (reference range: 110-150 g/L), hematocrit (HCT) was 38.2% (reference range: 35-45%), white blood cell (WBC) was $2.9 \times 10^{9}$/L (reference range: 3.5-9.5 $\times 10^{12}$/L), percentage of granulocyte (GR%) was 63.0% (reference range: 45-75%), total bilirubin (TBIL) was 62.2 μmol/L (reference range: 5.1-22.2 μmol/L), direct bilirubin (DBIL) was 18.7 μmol/L (reference range: 0-8.6 μmol/L), alanine amino-transaminase (ALT) was 30.43 U/L (reference range: 9-50 U/L), aspartate amino-transaminase (AST) was 61.38 U/L (reference range: 15-40 U/L), alkaline phosphatase (AKP) was 146.97 U/L (reference range: 45-125 U/L), γ-glutamyl transpeptidase (GGT) was 33.90 U/L (reference range: 10-60 U/L), prothrombin time (PT) was 21.5 seconds (reference range: 11.5-14.5 seconds), and international normalized ratio (INR) was 1.87. Abdominal computed tomography (CT) scans showed cirrhosis, splenomegaly, ascites, and left renal calculus (Figure 1). His Child-Pugh score was 12 points. Infusion of terlipressin 2 mg per 12 hours, esomeprazole 80 mg per 10 hours, polyene phosphatidylcholine 465 mg per day, isoglycyrrhizinate 150 mg per day, ademetionine 1,000 mg per day, and levofloxacin 0.5 g per day was given.

On June 19, 2018, the patient did not have hematemesis or melena. Laboratory tests demonstrated that WBC was $3.2 \times 10^{9}$/L, GR% was 63.0%, RBC was $3.85 \times 10^{12}$/L, Hb was 125 g/L, HCT was 36%, TBIL was 78.8 μmol/L, DBIL was 36.2 μmol/L, ALT was 29.03 U/L, AST was 53.49 U/L, AKP was 115.6 U/L, GGT was 31.46 U/L, albumin (ALB) was 31.3 g/L (reference range: 40-55 g/L), PT was 23.1 seconds, and INR was 2.04. Endoscopy showed three visible thrombi on the surface of the esophageal varices (Figure 2). Sclerotherapy with lauromacrogol 5 mL followed by tissue adhesive 0.5 mL was successfully performed by our endoscopist (Figure 2). After endoscopic treatment, terlipressin and esomeprazole were discontinued. Oral propranolol 10 mg per 12 hours was given.

On June 21, 2018, the patient developed hematemesis after sneezing. The volume of fresh blood vomited was about 100ml. Laboratory tests demonstrated that WBC was $4.8 \times 10^{9}$/L, GR% was 66.9%, RBC was $3.81 \times 10^{12}$/L, Hb was 117 g/L, HCT was 35.8%, TBIL was 53.9 μmol/L, DBIL was 34.5 μmol/L, ALT was 25.55 U/L, AST was 37.92 U/L, AKP was 124.34 U/L, GGT was 33.1 U/L, and ALB was 29.8 g/L. Infusion of somatostatin 3,000 u per 12 hours and esomeprazole 80 mg per 10 hours was given.

On June 22, 2018, endoscopy showed two ulcer lesions (Figure 3). At 15:00 o'clock, the patient developed hematemesis again. The volume of fresh blood vomited was about 100 mL. Laboratory tests demonstrated that WBC was $4.8 \times 10^{9}$/L, GR% was 76.1%, RBC was $3.65 \times 10^{12}$/L, Hb was 114 g/L, and HCT was 34.6%. Intravenous infusion of esomeprazole 80 mg per 10 hours was continued. The dosage of somatostatin was changed to 3,000 u per 6 hours. In addition, intravenous infusion of carbazochrome sodium sulfonate 80 mg per day and oral lyophilizing thrombin powder 5,000 u per day, norepinephrine 4 mg per day, and aluminum phosphate 20 g three times a day were given.

On June 24, 2018, the patient developed hematemesis again. The volume of fresh blood vomited was about 10 mL. Oral lyophilizing thrombin powder 5,000 u per day and norepinephrine 2 mg per day were given again.

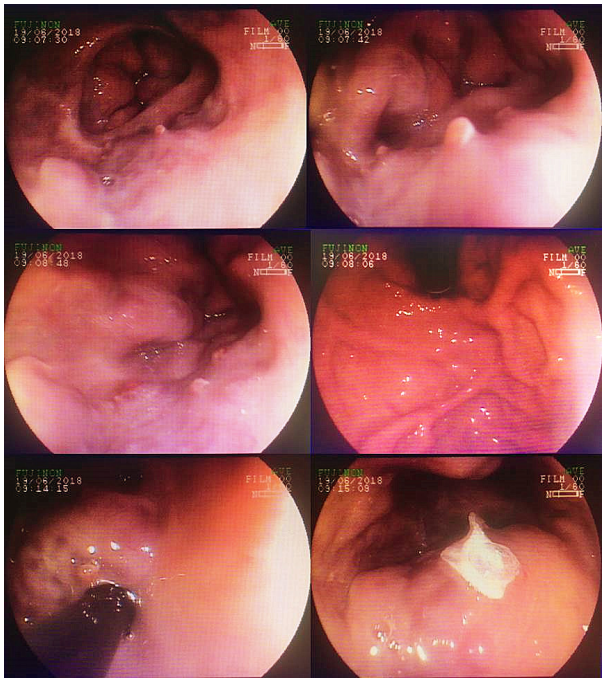

**Figure 2. Endoscopy on June 19, 2018 showed three visible thrombi on the surface of esophageal varices, and then EIS was performed.**

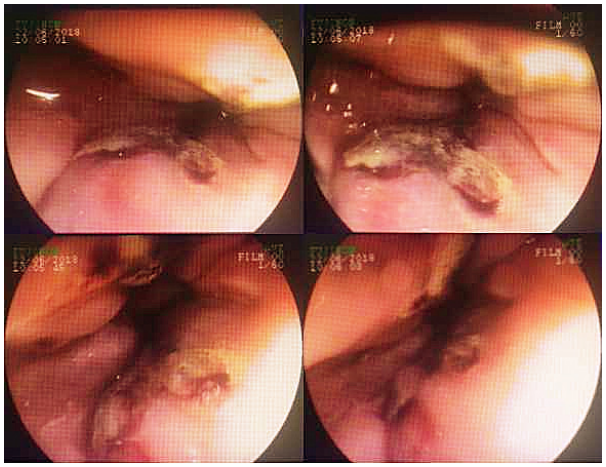

**Figure 3. Endoscopy on June 22, 2018 showed two ulcer lesions.**

On June 26, 2018, the patient did not have hematemesis or melena. Laboratory tests demonstrated that WBC was 4.1 × $10^9$/L, GR% was 71%, RBC was 3.63 × $10^{12}$/L, Hb was 116 g/L, HCT was 35.4%, TBIL was 45.5 μmol/L, DBIL was 28.0 μmol/L, ALT was 13.95 U/L, AST was 22.92 U/L, AKP was 108 U/L, GGT was 28.57 U/L, ALB was 27.0 g/L, PT was 23.4 seconds, and INR was 2.07. Isoglycyrrhizinate was discontinued. The dosage of somatostatin was changed to 3,000 u per 12 hours. Albumin 10 g per day was given. Oral Kangfuxin Ye, which is a traditional Chinese medicine drug for treatment of the damage of digestive tract mucosa, 10 mL per day was given.

On June 27, 2018, the patient developed hematemesis again. The volume of fresh blood vomited was about 30 mL. Intravenous infusion of somatostatin was changed to 3,000 u per 6 hours. Oral lyophilizing thrombin powder 5,000 u per day and norepinephrine 4mg per day were given again.

After that, he did not have hematemesis or melena. On June 30, 2018, laboratory tests demonstrated that WBC was 4.1 × $10^9$/L, GR% was 71%, RBC was 3.63 × $10^{12}$/L, Hb was 116 g/L, HCT was 35.4%, TBIL was 45.5 μmol/L, DBIL was 28.0 μmol/L, ALT was 13.95 U/L, AST was 22.92 U/L, AKP was 108 U/L, GGT was 28.57 U/L, ALB was 27.0 g/L, PT was 23.4 seconds, and INR was 2.07. The dosage of aluminum phosphate was changed to 20 g per day.

On July 1, 2018, the patient did not have hematemesis or melena. Somatostatin, levofloxacin, and carbazochrome sodium sulfonate were discontinued.

On July 4, 2018, the patient did not have hematemesis and then was discharged. Laboratory tests demonstrated that WBC was 3.2 × $10^9$/L, GR% was 77%, RBC was 3.37 × $10^{12}$/L, Hb was 111 g/L, HCT was 32.5%, TBIL was 31.7 μmol/L, DBIL was 21.4 μmol/L, ALT was 8.20 U/L, AST was 22.94 U/L, AKP was 103 U/L, GGT was 28.89 U/L, and ALB was 31.7 g/L. We recommended the patient to take medication at home, including oral Kangfuxin Ye 10 mL per day, aluminum phosphate 20 g per day, propranolol 10 mg twice a day, and polyene phosphatidylcholine 456 mg three times a day.

On August 6, 2018, the patient underwent follow-up endoscopic surveillance. Laboratory tests demonstrated that WBC was 2.2 × $10^9$/L, GR% was 54%, RBC was 4.05 × $10^{12}$/L, Hb was 129 g/L, HCT was 38.8%, TBIL was 39.9 μmol/L, DBIL was 23.3 μmol/L, ALT was 34.72 U/L, AST was 50.36 U/L, AKP was 159.70 U/L, GGT was 28.48 U/L, ALB was 36.1 g/L, PT was 19.5 seconds, and INR was 1.64.

On August 7, 2018, a follow-up endoscopy showed several esophageal varices with red color sign, and then EBL was performed. Mild varices were found in the gastric fundus (Figure 4).

On August 11, 2018, the patient did not have hematemesis or melena. Laboratory tests demonstrated that WBC was 2.5 × $10^9$/L, GR% was 56.2%, RBC was 3.67 × $10^{12}$/L, Hb was 117 g/L, HCT was 35%, TBIL was 38.4 μmol/L, DBIL was 21 μmol/L, ALT was 22.39 U/L, AST was 32 U/L, AKP was 170.49 U/L, GGT was 29.64 U/L, ALB was 32.1 g/L, PT was 20.6 seconds, and INR was 1.56. The patient was discharged. At the time of writing this manuscript, he is well without any other complaints.

## 3. Discussion

Currently, the first-line treatment option of acute variceal bleeding should be endoscopic treatment combined with vasoconstrictors (*20*). However, according to the current practice guideline, covered transjugular intrahepatic portosystemic shunt (TIPS) should be considered as the treatment of choice in the cases when endoscopic

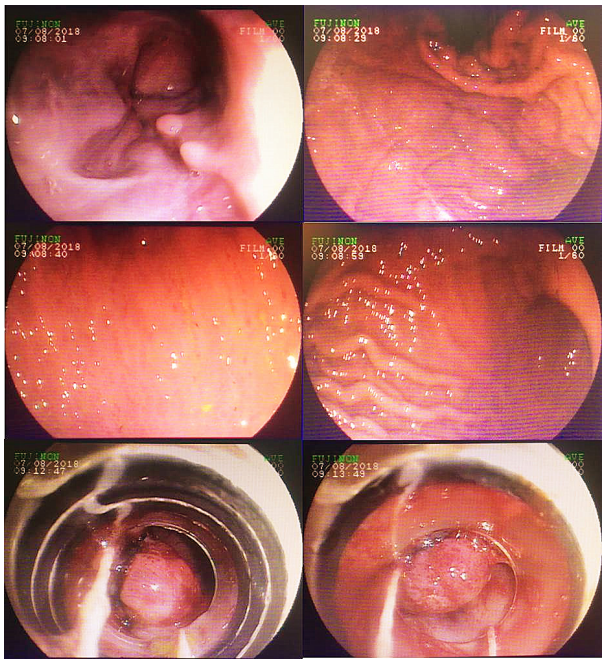

**Figure 4. Endoscopy on August 7, 2018 showed mild varices on esophagus and gastric fundus with red color sign, and then EBL was performed.**

treatment fails (*21*). Our case underwent endoscopic treatment for variceal bleeding many times. We recommended the use of TIPS, but he and his relatives refused.

EBL should be preferred, when endoscopic treatment is considered for the management of acute variceal bleeding in cirrhotic patients (*20,21*). Among the patients with acute esophageal variceal bleeding, the rate of re-bleeding in patients treated with EBL was lower than in those treated with EIS. The reason may be that EIS led to a sustained rise in hepatic venous pressure gradient, followed by an increased re-bleeding rate (*22*). A meta-analysis demonstrated that EBL was superior to EIS in terms of re-bleeding, complications, and variceal eradication (*23*). However, in our case, three visible thrombi were densely arranged on the surface of varices. Our endoscopist suggested that the ligation ring would pass over the thrombi and then lead to active bleeding during the procedure, if EBL was continued. Indeed, the American Society for Gastrointestinal Endoscopy (ASGE) guideline recommends that EIS may be performed in the case that EBL is technically difficult (*24*). After a comprehensive consideration, EIS was finally performed.

Adverse events of EIS include fever, retrosternal discomfort/pain, dysphagia, injection-induced bleeding, esophageal ulcers, esophageal strictures, esophageal perforation, pleural effusion, acute respiratory distress syndrome, and infection (*9,25*). Our case developed esophageal ulcer related bleeding after EIS (Figure 3). We reviewed the literature regarding the occurrence of re-bleeding secondary to esophageal ulcer after EBL or EIS (Table 1). As for most of esophageal ulcers

**Table 1. Re-bleeding secondary to esophageal ulcer after EIS or EBL.**

| | | | | EIS | | | EBL | | |
|---|---|---|---|---|---|---|---|---|---|
| First author | Year | Country | Number of total cases | Incidence of ulcer after EIS | Incidence of re-bleeding after EIS | Re-bleeding caused by ulcer after EIS | Incidence of ulcer after EBL | Incidence of re-bleeding after EBL | Re-bleeding caused by ulcer after EBL |
| Laine | 1993 | USA | 77 | NA | 43.6% (17/39) | 12.8% (5/39) | NA | 26.3% (10/38) | 2.6% (1/38) |
| Lo | 1995 | China Taiwan | 120 | NA | 50.8% (30/59) | 8.5% (5/59) | NA | 32.8% (20/61) | 1.6% (1/61) |
| Baroncini | 1997 | Italy | 111 | NA | 18.5% (10/54) | 5.6% (3/54) | NA | 15.8% (9/57) | 8.8% (5/57) |
| Lo | 1997 | China Taiwan | 71 | 8.8% (3/34) | 33% (10/30) | NA | 2.7% (1/37) | 6/36 (17%) | NA |
| de la Peña | 1999 | Spain | 88 | 4.3% (2/46) | 50% (23/46) | 4.3% (2/46) | 7.1% (3/42) | 28.6% (12/42) | 7.1% (3/42) |
| Al Traif | 1999 | Saudi Arabia | 60 | NA | 17% (5/29) | 6.9% (2/29) | NA | 23% (7/31) | 9.7% (3/31) |
| Masci | 1999 | Italy | 100 | 18% (9/50) | NA | NA | 8% (4/50) | NA | NA |
| Robert | 2001 | USA | 111 | 25.4% (15/59) | NA | NA | 5.7% (3/52) | NA | NA |
| Zargar | 2002 | India | 49 | 16.7% (4/24) | 25% (6/24) | 8.3% (2/24) | 4% (1/25) | 4% (1/25) | 0% (0/25) |
| Awad | 2012 | Egypt | 120 | 20% (12/60) | 13.3% (8/60) | NA | 16.7% (10/60) | 10% (6/60) | NA |
| Overall | | | | 4.3-25.4% | 13.3-50.8% | 4.3-12.8% | 2.7-16.7% | 4-32.8% | 0-9.7% |

Abbreviations: EIS, endoscopic injection sclerotherapy; EBL, endoscopic band ligation; NA: not available.

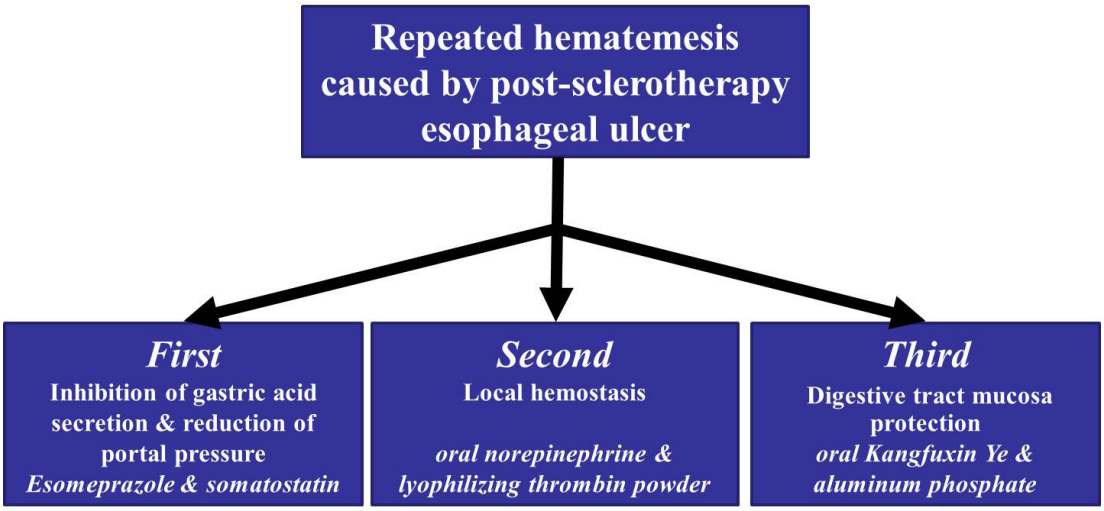


**Figure 5. Treatment procedure in this patient.**

without bleeding after EIS, no special treatment was required (*15*). The prophylactic use of acid suppression drugs after endoscopic treatment for gastroesophageal varices remains uncertain (*26*). By comparison, as for active bleeding secondary to esophageal ulcers, endoscopic injection of epinephrine might be useful for hemostasis (*15*). Our case had active ulcers bleeding after EIS. Our treatment strategy was as follows: the first was to inhibit gastric acid secretion and reduce portal pressure by intravenous infusion of esomeprazole and somatostatin, respectively; the second was local hemostasis by oral norepinephrine and lyophilizing thrombin powder; the third was to protect digestive tract mucosa by oral Kangfuxin Ye and aluminum phosphate (Figure 5). Despite his ulcer related bleeding stopped, the duration of treatment was long.

In conclusion, esophageal ulcer is a major cause of early re-bleeding after EIS. However, at present, there is no consensus regarding treatment strategy of esophageal ulcer related bleeding after EIS. Our successful treatment strategy may be validated in a large-scale study.

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Case Report

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# Necrotizing Autoimmune myopathy: A case report on statin induced rhabdomyolysis requiring immunosuppressive therapy

**Sandeep Kunwar*, Jai D Parekh, Ramya Sree Chilukuri, Venkata A. Andukuri**

*Department of Internal Medicine, Creighton University School of Medicine, Omaha, Nebraska, USA.*

**Summary** **Statins can cause a wide spectrum of muscular adverse effects ranging from asymptomatic elevation of Creatine Kinase (CK), myalgia and exercise intolerance to rhabdomyolysis. Most of these effects generally resolve on stopping the medication. However, statins can be associated with a unique autoimmune myopathy wherein symptoms persist or even progress after statin discontinuation and require immunosuppressive therapy. The case presented is a 60-year-old woman who was on statin treatment for a period of 2 years. She developed muscle weakness with a limb girdle distribution. She had persistent elevation of CK even after discontinuation of statin therapy. EMG done revealed irritable myopathy and muscle biopsy showed necrosis without inflammation. She subsequently tested positive for anti-3-hydroxy-3-methylglutaryl-coenzyme A (anti-HMG CoA) antibody which is found to be present in patients with statin-associated necrotizing autoimmune myopathy. Patient was started on steroid without much improvement in her symptoms. After a month of follow up, her upper extremity strength was back but lower extremity continued to be weak which prompted us to start her on Methotrexate and Azathioprine. Like our patient, there are rare subgroup of patients with an immune-mediated necrotizing myopathy that does not improve after discontinuation of the drug and requires aggressive treatment with immunosuppressive agents. Awareness and early recognition of this disease is very important in patients who continue to have CK elevation and weakness after discontinuation of statin therapy.**

***Keywords:*** Necrotizing autoimmune, myopathy, anti-3-hydroxy-3-methylglutaryl-coenzyme A (anti-HMG CoA) antibody

## 1. Introduction

Statins are some of the most widely prescribed medications, and though generally well tolerated, can lead to musculoskeletal side effects, with up to 20% patients experiencing myalgia's (*1*). There is a wide spectrum of muscular adverse effects associated with statins, from asymptomatic elevation of CK, myalgia and exercise intolerance to toxic necrotizing myopathy and rhabdomyolysis (*2*). In general, statins produce a self-limited myopathy that resolves within several months of medication cessation; however, they are also associated with increased incidence of inflammatory myopathies. The present case belongs to the group of inflammatory idiopathic myopathies (IIM), which is divided into four main groups: polymyositis (PM), dermatomyositis (DM), necrotizing autoimmune myopathy (NAM) and sporadic inclusion body myositis (SIBM) (*3*). NAM can be associated with connective tissue disorder but can also be triggered by viral infections such as HIV or malignancy, be statin induced, or be idiopathic. The absence or relative paucity of an inflammatory lymphocytic infiltrate is described as a pauci-immune necrotizing myopathy and distinguishes NAM from the characteristic histologic findings of PM or DM, which includes CD8+ or CD4+ T lymphocytes and B cells respectively (*3*). Recently, it has been reported that up to 20% of patients with diagnosis of an IIM have NAM (*4*). Marked elevations in CK is characteristic of NAM, with a mean value of 10,000 U/

**Address correspondence to:*
Dr. Sandeep Kunwar, Department of Internal Medicine, Creighton University School of Medicine, Omaha, Nebraska, 68105, USA.
E-mail: sandeepkunwar@creighton.edu

L compared to the self-limited form (*1*). NAM affects men and women equally and typically occurs in adults and the elderly (*4*).

## 2. Case Report

A 60-year-old female with past medical history of hypertension, diabetes and hyperlipidemia (treated with atorvastatin for the last 2 years), presented with proximal muscle weakness. She reported her weakness started 2 years ago mostly in the lower extremity progressing significantly for the past 6 months with recent involvement of her shoulder and arms as well. She initially had trouble getting out of her car seat, climbing steps and getting out of chair. Initially she was advised to lose weight and undergo rehabilitation. Gradually her weakness progressed to where she could barely climb one stair at a time. She was unable to place more than 3 plates in her cupboard above shoulder level due to weakness. She denied any associated muscle or joint pain, fever or chills, rashes, oral ulcers or any recent vision changes.

Physical examination revealed significant proximal upper and lower extremity muscle weakness. Muscle strength examination revealed decreased muscle bulk in the biceps and triceps. She was found to have significantly decreased strength in the hip flexors bilaterally, not capable of lifting her legs against minimal resistance from the examiner. She was unable to rise from a seated position. However, she had no findings on examination suggestive of a systemic autoimmune disorder and no cutaneous manifestations suggestive of DM.

Routine laboratory studies were normal except for a CK level of 12,387 U/L and aldolase of 48.4 U/L. Myoglobin in urine was 3,086 ng/mL. Her CK level remained relatively unchanged despite aggressive intravenous hydration and withholding her atorvastatin. Additional neurologic, serologic and musculoskeletal studies were performed. Thyroid function was normal. Nerve conduction studies and Electromyography (EMG) were consistent with an active, irritable myopathy of the proximal muscles. Given the persistent weakness and CK elevation, there was concern for possible statin associated NAM with consideration for other inflammatory myopathies such as DM and PM. Results for myositis- associated and connective tissue disease antibody including antinuclear antibody, anti-DNA ab, anti-Jo-1 antibody and Myositis panel for MI2 (Mi-2/nucleosome remodeling and deacetylase complex), ku, SRP (signal recognition particle), PL7 (threonyl) , PL-12 (alanyl), EJ (glycyl), and OJ (isoleucyl), and Jo 1 (antihistidyl-tRNA synthetase) were negative. The patient was tested for presence of novel anti-3-hydroxy-3-methylglutaryl-coenzyme A reductase (anti HMGCR) antibodies with a positive result. A muscle biopsy from left vastus lateralis was performed that showed necrotizing myopathy with minimal inflammation with prominent regeneration, necrosis, myophagocytosis, myofiber vacuolation and mild T cell cD3+ component. B cells were absent.

Given the connection between NAM and malignancy, cancer screening was reviewed. Colonoscopy, mammogram and cervical cancer screening performed in the past did not show any significant abnormalities. Computed tomography of the chest and abdomen was negative for malignancy. She was diagnosed with statin-associated NAM and started on prednisone 40 mg daily. After a month of follow up, her upper extremity strength was back but lower extremity continued to be weak. Patient was unable to perform lift off test from chair without hands. She was then started on methotrexate and azathioprine. Her repeat CK was 819 U/L and Aldolase was down to 11 U/L. Myositis specific antibodies were negative for SSA-52, SSA-60 and ribonucleic protein. Patient reported progressive improvement of her weakness to what she considers her baseline. On examination her strength was found to be 5/5 in both upper and lower extremities. Currently, after six months, the patient is on a tapering course of prednisone 30 mg/day. Her latest CK is 108 U/L.

## 3. Discussion

NAM is a recently recognized part of IIM. NAM has been associated with malignancies, HIV, antibodies to signal recognition particle, connective tissue diseases and certain medications, but it can occur in isolation (*3*). This under recognized condition was recently described in association with statin exposure. Christopher-Stine *et al*. (*5*) reviewed a cohort of patients with necrotizing myopathy on muscle biopsy and demonstrated that 16/26 patients' sera immune precipitated a pair of proteins with approximate sizes of 200 and 100 kDa. Sixty-three percent of these patients had statin exposure at some point prior to symptom onset. This antigen was later characterized as HMGCR, the pharmacologic target of statins. Further workup by this group has demonstrated that these antibodies are not present in healthy controls; in the majority of patients with DM, PM and SIBM; or in patients with statin exposure and isolated CK, myalgia, or self-limiting statin intolerance, thus suggesting they are highly specific for statin associated NAM (*5*). The novel anti-HMGCR antibody, which was discovered in 2010, is a promising diagnostic marker for statin-associated NAM (*6*). The reported sensitivity and specificity are 94.4% and 99.3% (*7*).

Patients generally present with significant proximal muscle weakness and marked elevation in CK levels, often greater than 10 times the upper limit of normal. EMG shows signs of irritable proximal myopathy, indicative of a severe muscle disease such as immune-mediated myopathy, compared to non-irritable myopathy such as a steroid induced myopathy. Muscle

biopsy has feature of prominent muscle necrosis with myofiber regeneration and minimal inflammation.

Symptoms of statin intolerance may occur at any time after commencement of treatment, with an average of 31 months in 1 series (range 0-84 months) (*1*). However, the immune-mediated muscle damage initiated in the presence of statins may be sustained long after statin cessation through persistently increased HMGCR expression in regenerating muscle fibers (*8*). However, once the immune system was activated, discontinuation of the statin at that time would not be sufficient to halt the ensuing muscle destruction. It is not likely to be related to cell mediated destruction of muscle fibers, as inflammatory cell infiltration is not a feature. it may be related to humoral factors such as cytokine or complement- mediated destruction of muscle fibers.

Currently, there are no controlled trials to guide treatment selection; thus all of the data available are from small retrospective studies or case reports. Initial treatment is generally high dose prednisone, but more aggressive immunosuppressive therapy may be needed in up to 77% of patients with this disorder, and an initial response with glucocorticoids takes 2 to 3 months (*9*). The observation that our patient improved only after addition of immunosuppressive agents suggest that NAM associated with statin use in our patient was also immune mediated. Methotrexate, azathioprine, cyclosporine, tacrolimus, rituximab, plasmapheresis, and IVIG are just a few of the immunosuppressive medications that have been reported to successfully treat anti-HMGCR and anti-SRP antibody-positive NAM (*9*). Relapses seem to be common when tapering glucocorticoids, potentially providing another reason to consider adding another immunosuppressive medication at disease onset (*10*). Patient comorbidities such as chronic infection, malignancy, chronic kidney disease, chronic liver disease, or diabetes mellitus may limit immunosuppressive choices.

In conclusion, statin use is associated with a NAM that does not respond to discontinuation of the offending agent. The main problem we have when we initially see a patient who has marked weakness and elevated CK while on statin is how to predict which patient will respond to just stopping the statin and which one will eventually require immunotherapy. We present this case with an aim to highlight that though statin-associated NAM is a relatively rare entity, it is an important consideration for the general internist in patients who continue to have CK elevation and weakness even after discontinuation of statin therapy. Early recognition of such symptoms is warranted for timely management and prevention of further complication. Awareness of this entity will help physicians who prescribe statins to take action to limit the associated morbidity.

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