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Volume 16, Number 5, 2022

Original Article

Brief Report

Letter to the Editor

Original Article

DOI: 10.5582/ddt.2022.01058

Surrounding gas composition affects the calling song development in the two-spotted cricket (*Gryllus bimaculatus*)

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SUMMARY Male crickets emit acoustic signals (*i.e.*, songs) by chirping using their forewings. Although the mechanisms and adaptive functions of these songs are well studied, knowledge about how songs develop within a generation is relatively scarce. Our previous work demonstrated a stable peak frequency at 5.7 kHz in the calling songs recorded from mature adult male crickets (*Gryllus bimaculatus*). In the present study, we monitored changes in the frequency component over time from the sexual maturity stage (early adult stage). We recorded 300 calling songs from a pool of 122 adults. The peak frequency distribution was lower and unstable (*i.e.*, greater coefficient of variance) in the early adult stage. The mean peak frequency was 4.9 kHz on day 3, but gradually converged to 5.8 kHz over the 2-week adult stage. Immature adult males (emitting immature songs) produced an appropriately tuned song with a peak frequency of 5.8 kHz in an environment of 80% helium and 20% oxygen. These results suggest that the frequency component of the calling song is acquired during the early to mid-adult stage, and may be related to sexual maturation in males. Findings from the helium substitution experiment revealed that physical resistance from surrounding gas molecules negatively affect the stability of male singing, and that muscle development and forewing hardening may contribute to the maturation of singing, suggesting that females may adaptively select sexually mature males based on song traits.

Keywords Cricket, *Gryllus*, calling, bioacoustics, development

1. Introduction

Acoustic signals are widely used for communication between members of the animal kingdom, including primates (*1,2*) , birds (*3-5*) , frogs (*6*) , and arthropods (*7-9*). In human clinical practice, abnormal acoustic signals (voice) are often found in patients with sicknesses. Such vocal abnormalities are due to physical disturbances in the vocal organs or functional disturbances in the neural circuits involved in vocalization. Furthermore, the tone of a person's speech reflects to some extent their mental health, and understanding the function, mechanism, and development of vocalization is important in achieving wellness for people.

The physical parameters that characterize acoustic signals are frequency (corresponding to pitch), amplitude (corresponding to volume), or more complex syllable patterns (*10-12*). The frequency spectrum is specific to each species (*13,14*), and closely related species produce songs with relatively different frequencies (*15*). The frequency components are continuously distributed to form a frequency spectrum, which is associated with premating isolation (*16,17*) such that females are likely to be attracted only to mating songs of the appropriate frequency, while ignoring other songs. In the two-spotted cricket *Gryllus bimaculatus*, we reported that the dominant frequency of song calling is tightly controlled and not affected by the size of the male's body or the size of the resonator (mirror and harp region of the forewing) (*9*). Based on the idea of directional hearing, this trait would be adaptive for males because songs with an inappropriate frequency may not transfer the information of the song emitter's (*i.e.*, male's) position to conspecific females. This function is especially critical in mating contexts, where females being attracted by male calling songs seek conspecific males on the basis of calling songs as a navigator. Another example of the biologic significance of frequency components is directional hearing. In crickets, a series of mechanistic studies demonstrated that the auditory system has directional hearing and can identify the direction of a sound source (*18-20*). Several physical models are proposed for

directional hearing, including amplitude, time, and phase differences between the left and right ears. Studies have demonstrated that such directionality is selectively observed near the dominant (main peak) frequency of the calling songs of crickets (*18,19*).

The detailed mechanisms by which crickets produce their specific chirping patterns are well documented in a number of studies. Theoretically, the harmonic frequency of a membrane instrument (*e.g.*, tympani) is affected by the membrane density, tension, and membrane diameter (size) (*21*). In a previous study, we found that the size (membrane area) of the 2 major sound sources (harp and mirror region of the forewings) of *G. bimaculatus* varies with the body size (*9*), suggesting that the constant frequency of cricket song is determined by other factors, such as membrane tension and density. Nonetheless, another approach to understanding the behavior (*i.e.*, the tight regulation of song frequency) is to calculate how it develops over the course of a generation (cf., Tinbergen's 4 questions). There are no reports on how the tightly regulated song frequency is acquired during the development in *G. bimaculatus*.

In the present study, we examined whether the characteristic song pattern of mature *G. bimaculatus* calling songs is age-dependent. Elucidation of the relationship between age and the song pattern may provide meaningful insights and new hypotheses for cricket ethology. By recording the mating songs of a large number of crickets, we found that adult male crickets gradually begin to emit mating songs from the day 3 after reaching adulthood, but it takes about 1-2 weeks for these mating songs to reach maximum activity. To provide a physical explanation for the song maturation, we conducted experiments in which the air was replaced by 80% helium (which has a lighter average molecular weight than air) and the songs were recorded. Although the neuromuscular development of the forewing control is likely to contribute to the song maturation, it is virtually impossible to directly manipulate these factors in *G. bimaculatus*. Hence, instead, we manipulated the molecular weight of the environmental gases that resist the movement of the forewings.

2. Materials and Methods

2.1. Crickets

Crickets were purchased from Tsukiyono Farm (Gunma, Japan) and reared at 28ºC on a 12-h light and 12-h dark cycle as previously reported (*22,23*). Food and water were provided *ad libitum.* Crickets were separated in plastic containers (1 cricket per container) at the last nymphal stage and recorded on the day of the last molt (designated as the first day of the adult stage). All crickets were maintained at 28°C. Three batches of crickets were purchased for this study. For quality control purposes, we checked the survival rates of the 3 batches and found no difference in survival rates among batches (Supplementary Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=120*).

2.2. Cricket song recordings

Cricket songs were recorded using a microphone (F-112, Sony Inc., Tokyo, Japan) connected to a linear pulse-code modulation (PCM) recorder (PCM-D100, Sony Inc.). The sampling rate was 48,000 Hz and the data were saved as uncompressed 16-bit waveform files. Crickets that emerged as adults on different days were recorded in a quiet room under white fluorescent light from January 23 to March 12, 2017. A sound recorder was placed in front of several cups containing crickets to record the crickets' songs. The experimenter sat in the room and waited for the crickets to chirp. When a cricket chirped, the experimenter recorded the cricket's identification (ID) number and the date and time it was recorded. If more than one cricket chirped at the same time, the experimenter rattled the container to silence them and then waited for each to chirp independently. Crickets whose songs were recorded for the day were transferred to an incubator (so that they would not be recorded again later that day). Recordings were made daily (3-6 hours per day), and chirps were recorded for 63 (52%) of the 122 adult males. The recorded audio files were divided into chunks (usually 5-10 seconds) and processed for later analysis. For this study, we used the calling songs of males during their first 30 days as adults, bringing the total number of recorded songs to 300 songs. The number of recordings at each age (including recordings of males more than 30 days after becoming adults) is shown in Figure 1C.

2.3. Computing environment

Songs were analyzed on a Macbook Pro (Retina, 15-inch, Mid 2015, OS X ver. 10.12.6, Apple Inc., Cupertino, California, USA) with R ver. 3.3.2, the R packages 'seewave' (ver. 2.0.5) and 'tuneR' (ver. 1.3.2), and Audacity version 2.1.1 (sound analysis software for Mac OS X; *http://audacityteam.org/*) was also used in this study.

2.4. Calculation of peak frequencies of calling songs

The recorded wave files were loaded into R using the 'seewave' package and a band-pass frequency filter (low-frequency cut-off 0.5 kHz, high-frequency cut-off 20 kHz) was applied. To obtain the peak frequency of the calling songs, the spectrum of each call was analyzed using the "spec" function of the package, which returned a value for the frequency and a corresponding value for the amplitude. The Nadaraya-Watson kernel regression estimation method was used to smooth the obtained data, and the frequency with the highest amplitude was taken

as the peak frequency. This method yielded peak values comparable to those obtained with Audacity (using 'Plot Spectrum' function, AM pers. obs.).

2.5. Figure drawing

We used R ver. 3.3.2 to draw figures shown in this paper. Details are provided in the figure captions. The moving median calculation was applied to some of the longitudinal data for smoothing. Moving medians (or moving averages) are commonly used to visualize general trends in fluctuating time series data, but for the sake of data transparency, we also included unsmoothed data in these figures.

2.6. Helium-substitution experiment

A mixture of 80% helium and 20% oxygen (Manyusha, Tokyo, Japan) was used. First, crickets were placed in a plastic bag filled with room air (1 cricket/bag) and their chirps were recorded. The crickets were then transferred to a plastic bag containing the helium-oxygen mixture (1 cricket/bag) and their chirps were recorded. In this study, we used crickets that were 5 days old (post-adult age).

3. Results

3.1. Post-adult life history of *G. bimaculatus* used in this study

The maximum lifespan of *G. bimaculatus* used in this study (days counted from the last molt date) was 68 days, but more than 60% of the crickets died within 30 days (Figure 1A). Forewing coloring (lacquering) occurred very early after the final molt, with forewings appearing for the first time (tender) on the day of the final molt and gradually lacquering out after 3 days (Figure 1B). The number of records (reflecting both chirping frequency and survival of male crickets) peaked at day 4 (Figure 1C), and after normalization for survival, peaked between days 10 and 20 (Figure 1D). After the peak, the number of records (both absolute and normalized) was overall decreased toward the latter half of the adult stage (Figures 1C and 1D). This observation suggests that male sexual activity peaks around the second or third week of adult life. On the basis of these results, we decided to track changes in vocalizations within 30 days of the last molt. The following analysis covers 300 calling songs recorded from 63 adult males.

3.2. Maturation of calling song

In this study, crickets began to chirp on day 2 (1 recording was obtained on day 2, 8 on day 3, 22 on day 4, and 17 on day 5 (Figure 1C)). The frequency peaks were lower in the early adult stage than in the later adult stage (Figure 2). The median peak frequency of the calling songs was around 4.9 kHz on day 3, and reached 5.8 kHz on day 17 (Figure 2). Also, the frequency varied minimally around week 3 (Figure 2). After day 22, there was a slight downward trend in peak frequency and an increasing trend in the variability (Figure 2). We further tracked a representative individual over time, finding a shift in the peak frequency between days 5 and 8 for the individual. The representative male shown in Figure 3 chirped with a peak below 5.0 kHz on days 4 and 5,

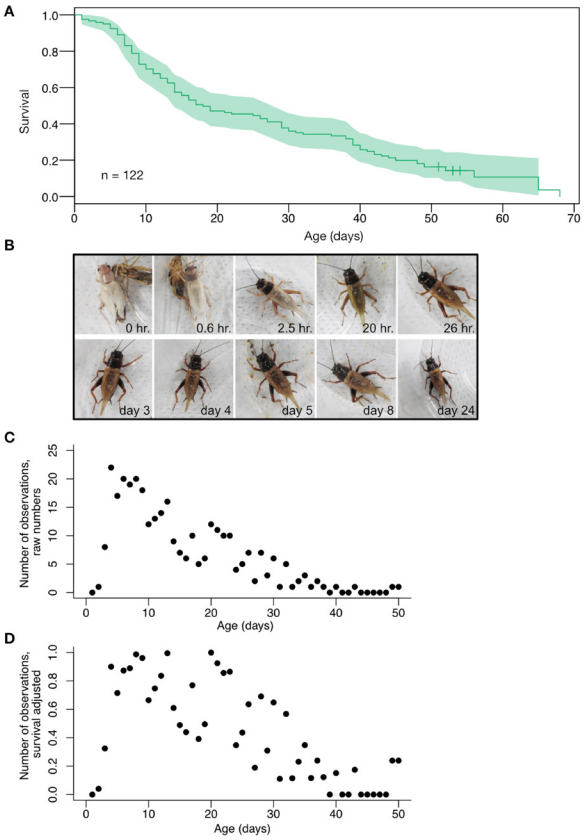

Figure 1. Survival and wing development of the crickets used in this study. (A) Survival curve of the crickets (*G. bimaculatus*) used in this study. The horizontal axis shows male post-adult age (counted from day of final molt), and the vertical axis shows survival. The green solid line shows the Kaplan-Meier curve of the crickets. Ticks indicate that one or more of the crickets were censored at that time-point (due to termination of experiment, or escaping). Green hatched area indicates 95% confidence intervals of the survival curve. This figure represents pooled survival data of 3 independent batches of crickets (n = 122). Survival curves separately drawn for each batch are shown in Supplementary Figure S1. **(B)** Wing hardening of a male cricket after its final molt. Photos of a representative male *G. bimaculatus* were obtained over time. The time at the lower right corner of each photo indicates the interval between the final molt and the time at which the photo was obtained. The peak frequency of the calling song of this male was 4.9 kHz on day 4, and 5.3 kHz on day 24. **(C)** Raw numbers of successful recordings are plotted in the chart. The x-axis represents age (days), and the y-axis represents the number of recordings at each age. **(D)** The number of recordings adjusted by survival is shown in the chart for each age. Because the raw recording numbers represent both singing activity and survival on each day, we used the survival information (shown in the Figure S1) to normalize the data to demonstrate the singing activity per cricket at each age. The maximum activity was set to be 1 (y-axis). The x-axis represents the age (days).

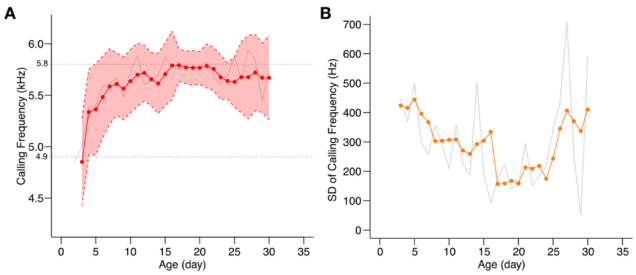

Figure 2. Change in peak frequency of calling songs observed at the population level. (A) Time-series data of the peak frequency of cricket calling songs. This chart represents 300 recorded calling songs from 63 males. The vertical axis shows peak frequency value of calling songs (kHz), and the horizontal axis shows cricket post-adult age (day of final molt = day 1). Red plots (connected by solid red lines) represent 3-day moving medians. The red-hatched region with red dashed lines indicates 3-day moving standard deviations. The number of samples (*i.e.*, recordings) obtained each day is shown in Figure 1C. The grey lines behind the red solid lines represent unsmoothed (*i.e.*, original time-series data of median peak frequency of calling songs. **(B)** Plot of standard deviation of peak frequency. The vertical axis indicates standard deviation of the peak frequency value (Hz), and the horizontal axis indicates the post-adult age of crickets. The red plots connected by red solid lines represent 3-day moving medians. The original, unsmoothed data are also shown in the chart with grey lines. The number of samples (*i.e.*, recordings) obtained each day is shown in Figure 1C.

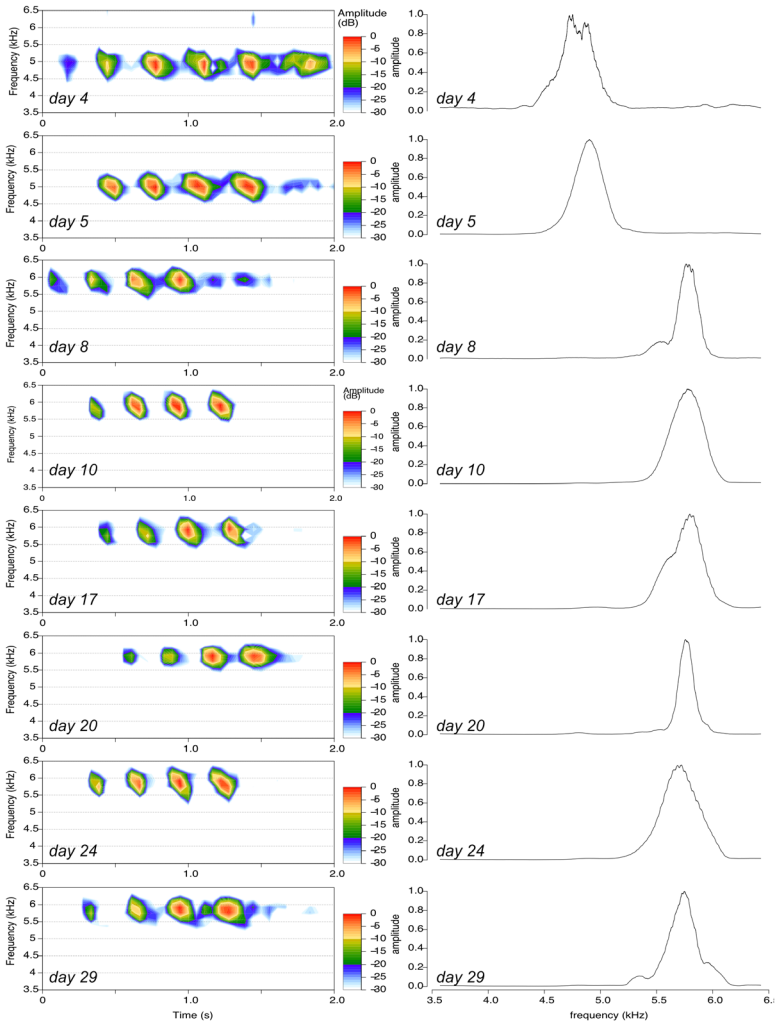

Figure 3. Change in peak frequency of calling songs of a representative male. Left panels are spectrograms of calling songs from a representative male that were recorded on different days. A chirp in each song is shown in the figure. The horizontal axis indicates time (seconds), and the vertical axis indicates frequency value (kHz). Color indicates relative amplitude of the frequency component as shown in scales on the right side of each chart. Figures were drawn by the 'spectro' function of the Seewave package running on R. The male ages for each chart are indicated at the lower left corner of each chart. Right panels demonstrate the distribution of the frequency components. The horizontal axis shows frequency values (kHz), and the vertical axis indicates the relative amplitude of each component. The amplitude values were normalized such that the maximum values = 1.0. The date at the lower left corner on each chart indicates male age. The distribution was calculated from a set of calling songs on each day (typically 10 seconds of recorded calling songs), using the 'spec' function of the Seewave package. Data were then smoothed for presentation purposes.

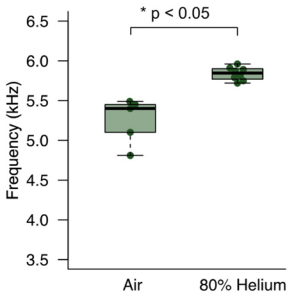

Figure 4. Change in peak frequency of calling songs of young males in a helium-substituted environment. The vertical axis shows the peak frequency value of the calling song. The labels below the horizontal axis indicate the group: 'Air' represents the calling songs recorded in normal room air conditions, and '80% Helium' represents calling songs recorded the helium-substituted condition. Males on day 5 were used for this experiment. Data from multiple different males were pooled (not all males emitted calling songs in both conditions, as sexual activity is low in early adult phase), and used for the analysis. Five samples were obtained for the normal condition and 8 were obtained for the helium-substituted condition. The peak frequency differed significantly between the 2 groups ($p < 0.05$ in Wilcoxon rank sum test).

and a peak around 5.8 kHz from day 8, a level that was maintained thereafter (Figure 3).

3.3. Effect of physical resistance on the peak frequency

The above finding that immature songs contain a low frequency peak indicates that the forewing membrane vibrates more slowly in immature males. We hypothesized that this was because some physical factor was preventing proper movement of the forewings. If this hypothesis is correct, then modifying the physical parameters that affect forewing movement should modify the peak frequency value of the chirp to an appropriate value. Thus, we tested whether crickets produce chirps with the proper pitch (peak frequency of 5.8 kHz) by reducing the physical resistance of the surrounding gas molecules. As shown in Figure 4, the chirps of young crickets (day 5) recorded in a helium-substituted environment (80% helium, 20% oxygen, average molecular weight (ignoring water content) = 9.6) were significantly higher than those recorded under normal air (Figure 4). The average frequency value of the songs recorded under helium replacement conditions was 5.8 kHz (Figure 4), which is comparable to that of the mature calling songs (see Figure 2A). This finding supports the idea that physical resistance from surrounding gas molecules affects the pitch regulation of cricket chirps, resulting in lower and more variable frequency peaks in the early adult male chirps.

4. Discussion

The present study demonstrated the development of song pitch in male calling songs during the post-adult maturation phase of *G. bimaculatus*, which reveals developmental aspects of our previous report that the frequency value of calling songs is robustly regulated during the late adult (7-14 days after final molt) phase (*9*). Female ears may be tuned to a certain pitch in orthopterans (*7,18-20*), suggesting that songs with improper pitches are ignored (or cannot be located) by females and thus have little reproductive contribution. Why then do immature males bother to sing if their songs will not be located? A possible explanation is that they learn to sing by producing sounds at an early stage (*e.g.*, it may require feedback from their auditory system). Crickets are capable of modifying their song frequencies by auditory feedback systems (*24*). From this perspective, an interesting question to address in future studies is whether interfering with the learning process (*e.g.*, damaging their auditory organs) disrupts song development.

Another explanation from an ethologic aspect is that songs from immature males may act as a decoy to predators. One of the reproductive costs of cricket songs is that they increase the predation risk (*25*). If the songs attract predators and only sexually mature males sing (and immature males keep silent), the predation risk should be biased toward older and sexually mature males. Being older means that they have survived several life-threatening risks, such as infection. Therefore, it seems advantageous that young, sexually immature males would sing even though their songs do not attract conspecific females, as they can act as decoys to protect more fit and sexually mature males from predators.

What molecular and physiologic/biophysical mechanisms explain song development? The sound producing mechanism in crickets demonstrated in previous studies (*8,24*) suggests that muscle development, tegmina condition (*e.g.*, moisture content or tension), developmental state of the neural circuit, and other factors affect cricket song frequency. In a simple biophysical model, the parameters that determine the vibration frequency of membrane instruments are density, tension, and size (diameter). Also, the shape of the membrane and how the initial vibration is given could affect the final vibration pattern. In the song development demonstrated in this study, a decrease in moisture content (*i.e.*, sclerotization) of the forewings may help to stabilize vibration and explains the robust song pitch at 5.8 kHz. The helium substitution experiment in this study demonstrates that physical resistance from surrounding gas molecules negatively affects the stabilization of song pitch. As a future study, vibratory properties of forewings could be observed using a laser Doppler vibrometer either in normal air or in a helium-substituted condition to further confirm this speculation.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Original Article

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Generic selection criteria for safety and patient benefit [XI]: Usability scores of brand-name and generic tapes containing sodium diclofenac by questionnaire survey

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SUMMARY The aim of this study was to evaluate patient satisfaction and usability scores of different tape treatments and examine the scores in relation to the mechanical characteristics of the tape formulation. A questionnaire for the assessment of comfort level and satisfaction with two brand-name (Naboal, Voltaren) and four generic tapes (Yutoku, Teikoku, Rakool, Towa) containing sodium diclofenac was developed and then applied to 12 healthy volunteers. Results showed that Voltaren was difficult to apply to the skin and easier to peel off the skin than Naboal ($p < 0.01$). Moreover, Rakool was easier to peel than Naboal ($p < 0.05$). The mechanically measured peeling force was associated with pain during peeling off ($r = –0.65$), and the measured value of bending rigidity was associated with ease of peeling off ($r = –0.97$). The knowledge obtained regarding the influence of pharmaceutical properties on the degree of satisfaction with and usability of different tape formulations may be useful for supporting the selection of generic tapes tailored to individual needs or pharmacist preferences, and thus improve treatment adherence and clinical outcomes.

Keywords Brand-name drug, generic drug, tape, satisfaction research, usability

1. Introduction

In recent years, the active use of generic drugs has been promoted for the purpose of reducing medical costs, and the volume share of generics in Japan is currently 79.0% (*1*), with the Ministry of Health, Labor and Welfare requesting an even higher share of over 80%. In contrast, according to a survey of 870 insurance pharmacies conducted by the Central Social Insurance Medical Council, the most common reason given for returning to brand-name drugs ($n = 585$) from generics was that generics did not suit the patient's needs (30%), suggesting that generics should be selected based on patient comfort and ease of use in order to further promote their use (*2*).

The most common reason for switching from generic to brand-name drugs was "ease of peeling during motion", followed by "comfortability of applying" and "difficulty of applying", indicating that the difference in feel from brand-name drugs is a major issue (*3*). Pharmacists are then required to take into account the characteristics of each product, select the one that best suits the patient's needs, and explain the product to the patient. However, there have been few reports on the usability characteristics that serve as selection criteria for various formulations (*4*,*5*), and they have not been fully utilized in the medical field. Although some studies have predicted usability based on physicochemical measurements (*6*-*9*), few have investigated the relationship between actual usability and physicochemical measurements.

In the present study, we evaluated the usability of diclofenac sodium-containing tape formulations of brand-name and generic drugs through the use of a questionnaire survey. In addition, we analyzed the correlation between physicochemical properties of the drug products and evaluation results of their physicochemical properties (*10*), the latter of which we have previously reported.

2. Materials and Methods

2.1. Materials

Six different diclofenac sodium tape (7 × 10 cm)

products were purchased for evaluation: two brand-name drugs, Naboal® tape 15 mg (Hisamitsu Pharmaceutical Co., Inc., Tokyo, Japan) and Voltaren® tape 15 mg (Dojin Iyaku-kako Co., Ltd., Tokyo, Japan); and four generic drugs: diclofenac sodium tape 15 mg "Yutoku" (Yutoku Pharmaceutical Ind. Co., Ltd., Saga, Japan), diclofenac sodium tape 15 mg "Teikoku" (Teikoku Seiyaku Co., Ltd., Kagawa, Japan), diclofenac sodium tape 15 mg "Rakool" (Mitomo Yakuhin Co., Ltd., Tokyo, Japan), and diclofenac sodium tape 15 mg "Towa" (Towa Pharmaceutical Co., Ltd., Osaka, Japan). Table 1 shows the classification, product name, abbreviated name, company name, and lot number of each diclofenac sodium tape used in this study.

2.2. Usability survey

Subjects were 12 individuals (mean age 23 ± 1.5 years, all female) who gave consent to participate in the study. Two brand-name and four generic formulations of the target drugs (Table 2) were assigned A - F and blinded. Subjects applied one tape agent symmetrically to each

Table 1. Product name, abbreviated name, company name, and lot number of the drugs used in this study

Class	Product name	Abbreviated name	Company name	Lot number
BN	Naboal® tape 15 mg	Naboal	Hisamitsu Pharmaceutical Co., Inc.	50402
BN	Voltaren® tape 15 mg	Voltaren	Dojin Iyaku-kako Co., Ltd.	40420
GE	Diclofenac sodium tape 15 mg "Yutoku"	Yutoku	Yutoku Pharmaceutical Ind. Co., Ltd.	5C150
GE	Diclofenac sodium tape 15 mg ""Teikoku"	Teikoku	Teikoku Seiyaku Co., Ltd.	4J150
GE	Diclofenac sodium tape 15 mg "Rakool"	Rakool	Mitomo Yakuhin Co., Ltd.	B191S
GE	Diclofenac sodium tape 15 mg "Towa"	Towa	Towa Pharmaceutical Co., Ltd.	A102

BN: Brand-name drug; GE: Generic drug.

Table 2. Description of questionnaire for the evaluation of the degree of satisfaction with various tapes

Tapes	Tape A	Tape (B • C • D • E • F)	
When applying			
(1) Ease of application to the skin	1. Very difficult to apply 2. Difficult to apply 3. Easy to apply 4. Very easy to apply	1. Very difficult to apply 2. Difficult to apply 3. Easy to apply 4. Very easy to apply	1. More difficult to apply than A 2. Slightly more difficult to apply than A 3. Same as A 4. Slightly easier to apply than A 5. Easier to apply than A
While applying			
(2) Ease of moving of joint with tape affixed	1. Difficult to move 2. Slightly difficult to move 3. Slightly easier to move 4. Easy to move	1. Difficult to move 2. Slightly difficult to move 3. Slightly easier to move 4. Easy to move	1. More difficult to move than A 2. Slightly more difficult to move than A 3. Same as A 4. Slightly easier to move than A 5. Easier to move than A
(3) Cooling sensation	1. Too cold, too painful 2. Very cold 3. Cold 4. Not too cold 5. Not cold	1. Too cold, too painful 2. Very cold 3. Cold 4. Not too cold 5. Not cold	1. Colder than A 2. Slightly colder than A 3. Same as A 4. Slightly warmer than A 5. Not colder than A
(4) Peeling resistance	1. Most of the tape peels off 2. Part of tape peels off 3. No peel off at all	1. Most of the tape peels off 2. Part of tape peels off 3. No peel off at all	1. Easier to peel than A 2. Slightly easier to peel than A 3. Same as A 4. Slightly harder to peel than A 5. Harder to peel than A
When peeling			
(5) Ease of peeling off	1. Very difficult to peel 2. Difficult to peel 3. Easy to peel 4. Very easy to peel	1. Very difficult to peel 2. Difficult to peel 3. Easy to peel 4. Very easy to peel	1. More difficult to peel than A 2. Slightly more difficult peel to apply than A 3. Same as A 4. Slightly easier to peel than A 5. Easier to peel than A
(6) Pain during peeling off	1. Very painful 2. Painful 3. Slightly painful 4. Painless	1. Very painful 2. Painful 3. Slightly painful 4. Painless	1. More painful than A 2. Slightly more painful than A 3. Same as A 4. Slightly less painful than A 5. Less painful than A
(7) Skin stuffiness after peeling off	1. Very moist 2. Moist 3. Normal	1. Very moist 2. Moist 3. Normal	1. More moist than A 2. Slightly more moist than A 3. Same as A 4. Slightly less moist than A 5. Less moist than A
(8) Total score	1. Bad 2. Not good 3. Good 4. Very good	1. Bad 2. Not good 3. Good 4. Very good	1. Worse than A 2. Slightly worse than A 3. Same as A 4. Slightly better than A 5. Better than A

shoulder or knee (with tape A applied on one side and one of tapes B to F applied on the other side). The application site was always the same on the left and right sides of the body. After the application time of 12 h, the tapes were peeled off one side at a time. "Ease of application to the skin", "ease of moving joints with tape affixed", "cooling sensation", "peeling resistance", "ease of peeling off", "pain during peeling off", "skin stuffiness after peeling off", "total score" the results were evaluated using the questionnaire shown in Table 2. In addition, as an overall evaluation, a rating based on a 3 - 5 scale score (hereinafter referred to as "absolute scale") was performed. Usability evaluation was also performed in conjunction with a relative scale compared to the brand-name drug, Naboal tape. This study was conducted under the supervision of a physician and with the approval of the Ethics Review Committee within Meiji Pharmaceutical University (Reception No. 2855).

2.3. Relationship between physicochemical measurements and usability scores

The correlation coefficients between our previously reported evaluation results of the physicochemical properties (*10*) of the formulations and the questionnaire-based usability scores for each formulation obtained in the present study were calculated using the Statcel 3 statistical software (*11*).

2.4. Statistical analyses

For each result, the values were compared using *Dunnett's* test. A *p*-value of 0.05 (marked with * in figures) or 0.01 (marked with **) was regarded as significant.

3. Results

3.1. Questionnaire survey results for usability

The results of the "ease of application" evaluation of each formulation based on the questionnaire survey are shown in Figure 1 as the (A) absolute scale and (B) relative scale. The brand-name Voltaren was perceived as "difficult to apply" in absolute and relative evaluation compared to the brand-name Naboal, with a significant difference ($p < 0.01$) in the absolute evaluation results. On the other hand, for the four generic formulations tested (Yutoku, Teikoku, Rakool, and Towa), there were no significant differences from the brand-name Naboal.

The evaluation results of the "peeling resistance" of each formulation are shown in Figure 2. Compared to the brand-name Naboal, the five other formulations tended to be perceived as easy to peeling in both absolute and relative evaluation. In particular, Voltaren had the lowest scores in both absolute and relative evaluation, suggesting that patients find it easier to peel off Voltaren than other formulations.

First, the evaluation results of the "ease of peeling off" of each product are shown in Figure 3. Compared to the brand-name Naboal, the five other formulations tended to be perceived as "easy to peel" in both absolute and relative evaluation. In particular, Rakool showed high evaluation scores in both absolute and relative evaluation, and there was a significant difference ($p < 0.05$) in absolute assessment results between Naboal and Rakool.

Next, to examine what properties of a formulation contribute to the patient's overall evaluation of the formulation, we examined the overall evaluation score of each formulation (Figure 4). Voltaren tended to have a lower evaluation score than Naboal. On the other hand, no significant differences were observed among the four generic formulations. In addition, Table 3 shows the correlation coefficients between the usability evaluation scores from the questionnaire survey and the overall evaluation for each formulation. A formulation's "ease of application" ($r = 0.81$), "peeling resistance" ($r = 0.67$), and not giving patients a "cooling sensation" ($r = –0.86$) may lead to a high evaluation of the formulation.

3.2. Relationship between physicochemical measurements and usability scores

Table 4 shows the correlation coefficients between the experimental measurements (based on previously reported physicochemical properties of the formulations) (*8*) and the questionnaire survey results (evaluation scores of usability for each formulation).

First, the "elongatedness" of each formulation was calculated as the ratio of the length of the maximally elongated formulation divided by the length of the original formulation when the short end of each formulation was fixed to the test stand, and the other end was pulled with a 300 g suspended weight. As shown in Table 4, measured "elongatedness" had positive correlation trends with evaluation scores of "ease of application" ($r = 0.55$) and "peeling resistance" ($r = 0.63$), and a negative correlation trend with the evaluation score of "pain during peeling off" ($r = –0.69$) and "cooling sensation" ($r = –0.66$). We also examined scatter plots examining the relationship between the "elongatedness" and the "ease of movement" evaluation scores from the questionnaire survey and found no correlation ($r = 0.16$).

The ball tack adhesive force was measured according to the inclined ball tack test method (*12*) listed in the Japanese Pharmacopoeia, Eighteenth Edition. That is, a stainless ball was rolled onto a formulation fixed with the adhesive side up on an inclined plate with an angle of 30°, and the weight (g) of the largest ball that stopped on the adhesive side was used as the measurement value. As shown in Table 4, positive correlations were identified between the "adhesive force" measured by the ball tack test and the "cooling sensation" evaluation score ($r =$

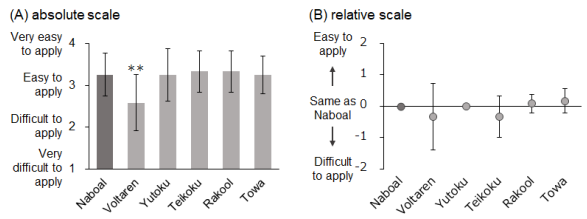

Figure 1. Satisfaction with the ease of application of each product to the skin. Results are shown as mean ± S.D. (n = 12) for the **(A)** absolute scale (**: $p < 0.01$; *Dunnett's* test *vs.* Naboal) and **(B)** relative scale to compare with the brand-name drug, Naboal.

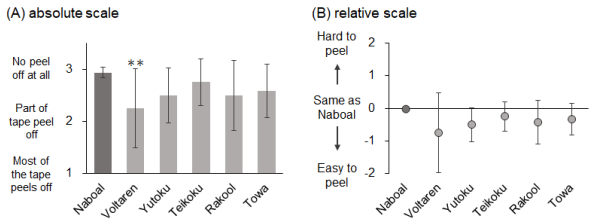

Figure 2. Evaluation results of peeling resistance of each formulation based on questionnaire survey. Results are shown as mean ± S.D. (n = 12) for the **(A)** absolute scale (**: $p < 0.01$; *Dunnett's* test *vs.* Naboal) and **(B)** relative scale to compare with the brand-name drug, Naboal.

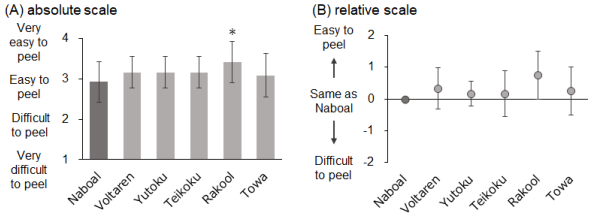

Figure 3. Satisfaction with the ease of peeling off of each product. Results are shown as mean ± S.D. (n = 12) for the **(A)** absolute scale (**: $p < 0.01$; *Dunnett's* test *vs.* Naboal) and **(B)** relative scale to compare with the brand-name drug, Naboal.

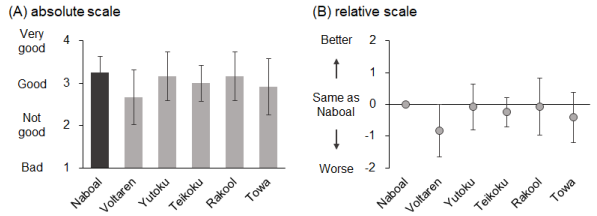

Figure 4. Total evaluation scores of each product. Results are shown as mean ± S.D. (n = 12) for the **(A)** absolute scale (*: $p < 0.05$, **: $p < 0.01$; *Dunnett's* test *vs*. Naboal) and **(B)** relative scale to compare with the brand-name drug, Naboal.

Table 3. Correlation coefficients between usability evaluation scores and total score based on questionnaire surveys for tape preparations

	Ease of application	Peeling resistance	Ease of peeling off	Pain during peeling off	Ease of movement	Cooling sensation	Skin stuffiness after peeling off
Total score	**0.81**	**0.67**	-0.05	-0.41	-0.14	**-0.86**	0.27

Numbers in bold font indicate weak correlations, whereas numbers in bold font and with underline indicate strong correlations.

Table 4. Correlation coefficients between usability evaluation scores and physical property measurements based on questionnaire surveys for tape preparations

Physicochemical properties	Patient satisfaction and usability scores in absolute scale (parentheses are relative scale)							
	Ease of application	Peeling resistance	Ease of peeling off	Pain during peeling off	Ease of movement	Cooling sensation	Skin stuffiness after peeling off	Total score
Elongatedness	0.55	**0.63**	-0.32	**-0.69**	0.16	**-0.66**	0.07	0.66
	(-0.11)	**(0.52)**	(-0.58)	**(-0.69)**	(0.49)	**(-0.65)**	(-0.70)	(-0.18)
Adhesive force	-0.32	-0.22	-0.29	0.19	-0.14	**0.54**	0.42	-0.14
	(0.59)	(-0.11)	(0.01)	(0.25)	(-0.44)	**(0.77)**	(0.04)	(-0.34)
Peel force	0.65	0.51	0.15	-0.47	-0.15	**-0.74**	-0.37	0.42
(after 0 h)	(-0.28)	(0.43)	(-0.13)	(-0.34)	(0.50)	**(-0.74)**	(-0.36)	(0.24)
Peel force	0.39	0.36	-0.15	**-0.65**	0.21	-0.46	-0.24	0.37
(after 24 h)	(-0.35)	(0.23)	(-0.50)	**(-0.75)**	(0.28)	(-0.59)	(-0.55)	(0.13)
Inflexibility	0.02	0.34	**-0.68**	**-0.74**	-0.08	0.12	-0.10	-0.18
(horizontal)	(-0.38)	(0.23)	**(-0.87)**	**(-0.93)**	(-0.37)	(0.07)	(-0.35)	(-0.12)
Inflexibility	0.02	**0.58**	**-0.97**	**-0.53**	0.06	0.00	**0.68**	0.24
(vertical)	(0.13)	**(0.54)**	**(-0.89)**	**(-0.63)**	(-0.05)	(0.13)	**(-0.44)**	(-0.79)
Water-vapor permeability	0.03	**0.53**	**-0.79**	-0.34	-0.46	0.14	0.45	-0.11
	(0.19)	**(0.56)**	**(-0.58)**	(-0.27)	(-0.28)	(0.40)	(-0.10)	(-0.62)

Numbers in bold font indicate weak correlations, whereas numbers in bold font and with underline indicate strong correlations. The numbers in the table represent absolute scale, and the numbers in parentheses represent relative scale.

0.54) as well as the "skin stuffiness after peeling off" evaluation score ($r = 0.42$).

Next, scatter plots representing the relationship between the "peel force" (at 0 or 24 hours) and the "pain during peeling off" evaluation scores from a questionnaire survey were examined. That is, the peel force of each formulation was measured by applying each formulation (cut to 30 mm × 52 mm) to a test plate wrapped with a polymer (ethylene-vinyl acetate; EVA) film, at a specified time (0 h or 24 h after application), using a digital force gauge ZTA-50N (IMADA Co., Ltd., Aichi, Japan) at a speed of 60 mm/min, and peeled off at an angle of 90 degrees. As shown in Table 4, there was a negative correlation between the "peel force" (after 0 h and 24 h) and the "pain during peeling off" score from the questionnaire (r = –0.47 and –0.65, respectively).

The rigidity and softness of each formulation was measured by a partially modified version of the 6.7 Rigid-softness cantilever method (*13*) from the Japanese Industrial Standards (JIS) "JIS L 1913:2010 General Nonwoven Fabrics Testing Methods". Briefly, the tape material (20 mm × 100 mm) was cut with the adhesive side facing upward and placed in a device with a horizontal portion and a 30° sloping portion aligned with the edge of the horizontal portion. The tape was then moved gradually toward the sloping portion and the distance traveled (mm) until the edge of the tape agent touched the slope was measured. The higher the rigidity measurement, the stiffer and harder the tape agent is to bend. As shown in Table 4, negative correlation trends were observed between stiffness (horizontal and vertical) and the "ease of peeling off" evaluation score ($r = –0.68$ and –0.97, respectively), as well as between stiffness (horizontal and vertical) and the "pain during peeling off" score ($r = –0.74$ and –0.53, respectively).

The "water-vapor permeability" of each formulation was measured by covering the opening of a 20 mL triangular flask containing 10 mL purified water with a formulation cut into a 25 mm diameter circle and measuring the weight after 24 h at 25°C and 55% relative humidity to determine the amount of purified water loss. A positive correlation ($r = 0.45$) was found between the measured "water-vapor permeability" and the "skin stuffiness after peeling off" evaluation score (Table 4).

4. Discussion

Fujino reported that the most common reason for switching from a generic to a brand-name tape was "ease of peeling during motion", followed by "comfortability of applying" and "difficulty of applying" (*3*). Therefore, we conducted a questionnaire survey on the usability of each tape formulation and evaluated it against the brand-name Naboal.

Figure 1 shows that the brand-name Voltaren was significantly ($p < 0.01$) "harder to apply" than the brand-name Naboal. This result suggests that patients who have difficulty applying the brand-name Voltaren may find it easier to apply the brand-name Naboal or one of the generic products, Yutoku, Teikoku, Rakool, or Towa. Switching from Voltaren to Naboal or one of the generic products may improve ease of application and reduce or eliminate the problems associated with using Voltaren.

In Figure 2, there was a significant difference ($p < 0.01$) between Voltaren and Naboal in the absolute evaluation results. This result suggested that patients using Voltaren who have problems with easy peeling may be more likely to switch to the brand-name Naboal or the generic Teikoku to reduce peeling and increase their satisfaction with the tape.

In addition, a questionnaire survey was conducted on other potentially problematic sensations: "ease of peeling", "pain during peeling off", "ease of moving joint with tape affixed", "cooling sensation", and "skin stuffiness after peeling off".

The evaluation results of the "ease of peeling" of each product are shown in Figure 3. There was a significant difference ($p < 0.05$) in absolute assessment results between the generic Rakool and the brand-name Naboal. Based on these results, patients who find Naboal "hard to peel off" could be recommended to switch to the generic Rakool.

Next, the "pain during peeling off" evaluation results of each formulation were examined. The results showed that there was no significant difference between the brand-name Naboal and the generics Yutoku, Teikoku, and Towa. On the other hand, Voltaren and Rakool tended to be perceived as less painful to peel off in both absolute and relative evaluations.

The evaluation results of "ease of moving joint with tape affixed" showed no significant difference between Voltaren, Yutoku, Teikoku, and Rakool compared to Naboal. Towa, on the other hand, tended to be it difficult to move in both absolute and relative evaluations.

Next, we examined the evaluation results of "cooling sensation" while each preparation was applied. There were no significant differences between Voltaren, Yutoku, Teikoku, and Rakool compared to the brand-name Naboal.

The results of the "skin stuffiness" evaluation after each formulation was peeled off show that, compared to Naboal, all five other formulations felt "moist" in absolute evaluation. On the other hand, the relative evaluation showed that patients felt that all five formulations were similar to "moist" when compared to Naboal. And the results of the "stuffiness" evaluation showed a large standard deviation, which indicates high variability compared to the other evaluation items. This may be because the "stuffiness" condition is affected by various factors such as the temperature and humidity on the day as well as the patient's health. Many patients also commented that it was difficult to assess whether the skin surface was moist or not.

Next, Table 3 shows the correlation coefficients

between the usability evaluation scores from the questionnaire survey and the overall evaluation for each formulation. "Ease of application" (r = 0.81), "peeling resistance" (r = 0.67), and "cooling sensation" (r = –0.86) were considered to have a significant influence on the overall evaluation score. In other words, the easy to apply and the hard to peel the formulation, the higher the overall evaluation tended to be. Moreover, the more difficult it was to peel off a formulation after application, the higher the overall evaluation tended to be. That is, a formulation's ease of application, capacity to provide patients with a cooling sensation, and ability to remain adhered to the skin after application may lead to a high evaluation of the formulation.

Table 4 shows the correlation coefficients between usability evaluation scores and physical property measurements based on questionnaire surveys for tape preparations.

First, regarding the elongation of the formulation, the easier it is to apply to non-flat areas, such as joints, and the more difficult it is to peel off because the formulation expands and contracts with the movement of the joint during application. Thus, upon peeling off a tape formulation, the peeling force stretches the tape formulation, thus reducing the force spent on peeling, which increases the peeling force and resulting in pain. We also examined scatter plots examining the relationship between the measured elongation and the "ease of movement" evaluation scores from the questionnaire survey and found no correlation (r = 0.16). This finding suggests that the degree of elongation is not an indicator of the ease of movement of the joints.

Next, scatter plots representing the relationship between the "adhesive force" determined by the ball tack test and the "cooling sensation" evaluation scores or "skin stuffiness after peeling off" evaluation scores from a questionnaire survey were examined. That is, adhesive force in the ball tack test was evaluated by stopping the force of the ball rolling over the adherend of the tape agent by its adhesive strength, and is known to be affected by the amount of "wetting" of the adhesive to the surface of the adherend (adhesion of the interface) (*10*). Therefore, a highly-adhesive formulation increases interface adhesion between the skin and the formulation, resulting in skin wetting. Furthermore, the high adhesiveness of the interface may facilitate in the permeation of ingredients from the formulation to the skin, allowing menthol (the main ingredient that causes a cooling sensation) to penetrate the skin and resulting in a stronger cooling sensation. These findings support that formulations with high peel force values require a large amount of force to peel the tape from the skin, and that the skin is pulled while still adhering to the adhesive during peeling, resulting in a strong sensation of pain. Therefore, peel force measurement may be used as an indicator of "pain during peeling off". Thus, considering that a formulation is pulled in a bent state when peeled off, the peel-off may increase pain doe to the bending rigidity of the formulation.

In addition, the negative correlation trends were observed between "inflexibility" (horizontal and vertical) and the "ease of peeling off" evaluation score, as well as between "inflexibility" (horizontal and vertical) and the "pain during peeling off" score. This suggests that the inflexibility of the support makes it difficult to peel off and causes pain when peeling off.

Furthermore, a high negative correlation trend was observed between "water-vapor permeability" and "ease of peeling off" evaluation scores. This suggested that the lower the water-vapor permeability, the easier it was to peel off. The reason for this was thought to be that moisture accumulated due to steam between the skin and the tape, which made it easier to peel off.

These results of this study may be useful as a basis for health care providers in selecting a suitable formulation and in selecting a generic drug according to the desired use of each individual patient. The findings also suggest that the physicochemical measurements of a formulation may predict the feel of the product. However, while these are useful findings, they are limited by including only a small number of young women. This does not ensure these interventions would have similar results in other ages and genders. Further studies with a larger number of ages, genders, and races would help to further corroborate our findings.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Comparison of efficacy and safety in the combination therapies of duloxetine and S-flurbiprofen plaster, and of duloxetine and conventional NSAIDs for chronic pain in patients with osteoarthritis (OASIS DUAL study)

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SUMMARY The purpose of this study is to compare the efficacy and safety of pharmacotherapies for chronic pain due to osteoarthritis (OA) between a group with duloxetine (DLX) and S-flurbiprofen plaster (SFPP) (the SFPP group) and a group with DLX and conventional non-steroidal anti-inflammatory drugs (NSAIDs) (the control group). The subjects were 49 patients (17 men and 32 women). The evaluation of factors associated with treatment termination due to symptoms improvement showed that significantly more women terminated treatment than did men, and significantly more members of the SFPP group terminated treatment than did members of the control group. The visual analogue scale (VAS) score in the SFPP group was significantly improved from 6.6 ± 1.7 before treatment to 3.6 ± 2.1 one month later and showed significant difference until nine months later. The VAS score in the control group was significantly improved from 6.7 ± 1.9 to 4.1 ± 2.8 one month later. The VAS score improvement rate was significantly higher in the SFPP group than in the control group, suggesting that the DLX-SFPP combination had higher efficacy than the DLX-conventional NSAIDs combination. The incidence of adverse drug reactions was 55% in the SFPP group, which is not significantly different from 50% incidence in the control group. The treatment discontinuation rate due to adverse drug reactions, however, was 60% in the control group and 19% in the SFPP group. It was suggested that the efficacy and safety of the DLX-SFPP combination for chronic pain due to OA are equal to or higher than that of the DLX-conventional NSAIDs combination.

Keywords Chronic pain, osteoarthritis, pharmacotherapy, duloxetine, S-flurbiprofen plaster

1. Introduction

Osteoarthritis (OA) is a disease that causes osteoplastic change in joints in a non-inflammatory and progressive manner and adds inconveniences to the activities of daily living (ADL) because of pain (*1*). OA prevalence increases with age. According to the large-scale population-based cohort study, the number of patients (40 years of age or older) who have OA of the knee is estimated to be 25.3 million, that of patients who have lumbar spondylosis 37.9 million, and that of patients who have OA of the hip 12.0 million in Japan (*2*). Since OA progresses chronically, it often causes chronic pain. It influences various ADL, reduces healthy life expectancy (*3*), and raises mortality (*4,5*). Therefore, OA is a considerable social problem.

The pathology of chronic pain from OA is complex because acute pain is often involved in the course of chronic pain. It makes treatment difficult (*6*). It is expected that appropriate treatment of chronic pain not only extends healthy life expectancy, but also reduces healthcare costs and premiums for nursing-care insurance. Clinical Practice Guidelines for the Management of Chronic Pain strongly recommend duloxetine (DLX) for OA (*7*), but oral non-steroidal anti-inflammatory drugs (NSAIDs) are often used chronically. The use of oral long-term NSAIDs causes adverse drug reactions, including gastric mucosa injury (*8*), cardiovascular risk (*9*), and renal dysfunction (*10*). There is also a problem of polypharmacy in the elderly. Therefore, it is necessary to consider the details of drug prescriptions.

Topical NSAIDs are the most strongly recommended according to the latest guidelines by the Osteoarthritis Research Society International (OARSI), and oral NSAIDs are conditionally recommended (*11*). Among

topical NSAIDs, S-flurbiprofen plaster (SFPP) has a stronger analgesic effect than topical flurbiprofen (*12*). The efficacy of long-term SFPP administration has been reported (*13*), and the incidence of serious adverse drug reactions in the digestive system was relatively low (*14*). As a drug used in combination with DLX for chronic pain due to OA, SFPP is expected to be effective. There are, however, few reports of combination therapy with DLX and no data comparing DLX with conventional NSAIDs and DLX with SFPP. If the DLX-SFPP combination shows efficacy equal to that of the DLX-conventional NSAIDs combination, and if safety is confirmed and adverse drug reactions and polypharmacy are taken into account, treatment with DLX and SFPP can be a useful option.

The purpose of the present study is to compare the efficacy and safety of pharmacotherapies for chronic pain due to OA between the SFPP group and the control group.

2. Materials and Methods

2.1. Ethical approval of the study protocol

The present study was approved by the clinical research ethical review board of Shido, Inc. (#S20220131) and conducted in accordance with the Declaration of Helsinki and the ethical guidelines provided by the Ministry of Health, Labour and Welfare. Since my clinic does not have an ethics review committee, I asked an external committee to review this study. As it did not involve invasive procedures for the patients or other intervention and used only medical information, an opt-out was provided to patients for the disclosure of information.

2.2. Study design and subjects

The study was designed as a single-center, retrospective study in Japan. I included only the existing data in the analysis; no new data were collected. The subjects were 49 patients who received pharmacotherapy for chronic pain due to OA in my hospital from January 2018 to October 2021 and used oral DLX and oral or topical NSAIDs and whose visual analogue scale (VAS) scores were measured. Lumbar spondylosis was not included, since lower back pain is not caused by lumbar spondylosis alone, but is also often caused by more than one disease. There were 17 men and 32 women, and their mean age was 71.8 ± 10.2 years.

2.3. Investigation items

I speculated that improving chronic pain due to OA and terminating treatment early are important to improve the quality of life (QOL) of patients. Therefore, as part of the primary endpoint analysis, I evaluated sex, age, the presence or absence of SFPP treatment, and VAS score as factors associated with the termination of treatment due to the improvement of symptoms. Secondary endpoints were time to the termination of treatment due to the improvement of symptoms; changes in VAS score 1, 3, 6, 9, 12, 18, and 24 months from the start of administration; VAS score improvement rates before and after the start of treatment; the incidence of adverse drug reactions; and the treatment discontinuation rate due to adverse drug reactions.

2.4. Statistical analysis

The primary endpoint was an event of treatment termination due to the improvement of symptoms, and the time from treatment initiation to the occurrence of an event or censoring was evaluated using a Cox proportional hazards model with age, sex, the presence or absence of SFPP treatment, and VAS score as covariates. Regarding secondary endpoints, Kaplan-Meier curves of time from the start of treatment to the end of treatment in the SFPP group and the control group were drawn and tested using the log-rank test. Regarding changes in VAS score 1, 3, 6, 9, 12, 18, and 24 months from the start of treatment, intragroup comparison in the SFPP group and in the control group was tested using the Wilcoxon signed rank test and intergroup comparison was tested using the Wilcoxon rank sum test. Regarding the amount of change in the VAS score from the start of treatment to the end of treatment due to the improvement of symptoms, intragroup comparison in the SFPP group and in the control group was tested using the Wilcoxon signed rank test. Regarding the VAS score improvement rate, treatment outcomes were classified in order to investigate efficacy as follows: improved (improvement rate ≥ 50%), somewhat improved (improvement rate > 0% to < 50%), unchanged (improvement rate 0%), somewhat worsened (improvement rate < 0% to > −50%), and worsened (improvement rate ≤ – 50%). According to the formula of Hirabayashi (*14*), the VAS score improvement rate was defined as (VAS score before treatment – VAS score after treatment) × 100 / (10 – VAS score before treatment). The VAS score improvement rate, the incidence of adverse drug reactions, and the treatment discontinuation rate due to adverse drug reactions between the SFPP group and the control group were tested using Fisher's exact test. Regarding backgrounds of the subjects, variables on a nominal scale were compared using Fisher's exact test, and continuous variables were compared using Student's *t*-test. R version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria [*https://www.R-project.org/*]) was used for analysis.

3. Results

In the present study, the SFPP group had 29 patients (12

men and 17 women) with a mean age of 71.7 ± 10.1 years. The control group had 20 patients (5 men and 15 women) with a mean age of 72.1 ± 10.8 years. No significant difference was observed in men-to-women ratio or age between the two groups. A breakdown of primary diseases and previous histories of hypertension, hyperlipidemia, diabetes mellitus, and gout in both groups are shown in Table 1.

The VAS score before the start of treatment was 6.6 ± 1.7 in the SFPP group and 6.7 ± 1.9 in the control group, showing no significant difference. Regarding oral doses of DLX, the starting dose was 20 mg in both groups, the mean maximum dose was 43.3 ± 13.9 mg in the SFPP group and 40.0 ± 16.6 mg in the control group, and the mean maintenance dose was 38.6 ± 14.4 mg in the SFPP group and 38.9 ± 17.2 mg in the control group, showing no significant difference. In the SFPP group, all patients used SFPP concurrently, and the maximum dose was two plasters a day irrespective of application sites. In the control group, 14 patients used oral NSAIDs (celecoxib in eight patients, loxoprofen sodium in four patients, and diclofenac sodium in two patients) concurrently, and 20 patients used a topical NSAID (loxoprofen sodium hydrate patch in all patients) concurrently. The maximum doses of the oral NSAIDs were as follows: celecoxib 200 mg/day, loxoprofen sodium 180 mg/day, and diclofenac sodium 75 mg/day. The maximum dose of the topical NSAID, loxoprofen sodium hydrate patch, was one patch daily. The mean duration of drug administration was eight months in the SFPP group and ten months in the control group. Regarding concurrently used antiemetics, mosapride was significantly more common in the SFPP group (28 patients in the SFPP group and 12 patients in the control group) and metoclopramide was significantly more common in the control group (one patient in the SFPP group and eight patients in the control group).

Table 1. Primary diseases and previous histories in two groups

Items	SFPP group (n = 29)	Control group (n = 20)	p
Primary disease			0.865
Osteoarthritis of the cervical spine	1 (3.4%)	0 (0%)	
Osteoarthritis of the shoulder	2 (6.9%)	1 (5.0%)	
Osteoarthritis of the wrist	1 (3.4%)	0 (0%)	
Osteoarthritis of the hip	4 (13.8%)	5 (25.0%)	
Osteoarthritis of the ankle	4 (13.8%)	4 (20.0%)	
Osteoarthritis of the knee	17 (58.6%)	10 (50.0%)	
Previous history			
Diabetes mellitus	5 (17.2%)	1 (5.0%)	0.379
Hypertension	16 (55.2%)	13 (65.0%)	0.563
Hyperlipidemia	7 (24.1%)	6 (30.0%)	0.747
Gout	2 (6.9%)	3 (15.0%)	0.387

Data are n (%). N.S., Fisher's exact test. SFPP: S-flurbiprofen plaster.

Table 2. Multivariate analysis using a Cox proportional hazards model with discontinuation due to the improvement of symptoms as an event (n = 49)

Items	Hazard ratio	95% CI	p
Age	1.011	0.966 - 1.059	0.631
Sex (man:1, woman:0)	0.254	0.071 - 0.913	0.036*
Group (SFPP group:1, control group: 0)	4.034	1.156 - 14.076	0.029*
VAS	0.932	0.702 - 1.237	0.626

*p < 0.05, Multivariate analysis using a Cox proportional hazards model. SFPP: S-flurbiprofen plaster; VAS: visual analog scale.

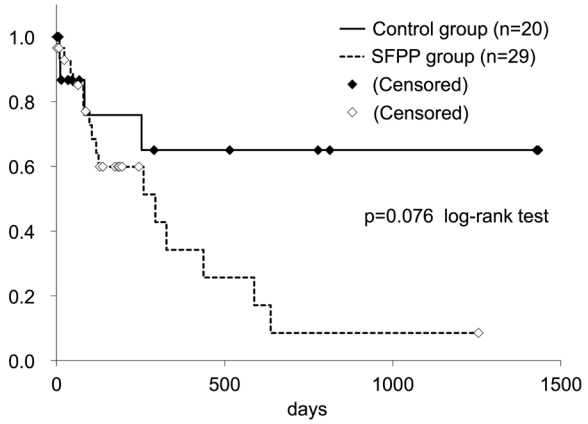

Figure 1. Kaplan-Meier curves with discontinuation due to the improvement of symptoms as an event.

3.1. Primary endpoint

The results of the evaluation of factors associated with the termination of treatment due to the improvement of symptoms showed that the termination of treatment was significantly more likely in women and the SFPP group (Table 2).

3.2. Secondary endpoints

Time to the termination of treatment due to the improvement of symptoms tended to be shorter in the SFPP group, but the difference between the two groups was not significant (Figure 1).

The mean VAS score in the SFPP group was significantly improved from 6.6 ± 1.7 before treatment to 3.6 ± 2.1 one month later and showed significant difference until nine months later (Figure 2a). Regarding the VAS score improvement rate in the SFPP group, scores were improved and somewhat improved in 86.2% of the patients and worsened and unchanged in 13.8%.

The mean VAS score in the control group was significantly improved from 6.7 ± 1.9 before treatment to 4.1 ± 2.8 one month later, but did not show significant difference after that (Figure 2b). Regarding the VAS score improvement rate in the control group, scores were improved and somewhat improved in 60%

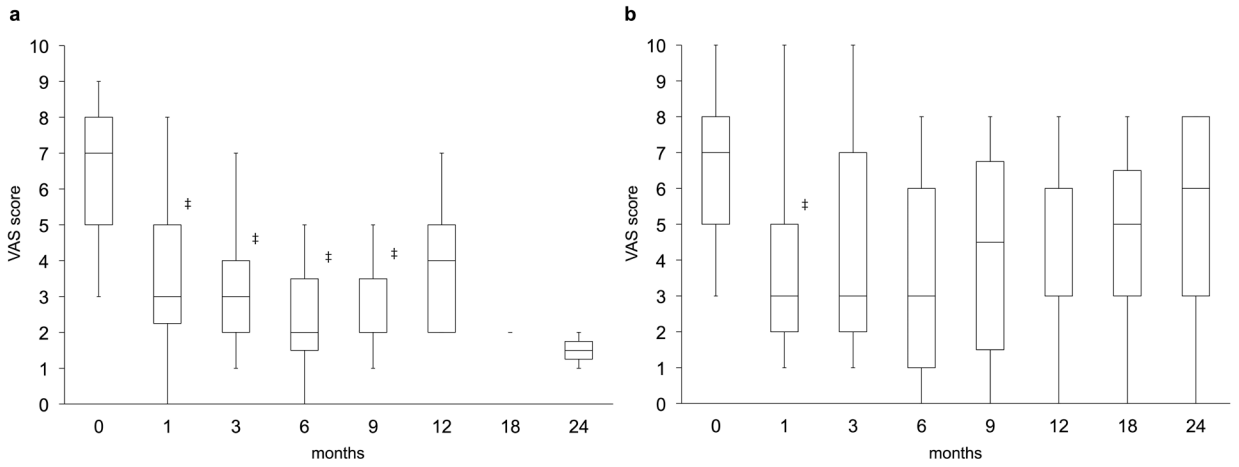

Figure 2. Change in VAS score. a: SFPP group. b: Control group. ‡$p < 0.05$ *vs.* 0 M. Wilcoxon signed rank test. Data are min-1stQ-median-3rdQ-max.

of the patients and worsened and unchanged in 40%.

When VAS scores were compared between the two groups, there was no significant difference during the follow-up period, including the observation period before treatment. Regarding the VAS score improvement rate, the proportion of patients who had improved and somewhat improved scores was significantly higher in the SFPP group, and the proportion of patients who had worsened and unchanged scores was significantly higher in the control group (Figure 3).

The incidence of adverse drug reactions in the SFPP group was 55% (16/29 patients), and the breakdown of the adverse drug reactions was as follows: nausea in seven patients, sleepiness in six patients, gastrointestinal disorder in five patients, constipation in one patient, thirst in one patient, and skin disorder in one patient. The rate of treatment continuation (defined as "treatment continuation or treatment termination due to improvement," and treatment termination due to any other reason was regarded as "treatment discontinuation") was 76%, and reasons for discontinuation were adverse drug reactions in three patients, worsening in one patient, and unknown in three patients. The treatment discontinuation rate among patients who developed adverse drug reactions was 19% (3/16 patients). The adverse drug reactions in patients who discontinued treatment due to adverse drug reactions were nausea in one patient, sleepiness in one patient, gastrointestinal disorder in two patients, and constipation in one patient, with one of the three patients discontinuing treatment due to more than one adverse drug reaction.

The incidence of adverse drug reactions in the control group was 50% (10/20 patients), and the breakdown of the adverse drug reactions were as follows: nausea in four patients, sleepiness in three patients, constipation in two patients, dizziness in

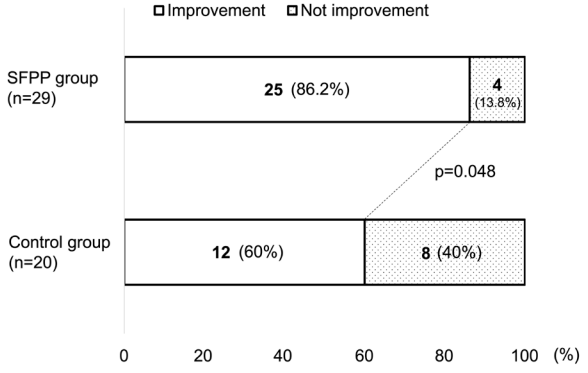

Figure 3. Degree of improvement in VAS score. $p < 0.05$ Fisher's exact test.

two patients, stomatitis in one patient, diarrhea in one patient, impaired urination in one patient, and headache in one patient. The treatment continuation rate was 45%, and reasons for discontinuation were adverse drug reactions in six patients, worsening in one patient, no change in two patients, and unknown in two patients. The treatment discontinuation rate among patients who developed adverse drug reactions was 60% (6/10 patients). Adverse drug reactions in patients who discontinued treatment due to adverse drug reactions were nausea in three patients, sleepiness in one patient, constipation in two patients, dizziness in one patient, stomatitis in one patient, diarrhea in one patient, impaired urination in one patient, and headache in one patient, with four of the six patients discontinuing treatment due to more than one adverse drug reaction.

There was no significant difference in the incidence of adverse drug reactions including gastrointestinal disorder and skin disorder due to plasters between the two groups (Table 3). In the SFPP group, oral NSAIDs were not concurrently used. Therefore, preventive

stomach medicines were not prescribed. In the control group, however, stomach medicines were prescribed for patients who were using oral NSAIDs, and the proportion of patients who were taking stomach medicines was significantly higher at 70%.

The treatment continuation rate was significantly higher in the SFPP group than in the control group, and the treatment discontinuation rate due to adverse drug reactions was significantly lower in the SFPP group than in the control group (Table 4).

Table 3. Adverse drug reaction data

Items	SFPP group (n = 29)	Control group (n = 20)	p
None	12 (41.4%)	9 (45.0%)	1.000
Gastrointestinal disorder	5 (17.2%)	0 (0%)	0.070
Skin disorder	1 (3.4%)	0 (0%)	1.000
Headache	0 (0%)	1 (5.0%)	0.408
Thirst	1 (3.4%)	0 (0%)	1.000
Sleepiness	6 (20.7%)	3 (15.0%)	0.720
Nausea	7 (24.1%)	4 (20.0%)	1.000
Constipation	1 (3.4%)	2 (10.0%)	0.559
Eyelid edema	0 (0%)	0 (0%)	-
Stomatitis	0 (0%)	1 (5.0%)	0.408
Impaired urination	0 (0%)	1 (5.0%)	0.408
Dizziness	0 (0%)	2 (10.0%)	0.162
Diarrhea	0 (0%)	1 (5.0%)	0.408
Unknown	1 (3.4%)	1 (5.0%)	1.000

Data are n (%). N.S., Fisher's exact test. SFPP: S-flurbiprofen plaster.

4. Discussion

Chronic pain is believed to have two disease states: one in which a noxious stimulus is continuously or repeatedly acting, and one in which a spontaneous pain continues even though there is no finding of tissue injury or the healing of tissue injury. The former is mainly nociceptive pain, which often responds to NSAIDs and surgical therapy. The latter often becomes involved with psychosocial factors. Therefore, NSAIDs and surgical therapy are ineffective in the elimination of pain (*15*). In addition, acute pain often occurs repeatedly during the course of chronic pain, which complicates the pathology (*6*).

According to a national questionnaire survey on the influence of chronic pain on ADL in 40,000 men and women 20 years of age or older, in all questions about ADL, 20%-30% of respondents replied that chronic pain always or often influences ADL, indicating that the impairment of ADL frequently occurs (Figure 4) (*3*).

Regarding the relationship between chronic pain and mortality, there is a report that patients 50 years of age or older who have pain in broad areas of the

Table 4. Continuation rate data

Items	SFPP group (n = 29)	Control group (n = 20)	p
Continuation/treatment termination due to improvement	22 (75.9%)	9 (45.0%)	0.038‡
Other reasons for treatment termination	7 (24.1%)	11 (55.0%)	
Adverse drug reaction	3 (10.3%)	6 (30.0%)	
Worsened	1 (3.4%)	1 (5.0%)	
Unknown	3 (10.3%)	2 (10.0%)	
Unchanged	0 (0%)	2 (10.0%)	

Data are n (%). ‡$p < 0.05$ Fisher's exact test. SFPP: S-flurbiprofen plaster.

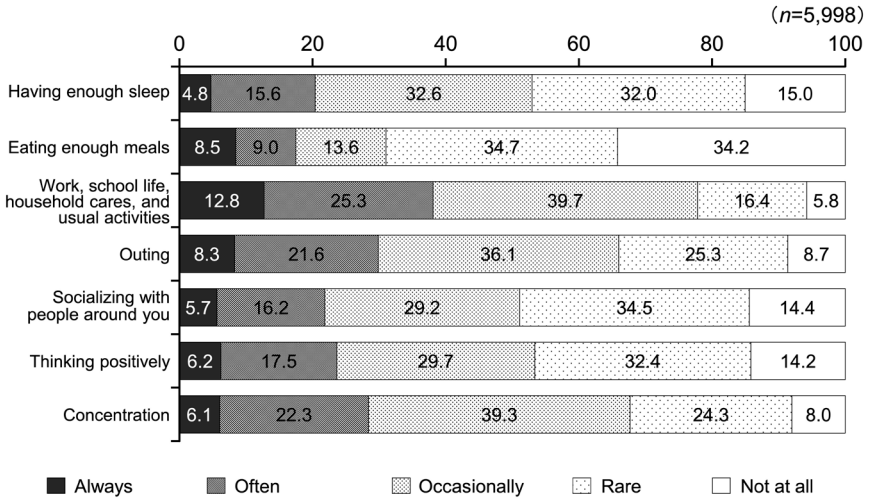

Figure 4. Influences of chronic pain on ADL. National survey in men and women 20 years of age or older (the number of respondents: first survey, n = 41,597; second survey, n = 5,998). Shoji Yabuki *et al.* Clinical Orthopaedic Surgery. 2012; 47:127-134.

locomotorium have high mortality, from cancer in particular (*4*). Also, in a report that pain in the locomotorium and death were correlated, the highest mortality occurred when pain was in the lower back, followed by pain in the hip joint and neck (*5*).

As mentioned above, chronic pain impairs many ADLs and raises mortality. Conditions derived from chronic pain not only worsen the physical function of the patient, but also induce an unhealthy mental state. Moreover, iatrogenic problems due to the treatment of chronic pain, such as adverse drug reactions and complications of invasive treatment, also increase distress. In patients with severe chronic pain, the mixing of chronic with acute pain make the condition refractory, which unfortunately induces some patients to commit suicide.

Due to the complexity of the pathology of chronic pain, treatment methods vary. In clinical practice, a combination of drugs is used according to Clinical Practice Guidelines for the Management of Chronic Pain with careful attention to adverse drug reactions. In the present study, pharmacotherapy using a combination of DLX and SFPP or conventional NSAIDs was performed.

Since reducing chronic pain due to OA and terminating the treatment early should be important to QOL improvement, I evaluated factors associated with the termination of treatment due to the improvement of symptoms. The results showed that women were significantly more likely than men to terminate treatment and the SFPP group was significantly more likely than the control group to terminate treatment. As a cause of the former result, it was reported that the tendency to develop depression is stronger in elderly women than elderly men and depression is related to sex differences in pain (*16*). It is presumed that oral DLX improved depression. Among the causes of the latter result are (a) improvement in the course of VAS score in the SFPP group compared to the control group up to nine months from the start of treatment and (b) significantly higher VAS score improvement rate.

When the efficacy data were compared, there was no significant difference in change in VAS score over time between the two groups, but significant improvement one month after the start of treatment in both groups. Only the SFPP group, however, showed significant improvement compared to the start of treatment between three months and nine months after the start of treatment. Moreover, the proportion of patients whose VAS score was improved was significantly higher in the SFPP group, and the proportion of patients whose VAS score was worsened or unchanged was significantly higher in the control group. Although short-term outcomes between these two groups did not differ, on the basis of these results, I thought that long-term outcomes might be better in the SFPP group. Even in the SFPP group, however, no significant improvement was observed 12 months or later after the start of treatment. This indicates that pain in patients who continue to receive analgesics may not be improved because a characteristic of analgesic treatment is that it is terminated when pain is improved.

Reasons for the significantly higher treatment continuation rate in the SFPP group is thought to solve the polypharmacy problem by prescribing plasters instead of oral drugs, which results in no increase in the number of orally administered drugs, and the comparability of the analgesic effect of SFPP to that of conventional NSAIDs. The efficacy of SFPP was supported by a study comparing the pharmacokinetics of SFPP and oral NSAIDs, which showed that SFPP concentrations in the synovium and the synovial fluid started to rise more slowly, but were maintained at high levels longer than NSAIDs concentrations (*17*), and by a study which confirmed the efficacy of SFPP irrespective of the concurrent use of oral NSAIDs (*18*).

When safety data were compared, there was no significant difference in the incidence of adverse drug reactions between the two groups. There was also no significant difference in the incidence of gastrointestinal disorder. The proportion of patients who were taking preventive stomach medicines, however, was significantly higher in the control group, so I thought the incidence of gastrointestinal disorder may have been suppressed in the control group. The treatment discontinuation rate due to adverse drug reactions was significantly lower in the SFPP group than in the control group, 19% and 60%, respectively. I thought that this was because the number of serious adverse drug reactions requiring the discontinuation of treatment was small in the SFPP group. There is also a report that the frequency of adverse drug events in elderly inpatients was significantly higher among those taking six oral drugs or more than among those taking five drugs or fewer (*19*). This led me to believe that no increase in the number of oral drugs and the solution of the polypharmacy problem influenced the treatment discontinuation rate in the SFPP group. While both (a) the incidence of gastrointestinal disorder associated with SFPP administration may be lower than with oral NSAIDs administration (*20*) and (b) the risk cardiovascular or renal disorder is not worrisome as an adverse drug reaction to SFPP (*21*) have been reported, there is also a report to the effect that the risk of NSAIDs use is similar whether topically or orally administered (*22*). Therefore, NSAIDs need to be administered more cautiously in patients with cardiovascular disease and renal disorder.

Limitations of the present study are as follows: this was a retrospective study with a small number (only 49) of patients; of the OA, lumbar spondylosis was not included; and there was a significant difference in the proportion of patients who were taking stomach medicines between the two groups.

5. Conclusions

In the present study, treatment outcomes for chronic pain due to OA were compared between two combination therapies: (a) DLX and SFPP and (b) DLX and conventional NSAIDs. The results showed that women (compared to men) and the SPFF group (compared to the control group) were significantly more likely to terminate treatment due to the improvement of symptoms. Time to the termination of treatment due to the improvement of symptoms tended to be shorter in the SFPP group, but the difference between the two groups was not significant. VAS scores in the SFPP group were significantly improved one month later and showed significant difference up to nine months later, while VAS scores in the control group were significantly improved one month later, but did not show significant difference after that. Regarding the VAS score improvement rate, moreover, the proportion of improved and somewhat improved patients was significantly higher in the SFPP group than in the control group, suggesting that the combination therapy of DLX and SFPP had higher efficacy than the combination therapy of DLX and conventional NSAIDs.

Although there was no significant difference in the incidence of adverse drug reactions between the two groups, the treatment discontinuation rate due to adverse drug reactions was significantly higher in the control group than in the SFPP group. Although no significant difference was seen between the control group and the SFPP group, skin disorder due to plasters was reported in the SFPP group, and I thought that appropriate management of this disorder would be needed. In the control group, since a significantly higher proportion of patients were taking preventive stomach medicines, I thought that the incidence of gastrointestinal disorder may have been suppressed in the control group.

It was suggested that the efficacy of combination therapy with DLX plus SFPP for chronic pain due to OA is equal to or higher than that of DLX plus conventional NSAIDs, and therefore that DLX plus SFPP can also be an effective option in terms of adverse drug reactions and polypharmacy.

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Conflict of Interest: The author has no conflicts of interest to disclose.

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Original Article

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Impact of inhaled ciclesonide on asymptomatic or mild COVID-19: A randomized trial

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SUMMARY The aim of this study was to determine the efficacy and safety of ciclesonide in the treatment of novel coronavirus disease 2019 (COVID-19) as gauged by pneumonia progression. This multi-center, open-label randomized trial was conducted with patients recruited from 22 hospitals across Japan. Participants were patients admitted with mild or asymptomatic COVID-19 without signs of pneumonia on chest X-rays. Asymptomatic participants were diagnosed after identification through contact tracing. Trial participants were randomized to either the ciclesonide or control arm. Participants in the treatment arm were administered 400 μg of ciclesonide three times a day over seven consecutive days. The primary endpoint was exacerbated pneumonia within seven days. Secondary outcomes were changes in clinical findings, laboratory findings, and changes over time in the amount of the viral genome. In the treatment group, 16 patients (39.0%) were classified as having exacerbated pneumonia compared to 9 (18.8%) in the control group. The risk ratio (RR) was 2.08 (95% confidence interval (CI): 1.15-3.75), indicating a worsening of pneumonia in the ciclesonide group. Significant differences were noted in participants with a fever on admission (RR: 2.62, 90% CI: 1.17-5.85, 95% CI: 1.00-6.82) and individuals 60 years of age or older (RR: 8.80, 90% CI: 1.76-44.06, 95% CI: 1.29-59.99). The current results indicated that ciclesonide exacerbates signs of pneumonia on images in individuals with mild or asymptomatic symptoms of COVID-19 without worsening clinical symptoms.

Keywords COVID-19, ciclesonide, randomized clinical trial, pneumonia

1. Introduction

Following initial reports of pneumonia related to novel coronavirus disease 2019 (COVID-19) from December 2019 onwards in Wuhan in Hubei Province, the People's Republic of China, similar reports were noted around the world, and the increasing number of reports signaled the advent of a pandemic. There are many patients with COVID-19 in Japan, but this is an emerging infectious disease, and there are limited treatments available for individuals with mild COVID-19. At the time of this research plan, standard treatment methods were limited

to symptomatic treatment (*1*).

Ciclesonide was first approved in Australia for indications of bronchial asthma in February 2004; as of March 2021, it was approved in 48 countries worldwide. In Japan, ciclesonide is manufactured and marketed by Teijin Pharma (Tokyo, Japan) under the trade name Alvesco, and it was first approved for indications of adult bronchial asthma in April 2007 and for children in January 2011. Ciclesonide's mechanism of action on asthma is through esterase activation in the lungs and alveoli, which then becomes the active metabolite desisobutrylciclesonide, which then binds to glucocorticoid receptors to control chronic inflammation in the alveoli.

In the wake of the COVID-19 pandemic, ciclesonide has been reported to possibly inhibit the Middle East respiratory syndrome coronavirus (MERS-CoV) in "Vero" cells (*2*). Basic research by the Coronavirus Laboratory at the National Institute for Infectious Diseases suggested that ciclesonide might have potent anti-viral activity against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (*3*). Ciclesonide's effects on preventing viral proliferation were either equivalent to or exceeded those of lopinavir in *in vitro* tests. Case reports indicated that the ideal period of ciclesonide administration should be in the early to intermediate stage of infection or during the initial phase of pneumonia before symptoms worsen (*3-6*). Beyond these, there have been no further reports on ciclesonide administration in patients with COVID-19.

The current study conducted a prospective randomized controlled trial to analyze ciclesonide's efficacy and safety in patients with COVID-19 in the early stages of infection. Assembling findings related to COVID-19 through this study should help to develop future countermeasures against this disease.

2. Materials and Methods

2.1. Participants

This multi-center, open-label randomized trial was conducted in Japan with patients recruited from 22 hospitals across Japan (Figure 1) between April 3, 2020 and September 18, 2020. The efficacy and safety of ciclesonide was assessed in patients with COVID-19 in the early stages of infection. Patients who tested positive for SARS-CoV-2 based on a polymerase chain reaction (PCR) or according to loop-mediated isothermal amplification were identified for recruitment. Since this study was conducted on asymptomatic or mildly ill patients, many of them were tested as close contacts or individuals with mild symptoms who tested positive. Patients admitted with COVID-19 were screened, and those with no signs of pneumonia on chest X-rays (CXR) were deemed eligible. Additional inclusion criteria included: age ≥ 20 years, had no clear indications of COVID-19-induced pneumonia on plain CXRs, individuals who could be hospitalized to receive the trial drug (*i.e.*, one week), and individuals who could inhale ciclesonide using inhalation assistance. Exclusion criteria were: a history of ciclesonide hypersensitivity, an infectious disease or deep-seated mycosis other than COVID-19 for which there is no effective antibacterial, a chronic respiratory disease such as chronic bronchitis, current treatment with inhaled or oral steroids, a history of a persistent fever of ≥ 37.5°C that lasted for over seven days, or current treatment with agents that are potentially efficacious in treating COVID-19 and that may affect assessments of efficacy, including remdesivir, lopinavir/ritonavir compound drugs, favipiravir, interferon, and hydroxychloroquine. Patients were randomized using the stratified block randomization method.

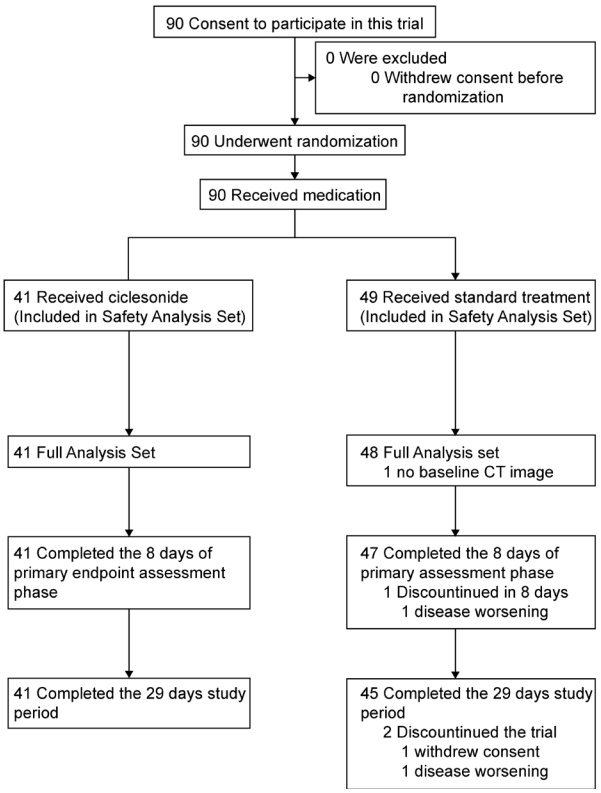

Figure 1. Flow diagram of participant recruitment and the phases of the RACCO randomized trial.

2.2. Protocol

Participants who were admitted to the hospital for 1-2 days of observation were identified prior to registration. Protocol details are provided in a previous paper (*6*). Demographic characteristics, vital signs, body mass index, samples and imaging, peak expiratory flow (PEF) measurements, and responses to a questionnaire were collected during the observation period.

Once enrolled, trial participants were randomized to either the ciclesonide group or a symptomatic treatment

group using the Electronic Data Capture (EDC) system. Participants in the ciclesonide group were administered 400 µg of ciclesonide in a pressurized metered-dose inhaler (pMDI) formulation three times a day with a spacer, over seven consecutive days. This dose was recommended for severe cases in a previous study, but this dose was chosen for the current study to avoid insufficient drug dosage to confirm antiviral activity (*2*). In contrast, the symptomatic treatment group received only symptomatic treatment for symptoms such as a fever and cough, *i.e.*, the study was open-label. The following information was obtained throughout the trial: demographic characteristics, a physical exam (including BMI), medical history (duration of hospitalization), hematological tests, blood chemistry, coagulation tests, infectious disease panel, and coronavirus tests (nasal and serum). A chest X-ray (CXR) was performed on days 1, 8, 15, 22, and 29, PEF was measured daily, and a questionnaire on appetite, fatigue, and coughing was administered on days 1-8, 15, 22, and 29. A complete listing of the study sites, data collected, and time points is shown in Supplemental Tables S1 and S2, and Supplemental Figure S1 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=123*).

2.3. Outcomes

The primary endpoint was exacerbated pneumonia within seven days of inhalation of ciclesonide, based on computerized tomography (CT) scans before drug administration and one week following treatment. Chest CT images within seven days of administration were assessed in the three following stages: remission or stabilization compared to the status before administration, potential exacerbation compared to the status before administration, or obvious worsening compared to the status before administration. Two radiologists independently interpreted images, and worsening signs of pneumonia on images were determined. Instances where the two radiologists disagreed were resolved by a specialist in pulmonary medicine. Efficacy was confirmed by checking for the exacerbation of pneumonia. Secondary outcomes were changes in clinical findings, laboratory findings, and changes over time in the amount of the viral genome.

2.4. Safety

The safety of this therapy was evaluated and the number of adverse events (AEs) was determined. AEs were defined as abnormal variations in clinical values and physiological function. The following information on AE was collected: incident duration, time to resolution, extent, treatment, outcome, assessment of its severity, correlation with the study drug, and predictability. The extent of an AE was categorized as mild (continued administration is possible with no treatment), moderate (continued administration is likely with some form of treatment), or severe (administration was or should be suspended). A severe AE were defined as the following: death, life-threatening complications, hospitalization or an extended stay in the hospital or other care facility, permanent or significant disability or functional limitation, or congenital illness/abnormalities affecting subsequent generations.

2.5. Statistical analysis

Based on the experience of the Center Hospital, 35% of patients who were deemed positive for COVID-19 without symptoms of pneumonia developed pneumonia during the follow-up period. Assuming that the incidence of pneumonia in the standard treatment and trial treatment groups would hypothetically be 35% and 10%, respectively, then the required sample size of 84 patients was calculated for both a power of 10% and 80% with a two-tailed alpha. The planned sample size was set at 90 to account for several dropouts.

The primary population used for the efficacy analysis was the complete analysis set, including all patients who had undergone randomization, whose baseline CT image data were acquired, and who had no severe protocol violations. Primary efficacy, *i.e.*, the proportion of patients with exacerbated pneumonia within seven days of administration, was compared between the groups using Fisher's exact test. In addition, the risk ratio (RR) and risk difference (RD) and their 90% and 95% confidence intervals (CIs) were calculated.

Predefined subgroup analyses stratified by age (< 60 or ≥ 60), smoking (yes or no), a fever on admission (< 37.5°C or ≥ 37.5°C) were performed in the same manner as the primary analysis. Analysis using the secondary efficacy outcomes (changes in clinical findings, laboratory findings, and changes over time in the amount of the viral genome) were performed, and summary statistics (number of cases, average value, standard deviation, minimum value, median value, and maximum value) were calculated for each group. Missing data for the primary endpoint or secondary efficacy outcome were not input. Other statistical methods and details regarding statistical assumptions are described in the Supplemental Appendix. Since the widths of the confidence intervals have not been adjusted for multiple comparisons, the intervals should not be used to infer definitive therapeutic effects for the secondary efficacy outcomes or subgroup analyses. All statistical analyses were performed by Y.U. using the software SAS, version 9.4 (SAS Institute).

2.6. Research ethics and disclosure

This trial was approved by the Clinical Research Review Board at The University of Tokyo (Protocol Number: 2019017SP) and it has been registered with the Japan

Registry of Clinical Trials (jRCT No: jRCTs031190269). The data obtained in this study will be registered in the "Registry research relating to COVID-19 (COVIREGI) (NCGM Ethical Inspection Committee approval No. NCGM-G-003494-00)".

3. Results

3.1. Patient characteristics

Eighty-nine patients were randomized in this study, 41 in the ciclesonide group and 48 in the symptomatic treatment group, with a mean overall age of 23.17 ± 3.88 years (Table1). The sociodemographic and clinical characteristics of the sample are shown in Table 1. Forty-four (49.4%) patients were male, with 20 (48.8%) in the treatment group and 24 (50.5%) in the observation group. There were no statistical differences between the groups.

The median time from onset to hospitalization was 5.0 days in the ciclesonide group and 5.5 days in the symptomatic treatment group. Most of the participants were Japanese, but three in the ciclesonide group and one in the symptomatic treatment group were non-Japanese. The comorbidities in the ciclesonide group were congestive heart failure (2 patients), mild liver disease (2 patients), mild diabetes (2 patients), obesity (2 patients), a peptic ulcer (2 patients), dementia (1 patient), cerebrovascular disease (1 patient), and those in the symptomatic treatment group were mild diabetes (3 patients), obesity (3 patients), a solid tumor (3 patients), mild liver disease (1 patient), and HIV infection (1 patient).

3.2. Primary and secondary outcomes

Univariate analysis compared exacerbated pneumonia in the two groups. Figure 2A shows the RRs while Figure 2B shows the RDs. In the treatment group, 16 patients (39.0%) were classified as having exacerbated pneumonia compared to 9 (18.8%) in the control group. The RR was 2.08 (95% CI: 1.03-4.20, Figure 2A). In the stratified analysis, results differed little depending on whether a patient had a history of smoking or not. In contrast, RR tended to differ significantly among patients with a fever on admission (RR: 2.62, 95% CI: 1.00-6.82) and individuals 60 years of age or older (RR: 8.80, 95% CI: 1.29-59.99). These point estimates are based on small numbers.

The overall RD was not significant (RD: – 0.20, 95% CI: 0.02-0.39, Figure 2B). Fever was not significant at the 95% confidence level but was at the 90% confidence level. A significant difference was not noted for individuals 60 and older (RD: 0.71, 95% CI: 0.32-1.00). However, those numbers are very small.

3.3. Safety

Table 2 shows the number of AEs in each group. AEs,

Table 1. Demographic characteristics and select clinical variables

Background factors	Ciclesonide group, *n* (%)	Symptomatic treatment group, *n* (%)	Total, *n* (%)
Number of subjects	41	48	89
Sex			
Male	20 (48.8)	24 (50.0)	44 (49.4)
Female	21 (51.2)	24 (50.0)	45 (50.6)
Age			
< 60 years (≥20 years)	36 (87.8)	37 (77.1)	73 (82.0)
≥ 60 years	5 (12.2)	11 (22.9)	16 (18.0)
BMI			
N	40	45	85
Mean	22.53	23.74	23.17
SD	3.38	4.22	3.88
Smoking			
Current smoker	10 (24.4)	11 (22.9)	21 (23.6)
Former smoker	7 (17.1)	8 (16.7)	15 (16.9)
Non-smoker	23 (56.1)	27 (56.3)	50 (56.2)
Unknown	1 (2.4)	2 (4.2)	3 (3.4)
Presence of comorbidities			
Yes	8 (19.5)	10 (20.8)	18 (20.2)
No	33 (80.5)	38 (79.2)	71 (79.8)
Presence of immunosuppressive conditions			
Yes	0 (0)	0 (0)	0 (0)
No	41 (100)	48 (100)	89 (100)
Chest CT image			
Signs of pneumonia	18 (43.9)	24 (50)	42 (47.2)
No signs of pneumonia	23 (56.1)	24 (50)	47 (52.8)
Plain chest X-ray			
No abnormalities evident	41 (100)	46 (95.8)	87 (97.8)
Signs of pneumonia (including infiltrative opacities/interstitial opacities)	0 (0)	0 (0)	0 (0)
Abnormal opacities other than signs of pneumonia	0 (0)	1 (2.1)	1 (1.1)
No X-ray	0 (0)	1 (2.1)	1 (1.1)

with no deaths, were observed in 15 patients (36.6%) in the ciclesonide group and 18 patients (36.7%) in the symptomatic treatment group. There were no significant differences in the number of AEs between the two groups. There was one serious AE in the symptomatic treatment group, which was an exacerbation of COVID-19 pneumonia, and the patient recovered after stopping the study and receiving antiviral treatment. AEs that were observed in more than 5% of patients included liver disfunction, an elevated sedimentation rate, a decrease in creatine phosphokinase in the blood, and headaches, all of which were not causally related to the study drug.

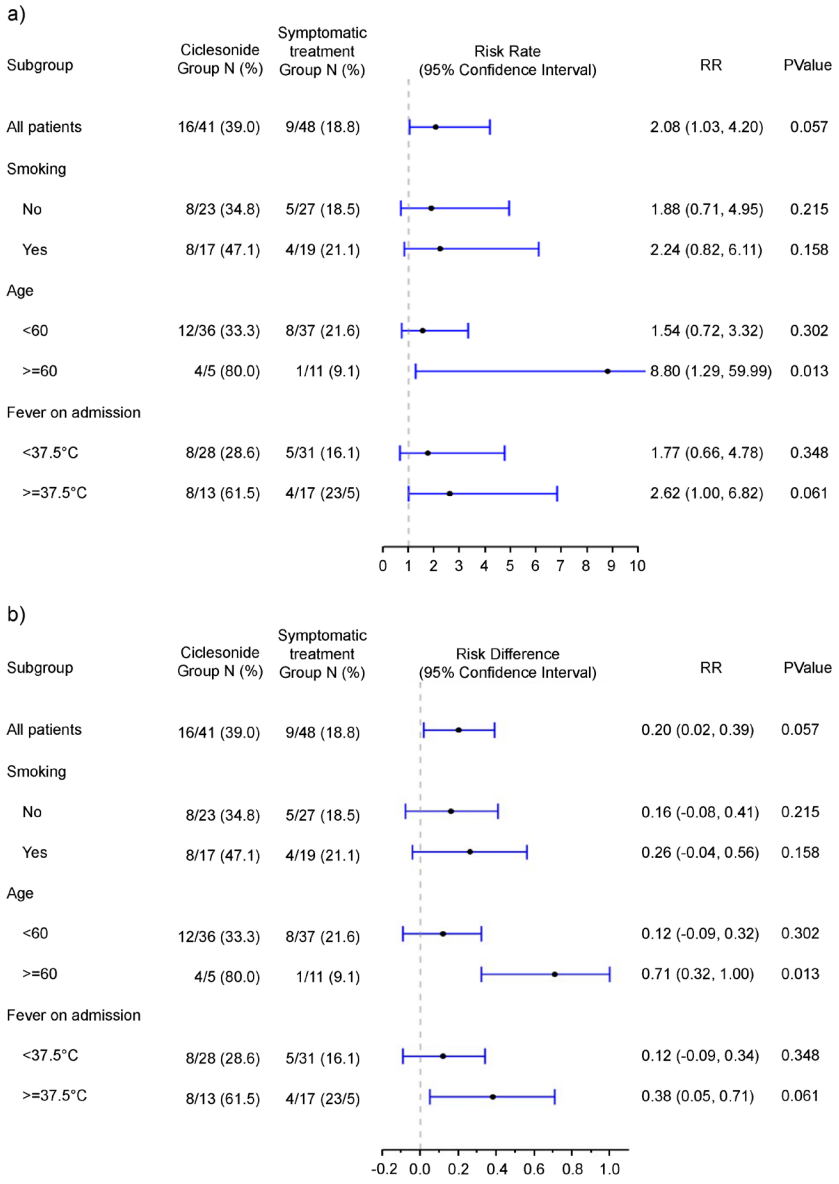

Figure 2. Primary outcomes and subgroup analysis. (**A**) shows the risk rate and (**B**) shows the risk difference. In both analyses, the ciclesonide group tended to have worsening opacities on CT images. Subgroup analysis revealed a similar trend regardless of smoking history or the presence or absence of a fever at admission. Elderly patients had particularly poor results in the ciclesonide group, but caution should be exercised in interpreting the results due to the small number of patients.

Table 2. The incidence of adverse events/side effects

Items	Ciclesonide group (n = 41)			Symptomatic treatment group (n = 49)		
	Number of events	Number of subjects (%)*	95% CI for incidence**	Number of events	Number of subjects (%)*	95% CI for incidence**
Adverse Events	26	15 (36.6)	0.22-0.53	34	18 (36.7)	0.23-0.52
Serious adverse event	0	0	0	1	1 (2.0)	0.00-0.11
Side effect	3	3 (7.3%)	0.02-0.20	0	0 (0.0%)	0.00-0.07
Any serious side effect	0	0 (0.0%)	0.00-0.09	0	0 (0.0%)	0.00-0.07

CI: confidence interval. *The sum of the number of subjects in whom a serious event occurred; if a corresponding event had occurred no less than once in the same subject, the event was counted as a single event. **The confidence intervals were calculated using the Clopper & Pearson method. ***Intergroup comparison was performed using Fisher's exact test.

3.4. Viral load

Figure 3 shows the viral load for each of the groups on Day 1 and within 8 days. There were no significant differences in the viral load between the two groups at either time. The distribution of the viral load from the nasopharyngeal swab on days 1 and 8 is shown in Table 3. Inhalation of steroids did not increase the viral load.

4. Discussion

This study evaluated the efficacy of inhaled ciclesonide in patients with COVID-19 by comparing the rate of exacerbated pneumonia in the inhaled ciclesonide

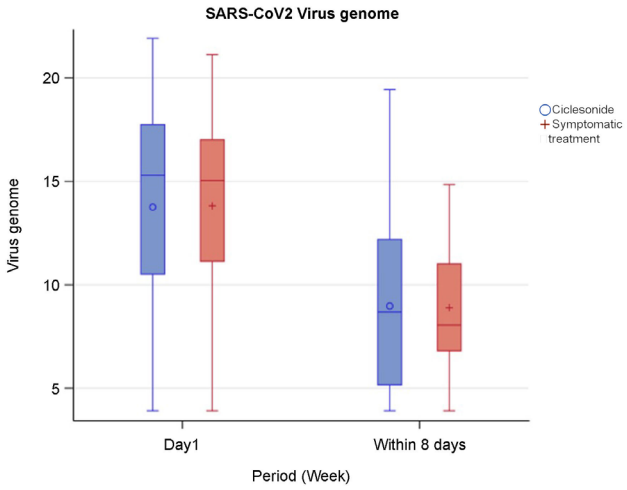

Figure 3. Viral load. Shown here is the amount of viral genome in nasopharyngeal swabs from Days 1 and 8. The amount of viral genome did not differ between the two groups.

Table 3. Nasopharyngeal viral load on days 1 and 8

Full Analysis Set

Treatment group	Summary statistics								*p*-value*
	Number	Average	Std Dev	Minimum	1st quartile	Median	3rd quartile	Maximum	
Ciclesonide group									
Day 1									
Viral load	37	13.76	4.96	3.9	10.5	15.29	17.7	21.9	0.983
Within 8 days									
Viral load	27	8.98	4.04	3.9	5.2	8.69	12.2	19.4	0.894
Variation	27	– 6.68	5.04	– 14.4	– 9.7	– 7.37	– 4.5	11.7	0.587
Rate of change	27	– 37.80	43.65	– 78.6	– 65.7	– 44.35	– 28.7	150.7	0.587
Symptomatic treatment group									
Day 1									
Viral load	39	13.82	4.79	3.9	11.1	15.04	17.0	21.1	
Within 8 days									
Viral load	26	8.90	3.09	3.9	6.8	8.05	11.0	14.8	
Variation	26	– 6.48	3.76	– 13.3	– 9.7	– 6.75	– 3.5	0.5	
Rate of change	26	– 40.20	21.52	– 70.5	– 57.4	– 44.76	– 27.1	7.5	

*Results were evaluating using the Wilcoxon rank-sum test.

Per-protocol Set

Treatment group	Summary statistics								*p*-value*
	Number	Average	Std Dev	Minimum	1st quartile	Median	3rd quartile	Maximum	
Ciclesonide group									
Day 1									
Viral load	36	13.61	4.95	3.9	10.0	15.04	17.6	21.9	0.841
Within 8 days									
Viral load	26	8.88	4.08	3.9	5.2	8.49	12.2	19.4	0.756
Variation	26	– 6.65	5.13	– 14.4	– 9.7	– 7.33	– 4.5	11.7	0.685
Rate of change	26	– 37.74	44.51	– 78.6	– 65.7	– 44.41	– 28.7	150.7	0.553
Symptomatic treatment group									
Day 1									
Viral load	38	13.87	4.84	3.9	11.1	15.24	17.0	21.1	
Within 8 days									
Viral load	25	8.95	3.14	3.9	6.8	8.09	11.0	14.8	
Variation	25	– 6.56	3.82	– 13.3	– 9.7	– 7.36	– 3.5	0.5	
Rate of change	25	– 40.32	21.96	– 70.5	– 57.4	– 45.25	– 27.1	7.5	

*Results were evaluating using the Wilcoxon rank-sum test.

group to that in the symptomatic treatment group. The primary outcome was the proportion of patients with worsening signs of pneumonia on CT images on day 7 in the ciclesonide inhalation group and in the conventional treatment group. Results indicated that the ciclesonide inhalation group had more exacerbated pneumonia than the symptomatic treatment group, even at the conservative two-sided significance level of 10%. Safety did not differ between the two groups, and there were no severe AEs. To the extent known, this is the first randomized clinical trial to yield these findings.

Previous studies have indicated that ciclesonide may be effective in inhibiting SARS-CoV-2 replication at the cellular level. Therefore, the current study hypothesized that inhibition of viral replication would directly inhibit the exacerbation of pneumonia and subsequent severe disease. As the results indicate, CT findings of pneumonia worsened in the ciclesonide group, but there were no instances of severe disease in either group, and clinical findings indicated that the incidence of fever tended to be higher in the ciclesonide group, but there was no exacerbation during the course of this study. There were no cases of severe illness requiring a ventilator and there was no relationship between the study drug and the exacerbation of pneumonia directly leading to severe illness, suggesting that ciclesonide administration temporarily aggravates pneumonia but does not affect the course of treatment.

Ciclesonide is commonly used in respiratory medicine as a treatment for asthma. Experimental data from the National Institute of Infectious Diseases reported that ciclesonide has an antiviral effect against novel coronavirus (SARS-CoV-2) (*7*). In Japan, a case report noted alleviation of pneumonia following the use of inhaled ciclesonide in three patients with COVID-19 (*2*).

However, ciclesonide appeared to exacerbate pneumonia in the current study. Results suggest that mild or asymptomatic cases are likely to improve spontaneously with symptomatic treatment alone.

In SARS, MERS, and influenza, systemic steroid administration is known to delay viral elimination. In SARS, viral replication peaks in the second week, but in SARS-CoV-2 replication peaks early; after the first week, the immunological component becomes the main factor, and this immunological response leads to the development of pneumonia.

However, virologically and epidemiologically, the infectivity of SARS-CoV-2 is considered to be from 2-3 days before to 10 days after the onset of the disease, which means that the signs of pneumonia on images on Day 8 that were assumed to be an immune response were not directly caused by the virus. The current understanding is that ground-glass opacities (GGOs) appear on chest CT images within 3 to 5 days of onset. This is seen in approximately 90% of symptomatic cases (*8*). Even in asymptomatic patients with COVID-19, CT imaging findings of GGOs predominate over consolidation in many patients, but the severity score was higher in symptomatic patients, suggesting that imaging and symptoms may diverge in asymptomatic or mildly symptomatic patients. Therefore, pneumonia may have developed irrespective of antiviral therapy, suggesting that it may have had little impact on the course of treatment in mild cases. GGOs are consistent with type II alveolar epithelial cells infected with SARS-Cov-2, and the opacities may represent inflammation of the structures surrounding these cells. Accordingly, improvement in the pneumogram is not directly related to ciclesonide treatment.

No other studies have assessed imaging to evaluate the efficacy of ciclesonide on COVID-19. In a study of the effect of inhaled ciclesonide on reducing the risk of AEs in COVID-19 outpatients at risk of severe disease, there were no differences in COVID-19 exacerbation by day 14 in the ciclesonide and control groups, and secondary outcomes were similar in both groups (*9*). Blinded, randomized, controlled trials in non-hospitalized patients with symptomatic COVID-19 have indicated that ciclesonide did not achieve its primary endpoint of a reduction in the time to relief of all COVID-19-related symptoms (*10,11*). An open-label phase 2 study indicated that inhalation of ciclesonide accelerated viral elimination from nasopharyngeal swabs on day 14, but there was no difference in the duration of hospitalization or the rate of alleviation of symptoms (*12*). The current study noted no differences due to the intervention between swabs on day 1 and those on day 8. There might have been differences due to the intervention if the swabs on day 1 were compared to those on day 14, but the clinical significance of this difference is uncertain.

The current trial had several limitations. One limitation stems from not knowing the ciclesonide dosage needed to ensure that patients achieved a cellular ciclesonide concentration equal to that found to induce antiviral action in preclinical studies. However, this study used a high inhalation dose, and administering a higher inhalation dose was not possible. In addition, the average duration of administration was five days after the onset of illness, so administering an antiviral so early after the onset of the disease may have been inefficacious, as is true with influenza. Treatments need to be adapted to changes in viral replication. The second limitation is that this study had a small sample size. However, this study had a high level of internal validity because participants were randomized from 22 facilities across Japan. In addition, this study included patients with a confirmed diagnosis according to PCR or antigen testing, and CT images were evaluated blindly by a third party, so this study also had a high level of external validity. The third limitation is that the findings of this study were diminished by not including a placebo control group. However, a placebo could not be prepared as an inhalant because it is very time-consuming and the study needed to start as soon as possible in response to

the rapid spread of COVID-19. The primary endpoint was assessed in a blinded setting, and presumably there is no bias in the results because of the fact that this study was open-label. The use of CT imaging as an endpoint is also controversial, but this study was designed in the very early days of the COVID-19 epidemic, and whether use of imaging would be appropriate had yet to be determined. Several studies on ciclesonide have been conducted, but the endpoints have varied.

In conclusion, ciclesonide was found to exacerbate signs of pneumonia on CT images without worsening clinical symptoms in individuals with mild or asymptomatic symptoms of COVID-19.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Original Article

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In vitro study about prevention of vascular reocclusion by low intensity ultrasonic irradiation

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SUMMARY For the treatment of acute ischemic stroke, the current standard of care is thrombolysis by the administration of intravenous (IV) recombinant tissue-type plasminogen activator (rt-PA). Although this approach is proven to be effective, reocclusion within 24 hours occurs in about 20% of patients who receive recanalization by rt-PA. In addition, the administration of anticoagulants within 24 hours after IV rt-PA increases the risk of intracranial hemorrhage; therefore, treatment with anticoagulants is contraindicated in this population. To address the need for an approach to sustain the effects of thrombolysis prevent blood vessel reocclusion without the use of anticoagulants, this study proposes a novel method using a low-intensity ultrasound (US) irradiation. An *in vitro* thrombus-growth model, in a latex rubber container was developed to study the effect of thrombus-growth suppression by US irradiation at 500 kHz in a 37°C water bath. The US acoustic intensity was set at or below 0.72 W/cm^2, which is the maximum allowed for noninvasive acoustic irradiation. Low-intensity US irradiation of the thrombus-growth model resulted in a remarkable suppression of thrombus growth (100.22 ± 10.1 mg *vs*. 50.22 ± 5.3 mg, $p < 0.0001$), and the clot-growth inhibition depended logarithmically on acoustic intensity. Thrombus growth can be suppressed by low-intensity US irradiation, opening a new way to combat vascular reocclusion after rt-PA treatment of acute ischemic stroke.

Keywords Acute ischemic stroke, reocclusion, prevention, non-invasive ultrasound, recombinant tissue-type plasminogen activator

1. Introduction

As the only approved therapeutic drug for patients with acute ischemic stroke (AIS), intravenous (IV) recombinant tissue-type plasminogen activators (rt-PAs) are widely used (*1,2*), yet still limited by many factors, including a narrow time window for treatment, the risk of systemic hemorrhage, and a high rate of non-recanalization (*3,4*). To enhance the effect of IV rt-PA and decrease its systemic adverse effects, many studies have shown that ultrasonic treatment may be a promising new regimen to either destroy clots directly with high intensity focused ultrasound (US) (*5-9*) or enhance chemical thrombolysis with relatively low intensity US (*10-12*).

Although, recanalization may be achieved successfully by IV rt-PA or other thrombolytic therapy, the development of blood-vessel reocclusion occurs with high probability (14-34% of cases) within 24 hours after IV rt-PA therapy (*10,13*). One of the main reasons for blood-vessel reocclusion is thrombus regrowth. When antiplatelet drugs are administered to prevent reocclusion within 24 hours after IV rt-PA therapy, the rate of symptomatic intracranial hemorrhage significantly (*4,13*). Consequently, the use of drugs such as anticoagulants to prevent reocclusion is prohibited.

In our previous work, we reported the use of low-intensity US to inhibit thrombus-growth. This method, which requires no surgery or medication, is a novel approach to overcome reocclusion after IV rt-PA therapy (*14*). In that study, thrombus-growth was inhibited by US irradiation administered at 0.72 W/cm^2 intensity in an *in vitro* thrombus-growth model, which was prepared using bovine plasma in a cylindrical acrylic container. This *in vitro* thrombus-growth model used clots for growth that included surplus coagulation factors. These coagulation factors were slowly released in plasma, thereby coagulating the surrounding plasma into new clots. The rate of thrombus-growth inhibition was calculated from the experimental results, and the

relationship between this rate and the US acoustic intensity was analyzed. Results indicated that the rate of thrombus-growth inhibition was approximately linear in acoustic intensity. However, the relationship exhibited a slightly S-shaped curve, suggesting the possibility that US reflection may have influenced the experimental results. In other words, we posited that our technique of administering US irradiation in a bovine thrombus-growth model in an acrylic cylindrical container may have resulted in the US waves being reflected from the container wall, thereby potentially significantly increasing the acoustic intensity in the center of the container. The main reason for this effect was that the dimension of the ultrasonic beam was enlarged because of the distance between the US transducer and the target, and thus, more reflection was likely to occur. In the previous study, the distance between the US transducer and the surface of the clot was 28 mm; in our subsequent study, the distance was corrected to 15 mm and the data were reacquired. In addition, by expanding the aqueous layer irradiated by US and using more US-absorbing materials than in the previous experiment, we reduced US reflections in the water tank. The overall result was thus less affected by US reflections.

In addition to these modifications, in this study, we used a latex rubber container, which has low US reflectivity, to hold the thrombus-growth model. The US reflectance of acrylic and latex rubber was calculated by the following equation:

$$I_r = (Z_1 - Z_2)^2 / (Z_1 + Z_2)^2,$$

where I_r is the reflectance of acoustic intensity, Z_1 is the acoustic impedance of plasma, and Z_2 is the acoustic impedance of acrylic or latex rubber.

The equation gives a reflectance of about 11.5% for acrylic and about 2% for latex rubber, so we posited that an experimental system fabricated from latex rubber would reduce US reflections. Therefore, by using a thrombus-growth model fabricated from a latex rubber tube, we verified the inhibition of thrombus growth resulting from US irradiation and its dependence on acoustic intensity.

As transmittance increases for lower-frequency US, clinical transcranial US diagnostic equipment often uses US frequencies around 2 MHz, which is a relatively low frequency used to penetrate cranial bone. However, as the transmittance through cranial bone is insufficient even at US frequencies around 2 MHz (*15-17*), we used 500 kHz as the US frequency. This frequency is reported to give superior cranial ultrasonic permeability and is likely safe for brain tissue (*18,19*).

We report herein that clot-growth is suppressed by US irradiation and that, based on an *in vitro* thrombus-growth model, the thrombus-growth rate depends logarithmically on the acoustic intensity within the setting of noninvasive acoustic intensity.

2. Materials and Methods

2.1. Preparation of thrombus-growth model

Citrated and freeze-dried bovine plasma (P4639-10ML, Sigma-Aldrich Japan K. K, Tokyo, Japan) was rehydrated and degassed at −0.04 MPa for 5 minutes. Clots for growth (growth-clots) were prepared from degassed plasma, 1 M calcium dichloride ($CaCl_2$) (FUJIFILM Wako Chemicals, Osaka, Japan), and thrombin (206-18411, FUJIFILM Wako Chemicals), with a final concentration of 50 mM $CaCl_2$ and 1 U/mL thrombin. The clots were incubated at 37°C for 60 min.

2.2. Acrylic plate used in thrombus-growth-suppression study

An acrylic plate cell was prepared as described previously (*11*). The design was a 15-mm discoidal hole drilled in the center of a 3-mm-thick acrylic plate, and a 0.3 mm-thick polycarbonate sheet was affixed to its back face for use as a clot cell (Nissindenki, Tokyo, Japan). A 535-μL discoidal growth-clot that had a 15-mm diameter and 3-mm-thick was prepared in the hole. After being covered with degassed bovine plasma contained in another clot cell, the discoid growth-clot was irradiated with US for 30 minutes. in a 37 °C water bath (Fiure 1a). The anti-thrombus-growth effect was evaluated as detailed in previous study (*11,12*). In brief, the optical density value was recorded before and after ultrasonication, and the clot thickness was calculated from the calibration curve.

2.3. Latex tube used in thrombus-growth-suppression study

A 200-μL growth-clot was prepared in the bottom of a latex tube (Finger Cots Unroll Type S, AS ONE, Osaka, Japan) with a 15-mm-inner-diameter. The latex tube was filled with degassed bovine plasma, which was then irradiated with US for 30 minutes in a 37°C water bath. The anti-thrombus-growth effect was evaluated based on the changes in clot mass on before and after ultrasonication (Figure 1b). The clots mass before ultrasonication was calculated by the following formula: clot with latex tube weight before ultrasonication (without plasma) minus empty latex tube weight. The clots mass after ultrasonication was calculated by the following formula: clot with latex tube weight after ultrasonication (plasma removed by micropipette) minus empty latex tube weight.

2.4. Ultrasound conditions

The US conditions were established as detailed in a previous study (*20*). In brief, a 10-mm-diameter US transducer (Honda Electronics, Aichi, Japan) was

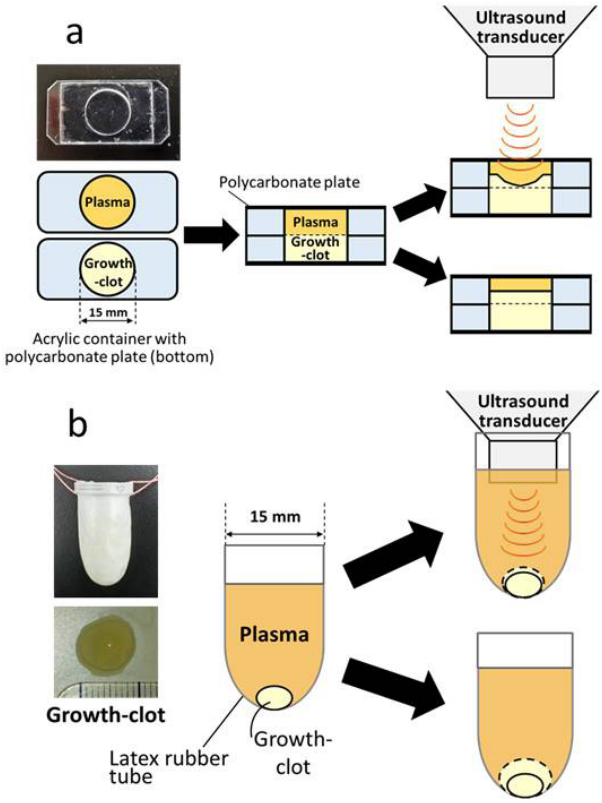
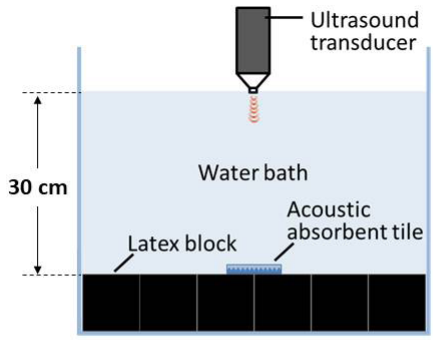


Figure 1. Schema of experimental apparatus for the anti-clot-growth model. (a) Fresh plasma and growth clot were prepared in two acrylic containers and were overlapped in such a way that no air was trapped between them. The clot was irradiated by US from the plasma side for 30 minutes. in a 37°C water bath. **(b)** Fresh plasma was poured around the growth clot prepared in a rubber latex tube, and the clot was irradiated by US in plasma for 30 minutes. in a 37°C water bath. The growth-clot in the rubber latex tube was about 9 mm in diameter, which is the same size as the acoustic-field distribution skirt of the US main robe. One scale of the measure in the picture of Growth-clot is 1mm. In both experimental conditions, the distance from the transducer to the surface of the growth-clot was adjusted to 15 mm.

operated at 500 kHz in the continuous-wave mode and at a maximum acoustic intensity of 0.72 W/cm^2. Measurements were made in a clear water tank using the Acoustic Intensity Measurement System (AIMS, Onda, Sunnyvale, CA, USA) with a 0.2-mm-diameter hydrophone probe (HNP-0200, Onda). The average acoustic intensity was measured to be 0.25 W/cm^2 at the maximum intensity 0.72 W/cm^2 by using a radiation method with the Ultrasound Power Meter (UPM-DT-1AV, Onda). An acoustic intensity of 0.72 W/cm^2 is the maximum average intensity allowed by the U.S. Food and Drug Administration (FDA) for diagnostic US equipment.

Rubber blocks with a side length of 10 cm were place on the bottom of the water bath, and an acoustic-absorbing tile (EPI_EUA101A, Onda) was placed under the transducer and on the rubber blocks. The distance between the US transducer and the acoustic-absorbing tile was about 30 cm (Figure 2).



Radial Distance from US transducer centerline (mm)	0	|0.5|	|1.0|	|1.5|	|2.0|	|2.5|	|3.0|	|3.5|	|4.0|	|4.5|
Acoustic intensity at 15 mm form US transducer (W/cm^2)	0.72	0.69	0.63	0.52	0.39	0.25	0.11	0.07	0.03	0.01

Figure 2. Experiment environment and acoustic-field distribution schema showing US irradiation environment. The water layer irradiated by US was designed to be less susceptible to the influence of US reflections. The acoustic-field distribution was measured by the AIMS at 15 mm from the US transducer, which is the distance from the US transducer to the clot surface. The acoustic intensity is shown in 0.5 mm intervals from the center of the US transducer. The average acoustic intensity was 0.2 W/cm^2.

2.5. Calculation of anti-clot-growth ratio

To evaluate the effect of US irradiation on the clot-growth rate, we calculated the anti-clot-growth ratio (R) at each acoustic intensity level using the following equation:

$$R = (\Delta \text{ non-US} - \Delta \text{ US}) / \Delta \text{ non-US} \times 100\%$$

where, Δ non-US (Δ US) is the clot size change in thickness (mm) or weight (mg) when not exposed to US irradiation for the experimental system with the acrylic container or the experimental system with the latex rubber container.

2.6. Statistical analyses

Five sets of two clots (for a total of 10 clots) were prepared for each intensity level. The differences in clot thickness or mass between the clots exposed and not exposed to US irradiation were examined using the paired Student's *t*-test. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Thrombus-growth-suppression effect by ultrasound in the thrombus growth model in acrylic container

Results of low-intensity US irradiation in a thrombus-growth model prepared in an acrylic container revealed that thrombus-growth was significantly suppressed at all measurement points, with an acoustic intensity of

0.01-0.72 W/cm^2 (Figure 3a). The rate of clot-growth suppression was about 50% at 0.72 W/cm^2, which is the maximum acoustic intensity of the ultrasonic diagnostic equipment allowed by the FDA. The rate of clot-growth suppression was found to depend logarithmically on the acoustic intensity, as indicated by the high correlation with the logarithmic formula (Figure 3b). Furthermore, based on its approximate expression, the acoustic intensity threshold above which clot-growth is suppressed by US irradiation was calculated to be close to 0 W/cm^2.

3.2. Thrombus-growth-suppression effect by ultrasound in the thrombus growth model in latex rubber container

US irradiation of the thrombus-growth model prepared in a rubber latex tube reveals that the clot-growth was remarkably suppressed at peak acoustic intensities of 0.18, 0.36, and 0.72 W/cm^2, representing average intensities of 0.06, 0.13, and 0.25 W/cm^2, respectively (Figures 4a and 4b).

The rate of clot-growth suppression was about 50% at an acoustic intensity of 0.72 W/cm^2. The acoustic intensity was not adjusted to be the average acoustic intensity of 0.25 W/cm^2, but instead to the maximum acoustic intensity of 0.72 W/cm^2 because use of the average acoustic intensity would lead to a maximum acoustic intensity exceeding the threshold allowed for noninvasive acoustic intensity. Analysis of the relationship between the rate of clot-growth suppression and acoustic intensity showed a high correlation with a logarithmic expression, as evident when using an acrylic container (Figure 4c). Furthermore, based on this approximate expression, the acoustic intensity threshold

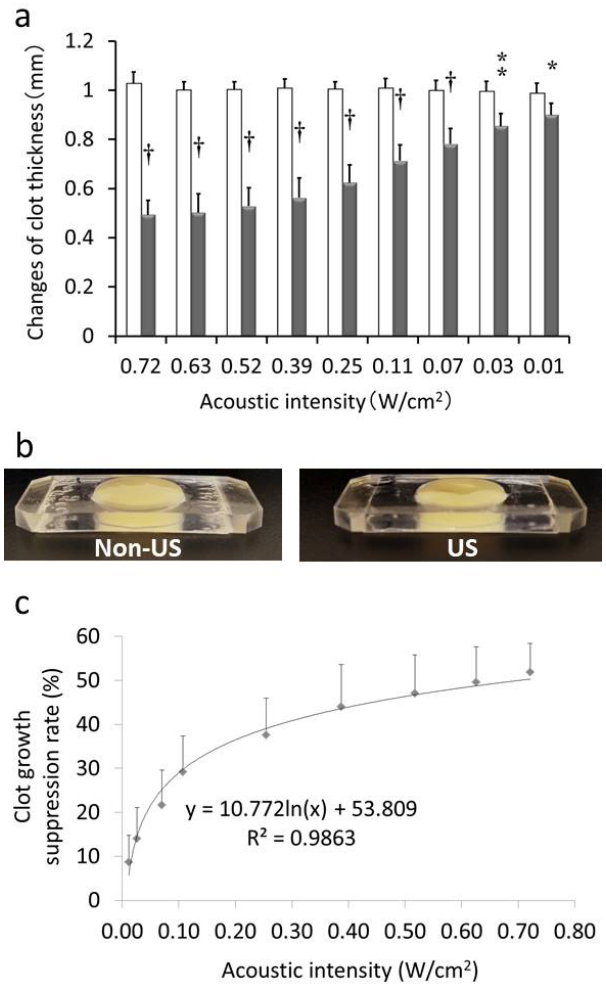


Figure 3. Clot-growth suppression in an acrylic plate. (a) The suppression of clot growth by US irradiation was evaluated in one dimension by calculating changes in clot thicknesses before and after US irradiation. **(b)** Post-experimental growth-clot photograph. **(c)** The relationship between clot-growth-suppression rate and acoustic intensity is plotted and fit with a logarithm. (■) Group irradiated by US, (□) group not irradiated by US. *$p < 0.05$, **$p < 0.001$, $^{\dagger}p < 0.0001$ vs non-US (Student's *t*-test), mean ± SD ($n = 5$ for each group).

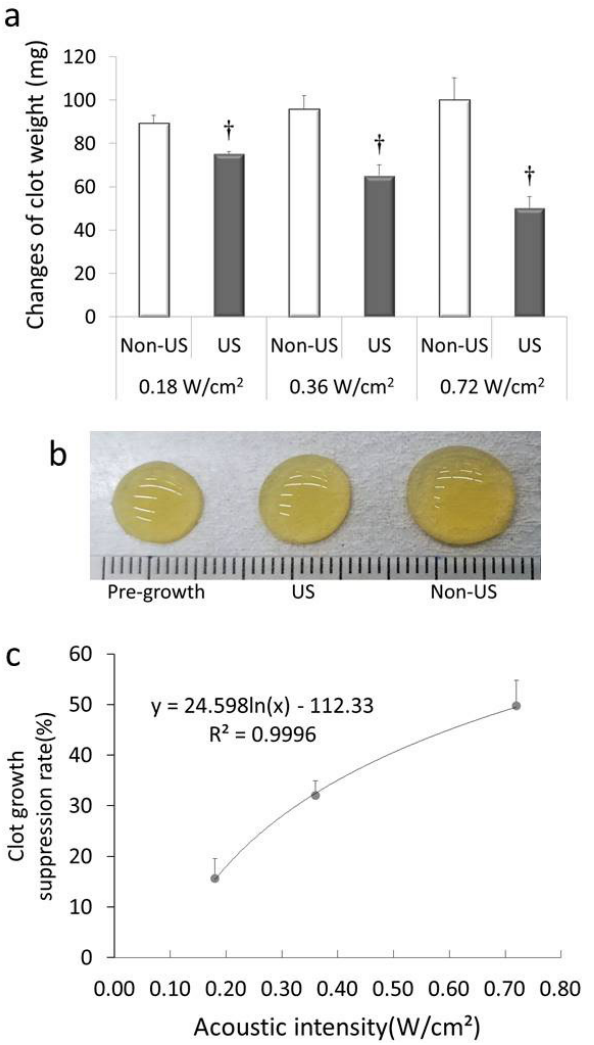


Figure 4. Clot-growth suppression in latex tube. (a) The suppression of clot-growth by US was evaluated in three dimensions by calculating the increase in thrombus mass. **(b)** Growth-clot photograph before and after the experiment with an acoustic intensity 0.72 W/cm^2. One scale of the measure is 1 mm. **(c)** The relationship between clot-growth-suppression rate and acoustic intensity is plotted and fit with a logarithm. $^{\dagger}p < 0.0005$ *vs.* non-US (Student's *t*-test), mean ± SD ($n = 5$ for each group).

above which clot-growth suppression occurs as a result of peak US irradiation was calculated to be about 0.1 W/cm^2 or an average acoustic intensity about 0.035 W/cm^2.

4. Discussion

This research shows that clot-growth can be suppressed by approximately 50% at most in a thrombus-growth model simply by exposing it to US irradiation at 500 kHz. These results were confirmed by using methods with noninvasive low acoustic intensity in two different experimental systems with acrylic and latex rubber containers.

Our results on clot-growth volume are similar to those obtained in the previous study on clot-growth suppression using acrylic containers. However, the present regression line that shows the relationship between the rate of clot-growth suppression and acoustic intensity differs from the line obtained previously. In the previous study, the relationship was linear, although with a slight S-shape, whereas in this study, the relationship is better approximated as logarithmic. This difference is tentatively attributed to the lower US reflectivity in this study, which minimized the influence of US reflection than that in the previous study. It should also be noted that in the previous experiment with the acrylic container, clot growth was evaluated based on clot length (mm), whereas in this study clot-growth was evaluated based on clot mass (mg) using the rubber latex tube. This difference is analogous to the difference between a one-dimensional and a three-dimensional evaluation, which may contribute to the difference between the results of these two models. Calculating the threshold value of the acoustic intensity and the thrombus-growth-suppression effect using the equations presented in Figure 3c and Figure 4c shows different values of about 0 and 0.1 W/cm^2, one-dimensionally and three-dimensionally, respectively. Therefore, it may be possible to suppress the growth of thrombus with a slight acoustic intensity over 0 W/cm^2, but to suppress reocclusion, it is necessary to suppress the thrombus-growth three-dimensionally, so the threshold value may be about 0.1 W/cm^2. In the experiment to measure clot thickness, an acrylic container with polycarbonate as the bottom material, which has a relatively close acoustic impedance to water among hard materials, was used to maintain the shape of the clots. In the experiment to measure the clot weight, latex rubber was used, which is a material with acoustic impedance closer to that of water. In addition, a large acrylic water bath was prepared to prevent the reflection of US, and a sound-absorbing material was placed on the bottom. However, this technique cannot completely prevent the reflection of US. We calculated that at least about 11.5% reflection occurred in the experiment using acrylic and about 2% in the experiment using latex rubber. However, most of the ultrasonic reflections may have been influenced by the bottom polycarbonate surface of the acrylic container by reducing the distance between the US transducer and the clot to suppress the reflection from the acrylic surface. In that case, US causes almost no diffuse reflection from the acrylic surface, and polycarbonate has an acoustic impedance closer to water than acrylic, so the reflection may have been suppressed even to a smaller degree. In addition, the experimental results using latex rubber with extremely low ultrasonic reflection (Figure 4c) and the experimental results using an acrylic plate (Figure 3b) showed that the thrombus-growth rate depends logarithmically on the acoustic intensity. These findings suggest that the experimental results using the acrylic plate did not cause diffuse reflection as in the previous experiment.

Reproducing the *in vivo* environment with the *in vitro* thrombus-growth model used in this study has some limitations. For example, in a living body, changes occur in the balance between the fibrinolytic system and the coagulation system and with the presence of endothelial cells, blood flow, pulsation, and blood pressure, among other factors, thereby preventing the *in vivo* environment from being completely reproduced in this *in vitro* study. It is unknown whether these factors affect thrombus-growth and the thrombus-growth-suppression effect achieved by US. Therefore, in future work, we will evaluate the preventive effect of US on vascular reocclusion in an animal model. Furthermore, we plan to use a thrombus-growth model with human plasma, fabricated in the same way as for the thrombus-growth model using bovine plasma discussed herein. We will then examine the preventive effect of US on vascular occlusion by studying clot-growth suppression as a function of various US parameters, such as frequency and continuous or pulsed wave, among other variables.

Many studies have been reported on the treatment of thrombosis with US, with or without rt-PA. In particular, magnetic resonance imaging guided high intensity focused US (MRg-HIFU) is expected to serve as a noninvasive method that can accurately target only the thrombus (*5*).

Focus US is used for clot breakdown and histotripsy thrombolytic therapy because it requires high acoustic intensity of several hundred watts, and cavitation is involved in the mechanism (*6-9*). Maxwell *et al*. reported that peak negative pressure of approximately 4 MPa is required for US histotripsy thrombolysis of whole blood clots in a model using dogs (*7*). In this study, the US conditions were 500 kHz, continuous-wave, maximum acoustic intensity 0.72 W/cm^2 (peak negative pressure 0.2 MPa), and it is unlikely that cavitation would be induced in degassed plasma. There was no difference in thrombus-growth weight (mg) that 0.72 W/cm^2 US was irradiated to the growth thrombus

using bovine serum instead of bovine plasma (non-US *vs*. US, 2.10 ± 0.557 mg *vs*. 2.07 ± 1.33 mg, mean ± SD, $p = 0.970$, $n = 3$ for each group, data not shown). The slight increase of thrombus weight (about 2 mg for each group) in this experiment seemed to be caused by serum that could not be removed with a micropipette. Therefore, it is thought that thrombus growth is suppressed by a mechanism different from those for clot breakdown and histotripsy thrombolytic models. This mechanism is currently under consideration, but is still unclear.

The use of 0.72 W/cm^2 US is an extremely noninvasive approach, but may require safety considerations when used intracranially. In a clinical study by Daffertshofer *et al.*, significant intracranial hemorrhage was caused when rt-PA was used in combination with US at 300 kHz and 0.7 W/cm^2 (*21*). It is believed that one of the causes of this finding is that cavitation was induced by hot spots caused by standing waves generated in the skull. Therefore, we consider it an important task to verify the inhibitory effect on thrombus-growth in the skull in a future study. However, according to the report by Shimizu *et al*., the safety of transcranial US at 500 kHz and 0.72 W/cm^2 was evaluated with the cynomolgus monkey, and no neurologic deficits were found on histologic evaluation. Furthermore, no neurologic deficits were observed when 500 kHz US and rt-PA were used in combination in a model using rhesus monkeys (*18*). This report suggests that low-intensity 500 kHz transcranial US has the potential to be safely applied to thrombus-growth suppression as well.

Thrombus-growth was suppressed by low-intensity US irradiation in an experimental setting with almost no reflection, and it was possible to measure the threshold acoustic intensity above which clot-growth is suppressed. We expect that this technology will continue to be developed until it can be used to prevent various thromboses, including reocclusion of blood vessels after rt-PA treatment of AIS.

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Brief Report

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Cross-infection risks of SARS-CoV-2 while playing catch using a baseball: Creating a safe sporting environment during the COVID-19 pandemic

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SUMMARY Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of the coronavirus disease (COVID-19), is found primarily in the respiratory tract secretions of infected individuals with one of the main routes of transmission being direct or indirect contact. In this study, using fluorescent paint, we evaluated the spread of contaminants while playing catch with a baseball. Fluorescent paint was applied to the right hand of a right-handed baseball player who then engaged in playing catch with 10 other right-handed players (partners) for 5 min each. The fluorescent paint was detected on the right hands (inside) and gloves (inside) of all the 10 partners as well as on the ball; in some partners it was also detected on the back of the right hands or the back of the gloves. However, except for their right hands, fluorescent paint was not detected on the surface of the bodies of the partners. These observations indicated that the fluorescent paint (mimicking virus-containing contaminants) on the hand spreads very efficiently from person to person during the throwing and catching of a baseball, suggesting that a thorough and frequent disinfection of the hands and equipment is important in the prevention of infections that may occur while playing baseball.

Keywords Cross-infection, playing catch, baseball, fluorescent paint, SARS-CoV-2

1. Introduction

The coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first reported in Wuhan, China in December 2019 (*1*) and has resulted in a global pandemic. Since SARS-CoV-2 is contained primarily in the airway secretions of infected persons, the main route of transmission is considered to be through droplets derived from the infected persons. In an enclosed environment, moreover, an aerosol infection may occur when aerosols derived from the infected persons float in the air for extended periods of time (*2*). Transmission may also occur through surfaces contaminated by direct or indirect contact with mucous membranes in the eyes, mouth, or nose of infected persons(*3*). SARS-CoV-2 has been reported to exist as an aerosol form for several hours and may be present on environmental surfaces for several days (*2*).

The measures taken to prevent SARS-CoV-2 infection include the use of appropriate preventive measures (such as personal protective equipment, and disinfectants) to contain the spread of the pathogen effectively (*4*). It has been recommended that contaminated environmental surfaces be treated with disinfectants and chemical agents, such as alcohol, which is considered to be highly effective (*5*). In the medical field, fluorescent paints and black light are used widely to visualize the effect of infection control measures for the purposes of research and to raise awareness (*6,7*). In this study, we investigated spread of a contaminant (source of infection) during a baseball game using fluorescent paint to visualize the process. In addition, we developed countermeasures to reduce the spread of the disease.

2. Materials and Methods

2.1. Study participants

The participants in this study were 11 right-handed male baseball players, with a mean age of 19.5 years,

who belonged to a university baseball team and who had been playing baseball for at least 10 years. The participants were informed in advance of the purpose and the method of the study, and the management of their personal information. All the participants provided written informed consent, prior to their participation in the study. This study was approved by the Ethics Committee of Chubu University (approval number 20200039). This study was conducted in accordance with the principles of the Declaration of Helsinki.

2.2. Evaluation methods

The study was conducted with one baseball player (regarded as a hypothetically infected person) and 10 other players (partners). With the assumption that the right hand of the hypothetically infected player would be covered with the infectious disease pathogen, fluorescent paint (Spectro-Pro Plus, Morain Cooperation Co., Ltd., Tokyo, Japan), which mimicked virus-containing contaminant, was applied to the right hand of the hypothetically infected player which could be detected using a black light illuminator (Stand Type Hand Wash Checker BLB Set, Saraya Co., Ltd., Osaka, Japan) (Figure 1A). The hypothetically infected player performed playing catch, with 10 different partners for 5 min each (Figure 1B). For every partner, the hypothetically infected player was applied with the fluorescent paint on the right hand and used a new ball. The partners were examined under black light and before and after playing catch.

3. Results and Discussion

In this study, we applied fluorescent paint, which mimicked virus-containing contaminant, to the hand of a baseball player (regarded as a hypothetically infected person), who then played catch with 10 partners for 5 min each. We next visualized the fluorescent paint on the surfaces attributable to contamination by using black light. Table 1 summarizes the results of the fluorescent paint detection on the surface areas (Figure 2) of the 10 partners. The fluorescent paint was detected on the inside of the right hands of all 10 of the partners ([1] and [2]), while it was not detected on the inside of the left hands of the partners ([3] and [4]). Interestingly, the fluorescent paint was detected on the back of the right hands ([5]and [6]) and gloves ([12] and [13]). On the other hand, the fluorescent paint was not detected on any surface area of the bodies of the partners (not shown in Table 1). These findings suggest that contaminant on the infected person's hand can spread to other person *via* a ball during playing catch.

The areas of the partners in which no fluorescent paint was detected were considered to be free from direct contact with the ball. However, fluorescent paint was detected in two locations on the back of the glove and two locations on the back of the right hand that were not in direct contact with the ball on some, though not all, partners (Table 1: [5], [6], [7], [8]). First, with regard to the back of the right hand; it is possible that the fluorescent paint adhering to the front of the glove may have been transmitted when the player gripped the ball in the glove. It is also expected that the act of hitting the front of the glove with the fist with the ungloved hand, which baseball players often do between ball catches, may have caused this secondary transmission. In addition, the detection of fluorescent paint at the back of the glove may be considered to be due to contact between the back of the glove and the right palm, which was covered with fluorescent paint. In brief, the contaminants were expected to be transferred from the hands of the hypothetically infected person to the ball, followed by the glove, palm, or fist of the partners, and subsequently to anything that came into contact with the infected body parts or surfaces, causing secondary or tertiary transmission.

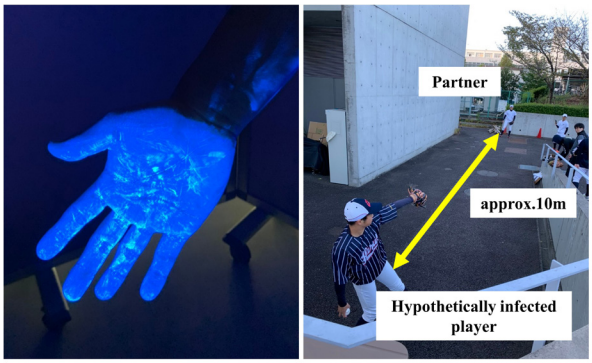

Figure 1. Evaluation method. (A) Fluorescent paint was applied to the right hand of the hypothetically infected player. The paint on the hand could be visualized as blue emissions under the black light. **(B)** The distance between the hypothetically infected player and his partner was approximately 10 m.

In the present study, no fluorescent paint was detected on the body; however, the body may not have been touched with the palm of the right hand because the exercise was continuous, *i.e.*, playing catch for 5 min. However, in a normal baseball practice or game, exercise is often interrupted; therefore, the contaminated hands may have been brought into contact with the eyes, nose, and mouth. The U.S. Centers for Disease Control and Prevention (CDC) have pointed out that people should be aware of contact transmission and should not touch their own eyes or nose to prevent new coronavirus infection. For example, if an infected person sneezes or coughs while covering the nose or mouth with his or her hand and then touches something around with that hand, the virus is transmitted *via* that hand, and then, if another person touches the same item the virus will stick to his or her hand. Now, if this person touches his or her eyes, nose, or mouth with the contaminated hand, the virus may then be transmitted

Table 1. Detection of fluorescent paint on the bodies and equipment

Evaluation areas	Partners										Positive rate (%)
	A	B	C	D	E	F	G	H	I	J	
Hand (inside) right											
[1]	+	+	+	+	+	+	+	+	+	+	100
[2]	+	+	+	+	+	+	+	+	+	+	100
Hand (inside) left											
[3]	-	-	-	-	-	-	-	-	-	-	0
[4]	-	-	-	-	-	-	-	-	-	-	0
Hand (back) right											
[5]	-	+	-	+	-	-	+	+	+	+	60
[6]	+	-	-	-	-	-	-	+	-	+	30
Hand (back) left											
[7]	-	-	-	-	-	-	-	-	-	-	0
[8]	-	-	-	-	-	-	-	-	-	-	0
Glove (inside)											
[9]	+	+	+	+	+	+	+	+	+	+	100
[10]	+	+	+	+	+	+	+	+	+	+	100
[11]	+	+	+	+	+	+	+	+	+	+	100
Glove (back)											
[12]	-	-	-	-	-	-	-	-	+	+	20
[13]	-	-	-	-	-	-	-	-	-	+	10
[14]	-	-	-	-	-	-	-	-	-	-	0

The 'catch' surface of the grove was defined as "inside" while the opposite side as "back." The evaluation of paint detection was performed by tat least three evaluators, and all the agreed-upon fluorescence-positive were marked with '+,' otherwise were marked '-.' The evaluation areas [1]-[14] correspond to those shown in Figure 2.

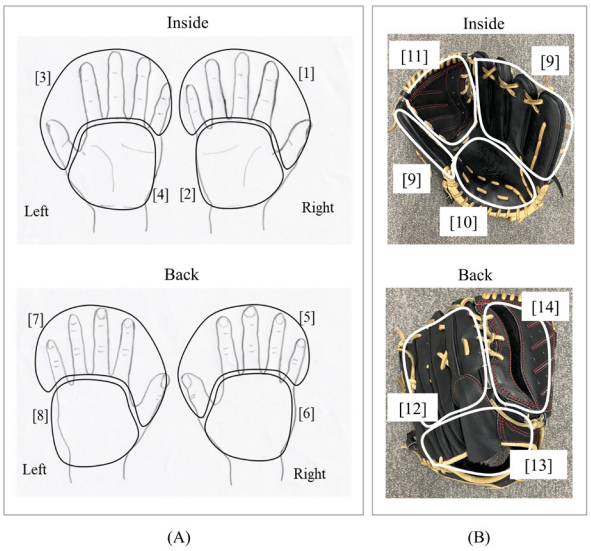

Figure 2. Evaluation areas. Hands (A) and glove (B) of partners.

through this person's mucous membranes. According to previous reports, people unconsciously touch their faces on an average of 23 times per hour, and approximately 44% of all cases touch the mucous membranes of the eyes, nose, and mouth (*8*). Since the results of this study suggest that transmission may occur from the hands of a hypothetically infected person to a hypothetically uninfected person *via* a contaminated ball, caution should be exercised.

The risk of COVID-19 infection has been considered to be low in sports that are conducted outdoors at a sufficient distance (*9*). The baseball game is usually performed outdoors at a sufficient distance. However, this experiment showed that contaminants may be transmitted from the hands of an infected person to the hands and glove of other players *via* the ball.

Although no previous studies on the transmission of contaminants *via* sports equipment have been found, an experiment has been reported, wherein, the SARS-CoV-2 virus was applied to the surfaces of 10 types of balls, including tennis balls; while the virus was not detected on any of the types of balls after 15 min, it was detected on seven of the 10 types after 1 min (*10*). Based on the results of this study, there is a risk of transmission *via* equipment such as balls, immediately after virus adherence. This suggests that the removal of contaminants from hands, balls, and other sports equipment may be an effective measure to prevent infection transmission during game play. Experiments applying high concentrations of SARS-CoV-2 to the surfaces of sports balls, particularly soccer balls (footballs), tennis balls, golf balls, and cricket balls, have shown that no traces of the virus were detected when the balls were wiped with dry or damp wet wipe, or dropped and rolled (*11*). Furthermore, there have been reports that despite the presence of infected participants there has been no evidence of potential surface infections in soccer club facilities where regular cleaning procedures were in place (*12*).

Cleaning and disinfection with alcohol has been reported to be effective in eliminating the microbial contamination of medical devices and other equipment

(*13*). Frequent hand washing with water and soap, and hand disinfection with 70% alcohol base are used as infection control protocols in medical settings (*14*). Therefore, the frequent wiping of balls, gloves, and other equipment, and hand washing during play is required to inhibit or control the spread of viruses and other infectious agents on the sports field. However, because it is impractical to use water and soap to wash hands on the baseball field during practice and games, alcohol disinfection may be effective and is recommended as an infection control measure.

The COVID-19 pandemic disrupted organized sports in communities indirectly by the enforcing of cancellations, drastic schedule alterations, or postponement of sports events. This had a significant impact on the psychosocial and physical development of athletes (*15*). Therefore, it was necessary to examine the infection control measures that may be used to achieve a safe environment for sport play and participation. Therefore, based on the findings of this study it is recommended that frequent hand alcohol disinfection during sporting activities is an effective method of creating a safe sports environment.

However, there were two limitations to this study. First, this study used fluorescent paint and not the actual virus. Second, since the experiment in this study was carried out individually with the 10 partners, we were unable to confirm the extent to which contaminants can be transmitted when playing with a large number of players. During actual games and practices, contaminants may infect other players *via* the balls that were touched by infected players.

We were able to visualize the gradual spread of the fluorescent paint that was applied to one player's hand and observe its transmission to another player's glove and hand *via* the ball while playing catch that occurs in baseball. This indicated the risk of viral transmission and other contaminants through sports equipment. Thus, the frequent disinfection of hands with alcohol during sport play is recommended as a countermeasure.

4. Conclusion

This study suggested the possibility of transmission of contaminants while playing catch that occurs in baseball. During the COVID-19 pandemic, the frequent disinfection of hands with appropriate disinfectants and chemical agents, such as alcohol, while playing sports is, therefore, recommended and may contribute to a safer sports environment.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Brief Report

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Cetirizine more potently exerts mast cell-stabilizing property than diphenhydramine

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SUMMARY Cetirizine, a second-generation antihistamine, and diphenhydramine, a first-generation antihistamine, are among the most widely used anti-allergic drugs. In addition to longer duration of action and less incidence of sedative side effects, recent clinical studies also indicate a higher potency of cetirizine than diphenhydramine in the treatment or prevention of allergic disorders. In the present study, using the differential-interference contrast (DIC) microscopy, we examined the effects of cetirizine and diphenhydramine (1 μM to 1 mM) on the degranulation from rat peritoneal mast cells. Using fluorescence imaging of a water-soluble dye, lucifer yellow, we also examined their effects on the deformation of the plasma membrane. At relatively higher concentrations (100 μM, 1 mM), both cetirizine and diphenhydramine significantly reduced the numbers of degranulating mast cells. Of note, at 1 mM, cetirizine more markedly reduced the number than diphenhydramine, almost entirely suppressing the degranulation of mast cells. Additionally, 1 mM cetirizine and levocetirizine, another second-generation antihistamine, almost totally inhibited the process of exocytosis in mast cells and washed out the trapping of the lucifer yellow on the cell surface, while diphenhydramine and chlorpheniramine, another first-generation antihistamine, did not. This study provided *in vitro* evidence for the first time that cetirizine more potently inhibited the process of exocytosis in mast cells than diphenhydramine, indicating its higher potency as a mast cell-stabilizer. Such mast cell-stabilizing property of cetirizine could be ascribed to its counteracting effect on the plasma membrane deformation in degranulating mast cells.

Keywords Cetirizine, diphenhydramine, exocytosis, mast cells, mast cell-stabilizing property

1. Introduction

Antihistamines are widely used in the treatment of allergic disorders, such as seasonal pollinosis, chronic rhinitis, urticaria and allergic conjunctivitis (*1*). Among them, diphenhydramine, a first-generation antihistamine, has commonly been used in clinical practice due to its prompt onset of action and readily availability (*2*). However, studies indicated that cetirizine, a second-generation antihistamine, is more effective and safer than diphenhydramine because of its longer duration of action and less incidence of sedative side effects (*3,4*). Additionally, recent studies in both humans and experimental animals revealed that cetirizine was more potent than diphenhydramine in the treatment or prevention of allergic reactions (*5,6*). Cetirizine and diphenhydramine primarily exert anti-allergic properties by antagonizing histamine H1 receptors in peripheral tissues (*1*). However, the difference in their pharmacological potency strongly suggests the presence of an additional mechanism by which they exert anti-allergic properties. In our previous studies, by continuously monitoring the process of exocytosis in mast cells, we provided *in vitro* evidence that anti-allergic drugs, anti-microbial drugs and corticosteroids exert mast cell-stabilizing properties (*7-11*). In our recent studies, we have additionally revealed that food constituents, such as vitamins, caffeine and catechin, also stabilize mast cells (*12,13*). In the present study, to elucidate the additional mechanism underlying the anti-allergic properties of antihistamines, we directly examined their effects on the degranulation from rat peritoneal mast cells. Here, this study provides *in vitro* evidence for the first time that cetirizine more potently inhibits the process of exocytosis in mast cells than diphenhydramine, showing its higher potency as a mast cell-stabilizer. This study also shows that the mast cell-stabilizing property of cetirizine may be attributable to its counteracting effect on the plasma membrane deformation in degranulating mast cells.

2. Materials and Methods

2.1. Cell sources and preparation

Male Wistar rats no less than 25 weeks old were purchased from The Jackson Laboratory Japan, Inc. (Yokohama, Japan). We profoundly anaesthetized the rats with isoflurane and sacrificed them by cervical dislocation. The protocols for the use of animals were approved by the Animal Care and Use Committee of Miyagi University. As we previously described (*7-14*), we washed rat peritoneum using standard external (bathing) solution which consists of (in mM): NaCl, 145; KCl, 4.0; $CaCl_2$, 1.0; $MgCl_2$, 2.0; HEPES, 5.0; bovine serum albumin, 0.01% (pH 7.2 adjusted with NaOH) and isolated mast cells from the peritoneal cavity. We maintained the isolated mast cells at room temperature (22-24°C) for about 8 hours until use. The suspension of mast cells was spread on a chamber placed on the headstage of an inverted microscope (Nikon, Tokyo, Japan). Mast cells were easily distinguished from other cell types since they included characteristic secretory granules within the cells (*7-14*).

2.2. Quantification of mast cell degranulation

Cetirizine dihydrochloride, purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and diphenhydramine hydrochloride, from Wako Pure Chem Ind. (Osaka, Japan), were separately dissolved in the external solution at final concentrations of 1, 10, 100 μM and 1 mM. Levocetirizine dihydrochloride (Tokyo Chemical Industry Co., Ltd.) and chlorpheniramine hydrochloride (Wako Pure Chem Ind.) were dissolved at final concentration of 1 mM. After we incubated mast cells in these solutions or a solution without the drugs, exocytosis was externally induced by compound 48/80 (Sigma-Aldrich Co., St. Louis, MO, USA; final concentration 10 μg/mL) (*7-14*). We obtained bright-field images from randomly chosen 0.1-mm^2 fields of view (10 views from each condition), as described previously (*7-14*). We counted the number of degranulated mast cells (definition; cells surrounded by more than 8 granules outside the cell membrane) and calculated their ratio to all mast cells.

2.3. Lucifer yellow trapping on the cell subsurface

After the mast cells were incubated in the external solutions containing no drug, 1 mM diphenhydramine, chlorpheniramine, cetirizine or levocetirizine for 10 min, exocytosis was externally induced by compound 48/80 (10 μg/mL). Then, the cells were incubated for 5 min at room temperature in the external solution containing a hydrophilic fluorescent dye, lucifer yellow (*7,8,10,14-16*) (Wako, Osaka, Japan; final concentration 10 μM), and washed thoroughly 2 or 3 times with dye-free external solutions. Fluorescent images were taken using a TE 2000-E Nikon Eclipse fluorescence microscope (Nikon, Tokyo, Japan).

2.4. Statistical analyses

Data were analyzed using Microsoft Excel (Microsoft Corporation, Redmond, Wash., USA) and reported as means ± SEM. Statistical significance was assessed by two-way ANOVA. A value of $p < 0.05$ was considered significant.

3. Results and Discussion

Mast cells incubated in the external solution alone or relatively lower concentrations of diphenhydramine (1, 10 μM) showed a lot of wrinkles on the cell surface and released secretory granules as a consequence of exocytosis (Figures 1Ab-d *vs*. 1Aa). However, in mast cells incubated in relatively higher concentrations of diphenhydramine (100 μM, 1 mM), these findings of exocytosis were partially or almost totally absent (Figures 1Ae and 1Af). Quantitatively, relatively lower concentrations of diphenhydramine (1, 10 μM) did not affect the numbers of degranulating mast cells (Figure 1B). In contrast, 100 μM diphenhydramine significantly decreased the number of degranulating mast cells (external solution, 84.0 ± 3.26% *vs.* 100 μM diphenhydramine, 73.8 ± 3.47%; $n = 13$, $p < 0.05$), and 1 mM diphenhydramine further reduced the number of degranulating cells (41.2 ± 8.50 %; $n = 13$, $p < 0.05$; Figure 1B).

Like the effects of diphenhydramine (Figure 1), relatively lower concentrations of cetirizine (1, 10 μM) did not affect the degranulation of mast cells (Figures 2Ac, 2Ad *vs.* 2Ab) and the numbers of degranulating cells were almost comparable to those incubated in the external solution alone (Figure 2B). However, relatively higher concentrations of cetirizine (100 μM, 1 mM) partially or entirely halted the process of exocytosis (Figures 2Ae and 2Af). Quantitatively, similarly to the effects of diphenhydramine (Figure 1B), 100 μM cetirizine significantly reduced the number of degranulating mast cells (external solution, 92.0 ± 0.99% *vs.* 100 μM cetirizine, 76.2 ± 4.42%; $n = 10$, $p < 0.05$; Figure 2B). However, differing from the effects of diphenhydramine (Figure 1B), 1 mM cetirizine showed more marked reduction, almost totally suppressing the number of degranulating mast cells (2.64 ± 1.03 %; $n = 10$, $p < 0.05$; Figure 2B).

In addition to the exocytotic release of chemical mediators, including histamine, leukotrienes and serotonin, mast cells produce various kinds of proinflammatory cytokines or growth factors (*17*). Therefore, to accurately determine the ability of drugs or substances on the stabilization of mast cells, the exocytotic process itself needs to be directly monitored,

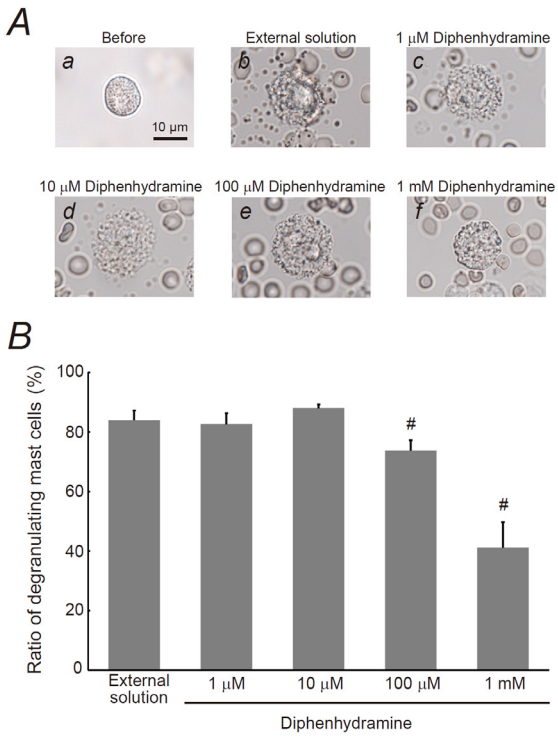

Figure 1. Effects of diphenhydramine on mast cell degranulation. **A**: Differential-interference contrast (DIC) microscopic images were taken before (*a*) and after exocytosis was externally induced by compound 48/80 in mast cells incubated in the external solutions containing no diphenhydramine (*b*), 1 μM diphenhydramine (*c*), 10 μM diphenhydramine (*d*), 100 μM diphenhydramine (*e*) and 1 mM diphenhydramine (*f*). **B**: After the mast cells were incubated in the external solutions containing no diphenhydramine or different concentrations (1, 10, 100 μM and 1 mM) of diphenhydramine, exocytosis was induced by compound 48/80. The numbers of degranulating mast cells were expressed as percentages of the total mast cell numbers in selected bright fields. $^{\#}p < 0.05$ *vs.* incubation in the external solution alone. Values are means ± SEM. Differences were analyzed by ANOVA followed by Dunnett's *t* test.

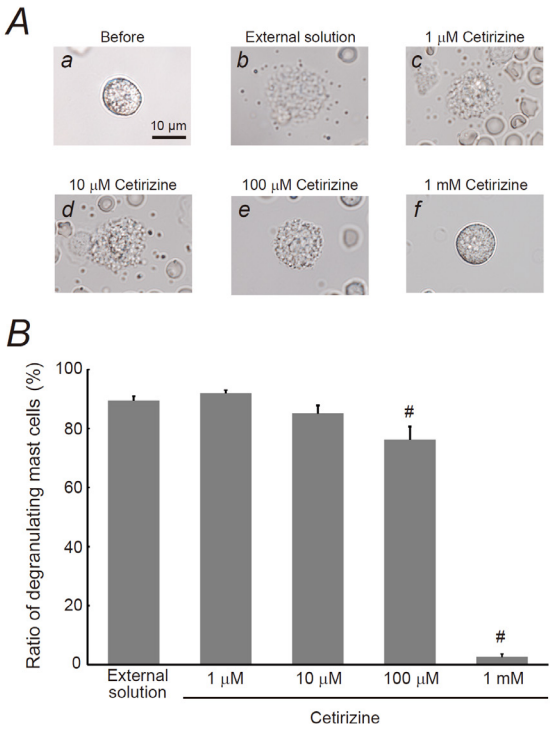

Figure 2. Effects of cetirizine on mast cell degranulation. **A**: Differential-interference contrast (DIC) microscopic images were taken before (*a*) and after exocytosis was externally induced by compound 48/80 in mast cells incubated in the external solutions containing no cetirizine (*b*), 1 μM cetirizine (*c*), 10 μM cetirizine (*d*), 100 μM cetirizine (*e*) and 1 mM cetirizine (*f*). **B**: After the mast cells were incubated in the external solutions containing no cetirizine or different concentrations (1, 10, 100 μM and 1 mM) of cetirizine, exocytosis was induced by compound 48/80. The numbers of degranulating mast cells were expressed as percentages of the total mast cell numbers in selected bright fields. $^{\#}p < 0.05$ *vs.* incubation in the external solution alone. Values are means ± SEM. Differences were analyzed by ANOVA followed by Dunnett's *t* test.

instead of just quantifying the amount of histamine alone (*7,8,10*). In our series of *in vitro* studies using rat peritoneal mast cells, we carefully observed the whole process of exocytosis under the microscope and actually counted the numbers of degranulating mast cells (*7-11*). Thus, we have provided *in vitro* evidence so far that anti-allergic drugs (tranilast, olopatadine, ketotifen, loratadine), anti-microbial drugs (clarithromycin), corticosteroids (hydrocortisone, dexamethasone) and catecholamines (adrenaline) exert mast cell-stabilizing properties (*7-11*). Additionally, we have revealed in our recent studies that food constituents, such as vitamins (ascorbic acid, pyridoxine), caffeine and catechin, also stabilize mast cells, and that such effects were synergistically enhanced by the combination of these constituents (*12,13*). In the preset study, using the same approach, we provided direct evidence for the first time that cetirizine and diphenhydramine dose-dependently inhibited the process of exocytosis. In addition to their primary pharmacological property of blocking histamine receptors, these antihistamines also exerted mast cell-stabilizing properties at high concentrations (Figure 4).

In humans, the serum concentrations of cetirizine and diphenhydramine reaches around 0.5 and 1 μM when physiological doses were orally administered (*18,19*). However, according to *in vitro* studies using microorganisms or cultured human epithelial cells, concentrations as high as 500 μM to 1 mM cetirizine and diphenhydramine were required to additionally elicit their antibacterial properties (*20,21*). Therefore, in the present study, we tried doses starting from 1 μM up to 1 mM. Mast cells that are derived from mucosal tissues, including conjunctiva, are known to produce larger amounts of chemical mediators than those from serosal tissues, including the peritoneal cavity (*22*). Therefore, in previous studies, mast cells derived from human conjunctiva actually required extremely high doses of antihistamines to effectively elicit their anti-allergic properties (*23*). In this regard, the present findings indicated the potency of cetirizine and diphenhydramine in the topical use for allergic conjunctivitis or urticaria.

From our results, at 1 mM, cetirizine more markedly

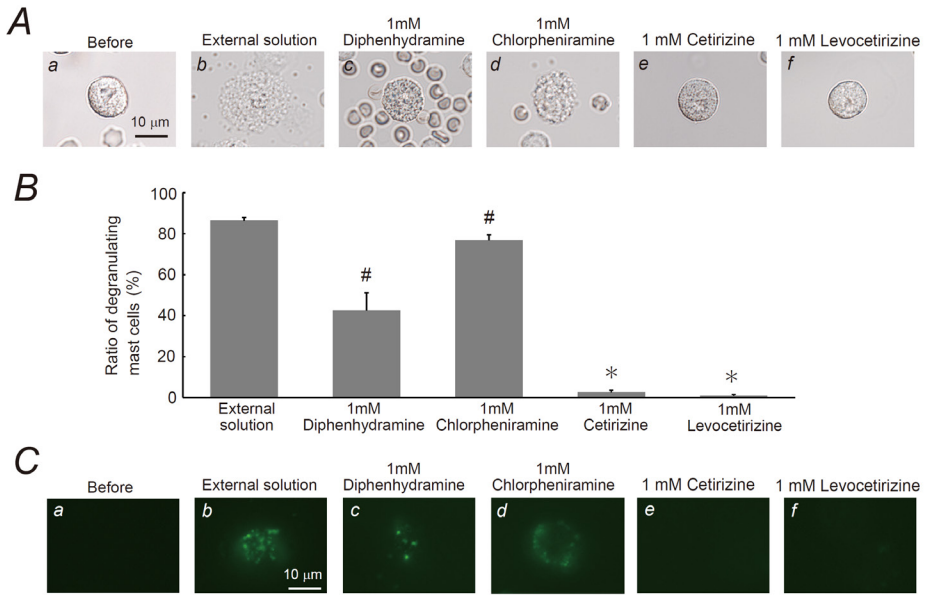

Figure 3. Effects of high concentrations of antihistamines on mast cell degranulation. A: Differential-interference contrast (DIC) microscopic images were taken before (*a*) and after exocytosis was externally induced by compound 48/80 in mast cells incubated in the external solutions containing no antihistamines (*b*), 1 mM diphenhydramine (*c*), 1 mM chlorpheniramine (*d*), 1 mM cetirizine (*e*) and 1 mM levocetirizine (*f*). **B**: After the mast cells were incubated in the external solutions containing no antihistamines or 1 mM antihistamines (diphenhydramine, chlorpheniramine, cetirizine or levocetirizine), exocytosis was induced by compound 48/80. The numbers of degranulating mast cells were expressed as percentages of the total mast cell numbers in selected bright fields. $^{\#}p < 0.05$ *vs.* incubation in the external solution alone. $^{*}p < 0.05$ *vs.* incubation in the external solution containing 1 mM diphenhydramine. Values are means ± SEM. Differences were analyzed by ANOVA followed by Dunnett's *t* test.

reduced the number of degranulating mast cells than diphenhydramine did (Figure 2B *vs.* 1B). To clarify the difference in the mast cell-stabilizing properties between first- and second-generation antihistamines, we also examined the effects of chlorpheniramine and levocetirizine, another first- and second-generation antihistamines, at 1 mM (Figure 3). Using the same approach in our established experimental settings, we've previously confirmed that several mast cell stabilizers, such as tranilast, adrenaline and olopatadine, markedly reduced the number of degranulating mast cells and thus inhibited the process of exocytosis (*7,8,11*). Therefore, these findings were regarded as the positive control for the following experiment. Similarly to the effects of diphenhydramine, 1 mM chlorpheniramine partially halted the process of exocytosis in mast cells (Figure 3Ac, 3Ad *vs.* 3Ab) and significantly reduced the numbers of degranulating cells (external solution, 86.5 ± 1.43% *vs.* 1 mM diphenhydramine, 42.4 ± 8.74%, $n = 13$, $p < 0.05$; 1 mM chlorpheniramine, 76.7 ± 2.68%, $n = 12$, $p < 0.05$; Figure 3B). In contrast, in mast cells incubated in 1 mM cetirizine or levocetirizine, the findings suggestive of exocytosis were almost completely absent (Figure 3Ae, 3Af) and the numbers of degranulating mast cells were almost entirely lost (1 mM cetirizine, 2.55 ± 0.99%, $n = 10$; 1 mM levocetirizine, 0.86 ± 0.61%, $n = 11$; Figure 3B). These findings strongly suggested that the second-generation antihistamines, such as cetirizine and levocetirizine, are highly potent as mast cell-stabilizers

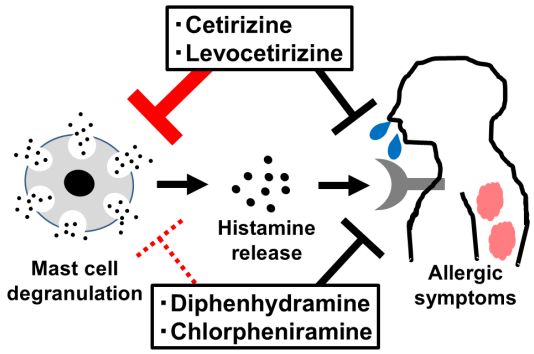

Figure 4. Mast cell-stabilizing properties of antihistamines. Allergic reaction consists of degranulation from mast cells (exocytosis), release of histamine and stimulation of tissue histamine H1 receptors. This causes allergic symptoms, such as sneezing, runny nose (allergic rhinitis), rash and itching (urticaria). In addition to their primary pharmacological property of blocking histamine receptors, antihistamines also exerted mast cell-stabilizing properties at high concentrations. Of note, the second-generation antihistamines, such as cetirizine and levocetirizine, are much more potent than the first-generation antihistamines, such as diphenhydramine and chlorpheniramine, in stabilizing mast cells.

(Figure 4), and that they are much more potent than the first-generation antihistamines, such as diphenhydramine and chlorpheniramine (Figure 4).

In recent studies in humans or experimental animals, cetirizine was more effective than diphenhydramine in the treatment or prevention of allergic reactions (*4-6*).

This may be attributable to the difference in the mast cell-stabilizing properties between these antihistamines as we demonstrated in the present study (Figures 3A and 3B). In addition to allergic reactions, mast cells were also responsible for the development and progression of organ fibrosis, such as liver cirrhosis, renal fibrosis and lung fibrosis (*24-26*). These studies indicated the pharmacological efficacy of suppressing the mast cell activity in the treatment or protection against organ fibrosis. In our previous study, tranilast, a potent mast cell-stabilizer, actually improved peritoneal fibrosis in rats under uremic condition (*27*). Given the highly potent mast cell-stabilizing property of cetirizine (Figure 4), the administration of this drug may also be beneficial in the treatment of organ fibrosis in addition to allergic diseases.

In our previous studies, drugs such as chlorpromazine, salicylate, olopatadine, ketotifen and clarithromycin, changed the plasma membrane curvature in rat peritoneal mast cells (*7,8,10,14*), and thereby regulated the process of exocytosis. In the present study, since 1 mM cetirizine and levocetirizine almost completely inhibited the exocytosis of mast cells (Figures 3A and 3B), the drug-induced alteration of the membrane architecture may also affect the exocytosis. To determine whether the wrinkles observed in the degranulating mast cells (Figure 3A) represented the membrane surface deformation caused by exocytosis, we finally used lucifer yellow (Figure 3C), a water-soluble fluorescent dye which is retained in the invaginated folds generated in the plasma membranes (*7,10,15,16*). In mast cells that were treated with external solution alone, 1 mM diphenhydramine or 1 mM chlorpheniramine, lucifer yellow was trapped almost entirely or at least partially on the cell surface area (Figures 3Cb-3Cd). Because the dye, which is usually membrane-impermeable (*28*), was almost totally absent in the cells before exocytosis was induced (Figure 3Ca), the staining indicated its retention in the opened pores created by exocytosis (*7,8,10,14,29*). However, after incubating mast cells in 1mM cetirizine or 1 mM levocetirizine (Figures 3Ce and 3Cf), the dye was almost completely washed out. These results indicated that cetirizine or levocetirizine inhibited the creation of the invaginated folds when they exerted mast cell-stabilizing properties. This suggested that these antihistamines counteracted the membrane surface deformation caused by exocytosis.

Cetirizine and levocetirizine are zwitterionic at physiological pH and less lipophilic compared to their cations (*30*). Therefore, they are less likely to be accumulated inside the plasma membranes (*31*). Instead, they can directly interact with the polar headgroups of phospholipids (*31*), and thus actually induced changes in the plasma membrane fluidity and its heterogeneity (*32*). In secretory cells, such as lung alveolar cells and mammary gland cells, the process of exocytosis can be modulated by mechanical stimuli to the membranes, including changes in the membrane tension, shear stress, hydrostatic pressure and compression (*8,10,33*). Therefore, such counteracting effects of cetirizine or levocetirizine on the plasma membrane deformation in degranulating mast cells were likely to be responsible for their mast cell-stabilizing properties.

In summary, this study provided *in vitro* evidence for the first time that cetirizine more potently inhibited the process of exocytosis in mast cells than diphenhydramine, indicating its higher potency as a mast cell-stabilizer. Such mast cell-stabilizing property of cetirizine could be ascribed to its counteracting effect on the plasma membrane deformation in degranulating mast cells.

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RF and AA performed the experiments and analyzed the data. MA helped analyze the data. IK designed the experiments, interpreted the results and wrote the paper. All authors read and approved the final manuscript. This study was performed in accordance with the guide for the care and use of laboratory animals of Miyagi University, which included ethical considerations.

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Letter to the Editor

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Percutaneous surgical repair for a patient with adult pararectal hernia caused by intractable ascites associated with liver cirrhosis

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SUMMARY Patients with liver cirrhosis are at increased risk of various visceral hernia because of persistent ascites and tissue fragility. Here we report successful treatment in a patient with pararectal hernia due to liver cirrhosis by a less invasive approach *via* para-anal region. The patient was a 73-year-old woman with a history of chronic hepatitis B that had been untreated for at least 20 years. At the age of 68 years, she was referred to our hospital for treatment of persistent ascites and thrombocytopenia due to advanced liver cirrhosis. Neither diuretics nor cell-free and concentrated ascites reinfusion therapy could decrease the ascites. She needed repeated paracentesis. She was referred to the surgical department due to the painful swelling of the left buttock which was diagnosed as the pararectal hernia. The welling was huge enough with the erosion of the covering skin. Surgery was planned in view of concern about the possible rupture of the hernia. Due to the massive ascites with the advanced liver cirrhosis, we were reluctant to do the laparotomic approach, and simple closure of the hernial orifice *via* direct approach from the cutaneous side was planned and performed. The patient was fortunately discharged seven days after the operation without any complications. One year later, there has been no recurrence of the hernia. Even in cases with massive ascites, direct simple closure of the hernia by percutaneous approach may be one of the options for the treatment of the pararectal hernia in case of urgent situation.

Keywords Liver cirrhosis, pararectal hernia, repair of hernia

Letter to the Editor,

Patients with liver cirrhosis accompanied by massive ascites and sarcopenia are at risk of visceral hernia. The incidence of hernia involving the abdominal wall is particularly high in these patients (*1*). Pararectal hernia tends to occur in older multiparous women and those who have undergone extensive resection of the pelvic floor. However, there have been no reports of pararectal hernia in patients with liver cirrhosis, and there is no established treatment. In this report, we describe a case of pararectal hernia in a patient with liver cirrhosis who was treated by the surgical repair *via* trans-cutaneous approach.

A 73-year-old woman with progressive and symptomatic enlargement of the pararectal hernia was referred to the surgical department by hepatologists in our hospital. She had two children born with normal vaginal delivery. She had a history of cholecystectomy 12 years ago, but had no history of operations of the pelvic organs. She had been treated for hepatitis B cirrhosis for more than 5 years with persistent ascites and thrombocytopenia. A nucleic acid analog could turn the hepatitis B virus DNA negative, but her ascites did not resolve and has persisted. Various kinds of diuretics, like the v2-receptor blocker (tolvaptan), furosemide, and spironolactone, were applied. They could slow down the increase of the ascites but were not possible to decrease. Three years ago, cell-free and concentrated ascites reinfusion therapy was attempted, but she developed fever as an adverse effect and could not try again. Some invasive treatments, like transjugular intrahepatic portosystemic shunt or peritoneovenous shunt, were rejected by the patient. Therefore, she had been managed by paracentesis almost every month, with around 1,500 ml drainage each time. The pararectal hernia had been diagnosed 14 months before the operation with the abdominal computed tomography examination, but at that time the surgical

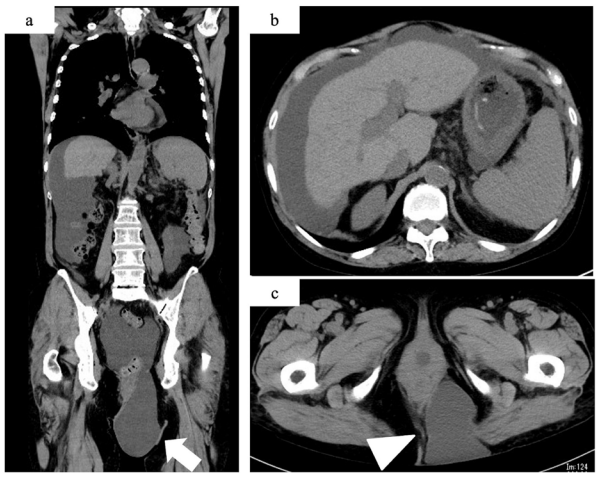

Figure 1. Preoperative computed tomography. a. Preoperative computed tomography (coronal section) demonstrating abdominal ascites and pararectal hernia (White arrow). **b.** Preoperative computed tomography demonstrating liver cirrhosis (atrophy of right robe and large amount of ascites). **c.** Preoperative computed tomography demonstrating pararectal hernia (White arrowhead).

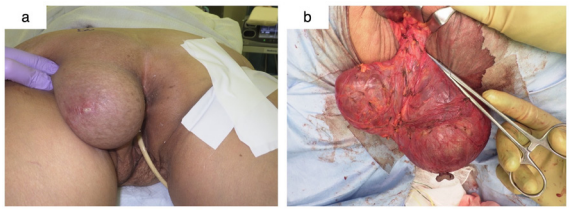

Figure 2. Intraoperative findings. a. Figure shows a pararectal hernia in the prone position. **b.** Figure shows a hernia sac after peeling to the hernia orifice.

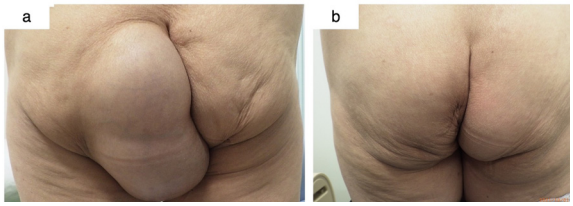

Figure 3. Patient's preoperative and postoperative appearance. a. Patient's preoperative standing position shows a huge mass of hernia sac. **b.** Patient's postoperative standing position shows disappearance of hernia sac.

repair had not been attempted because the hernia was small and her symptom was mild. In addition, there was concern about the possibility of postoperative infection or dehiscence by the laparotomic approach. However, the hernia gradually enlarged and she developed painful symptoms, including severe tightness in her left buttock (Figure 1). Her daily movement, including the sleeping, was much disturbed. Finally, skin erosion like a pressure ulcer appeared on the top of the swelling (Figure 2). Concerning the rupture, strangulation and sever symptoms, we decided to perform surgery. We selected the per-cutaneous approach from the peri-anal skin, considering to make the injured area as small as possible.

The patient was placed in the jack-knife position under general anesthesia (Figure 2). After making a spindle-shaped incision on the top of the swelling, cutaneous and connective tissue were meticulously peeled off from the hernia sac. While approaching the proximal side of the hernia, the hernia was completely isolated up to the orifice, with identifying the boundary between the adipose tissue of the pelvic floor and the levator ani muscle. The orifice was double-ligated with 4-0 prolene suture (Figure 2). The connective tissue and muscle were reinforced around the hernia orifice with a vertical mattress suture using 3-0 prolene. The skin was primarily closed. The operation time was 5 h 36 min, and blood loss was 30 mL. To decrease the abdominal pressure, ascites was drained by indwelling fine catheter for 3 days after the operation. The patient was discharged on postoperative day 7 with no complication. As of 1 year after the operation, no recurrence has been observed (Figure 3), although her ascites has been remained. Her subjective symptoms disappeared and she has maintained good quality of life.

Repair of the abdominal wall hernia with intractable massive ascites due to liver cirrhosis has been recommended only after or with the liver transplantation (*1*). However, in Japan, liver transplantation is currently limited, and impossible in elderly patient over 70 years. Considering that persistent ascites is a major risk factor for repair of the hernia, it is also recommended that any type of portosystemic shunt or peritoneovenous shunt should be performed before the radical hernia repair (*1*). However, under the limited chance of the liver transplantation, such shunt operations that has been used as the bridge to the transplant is rarely done. Therefore, surgical treatment of the hernia tends to be avoided in patients with massive ascites. However, hernias may be associated with several life-threatening events in cirrhotic patients, including incarceration and/or strangulation of the gastrointestinal tract, and then may lead to the fatal outcome. That is why the symptomatic hernia had to be treated surgically in selected cases.

Pararectal hernia is a kind of the internal hernia, and primary pararectal hernias are extremely rare hernia in the abdominal wall hernia (*2,3*). Because of its rarity, the best approach for the repair of the hernia has not been established among the abdominal, the perineal, and the combined abdominoperineal approaches (*2,3*). In addition, the repair under the massive ascites due to the liver cirrhosis has not been reported. Hernia repair consists of closure of the orifice and reinforcement of the surrounding tissue by the autologous tissue or artificial material. Simple closure only has a high recurrence rate especially under the remaining high pressure of the abdominal cavity. The risk of recurrence is naturally low with enforcement, and using the mesh is now quite popular in these days (*4*). However, in

the repair with massive ascites, concern about the postoperative infection is not small. Arroyo *et al*. reported that the recurrence rate after hernia repair in patients with cirrhosis was significantly lower using mesh, occurring in 11% of simple closures versus 1% of mesh-based procedures (*4*).

In our case, considering the balance between the risk and benefit of the surgery, simple closure using the perineal approach was preferred. The surrounding tissue including some pelvic floor muscles was used for coverage of the orifice, although it sounded palliative without enforcement by the mesh placement. The effectiveness of robotic-assisted pelvic floor hernia surgery has recently been reported (*5*). The benefits of an intra-abdominal approach include avoidance of organs near the hernia and reliable mesh deployment. However, we hesitated to do a laparoscopic approach in our case, because it would be too difficult to secure the visual field due to the massive ascites. Although the repair in our case was performed under direct vision, all procedures needed to be performed very carefully while identifying the boundary between the peritoneum and surrounding tissue using a magnifying glass. Furthermore, bleeding was thoroughly controlled using a ball-shaped electrode that was appropriate even for small blood vessels in the middle of surgical procedure. Although the procedure was challenging, it was useful for avoiding postoperative complications.

In conclusion, direct simple closure *via* perineal approach could be a safe option for the repair of pararectal hernia with intractable ascites associated with liver cirrhosis.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

Informed consent: Written informed consent was taken from the patient for publication of case details and photographs.

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Letter to the Editor

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Nevus of Ota on the auricle successfully treated with Q-switched ruby laser

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SUMMARY Nevus of Ota is a dermal melanocytosis that consists of blue-brown spots, patches and plaques along the distribution of the first and second branches of trigeminal nerve. The efficacy of Q-switched ruby laser treatment against nevus of Ota on dark skin has not been described. The present case, a 2-month-old Indonesian girl, showed rare auricular involvement. Because ear has complicated steric structure, whose skin is sensitive and thin, pain and inflammatory reaction are inevitable. We discussed the difficulty of laser treatments on auricular lesions.

Keywords Nevus of Ota, dark skin, ear, Q-switched ruby laser, dermal melanocytosis

Letter to the Editor,

A 2-month-old Indonesian girl visited our hospital for the treatment of her eruption, which was present at birth. The patient had no family history of pigment disorders, and her physical and mental development was normal. On physical examination, a blocky blue plaque with a number of brown spots covered the whole of the left auricle and around the ear (Figures 1a and 1b). There were no other changes in the skin, conjunctiva, or oral mucosa.

A biopsy from the affected skin of postauricular lesion showed the presence of dermal melanocytes in the upper and middle dermis as well as basal pigmentation (Figures 1c, 1d, and 1e). Written informed consent was obtained from the patient for publication of this case report and accompanying images.

Based on above clinical and histopathological findings, we diagnosed this case as having nevus of Ota. Treatment was performed with Q-switched ruby laser at under the topical anesthesia. Because blistering was observed as the adverse effect even with the lowest energy (4 J/cm^2), the treatments were performed every 3-4 months, and the postoperative icing and topical corticosteroids were used more frequently. The pigmentation has improved gradually after 4th laser treatments (Figure 1g).

We considered ectopic mongolian spot or blue nevus as the differential diagnoses of the eruptions seen in our patients. Blue nevus was denied by the absence of tumoral proliferation of dermal melanocytes histopathologically. Mongolian spot is different from Nevus of Ota in terms of the absence of color variation in the lesion clinically and basal pigmentation histopathologically. Furthermore, dermal melanocytes are located in the middle-deep dermis in mongolian spots. Nevus of Ota usually affects the forehead, temples, eyelids, cheeks, and nose, but in this case, they unusually appeared on the auricular, which has been described by a few previous reports (*1*).

Laser treatment on auricular lesions is sometimes difficult because of the complicated steric structure. Furthermore, because ear skin is sensitive and thin, pain and inflammatory reaction are inevitable. We could not find description about the tips of laser treatments on auricular in textbooks and previous literatures. In our case, treatments were performed carefully to avoid uneven laser irradiation due to the complicated structure. Furthermore, to reduce pain and inflammatory reaction, treatment area in each session was divided into two or three parts, and the postoperative icing and topical corticosteroids were used more frequently for longer duration.

Nevus of Ota typically affects the Asian population (*2-4*), therefore efficacy has been well established for the laser treatment of Fitzpatrick skin phototype III to IV (*5*). On the other hand, in individuals with baseline dark skin pigmentation (*e.g.*, Fitzpatrick skin phototype V to VI), clinical data about laser efficacy is still lacking. Postinflammatory hyperpigmentation or hypopigmentation occurs most commonly in individuals with dark skin, because normal distribution of melanin, the target of ruby laser, in the epidermis may be adversely affected. Accumulation of similar cases is necessary to evaluate the efficacy and safety of

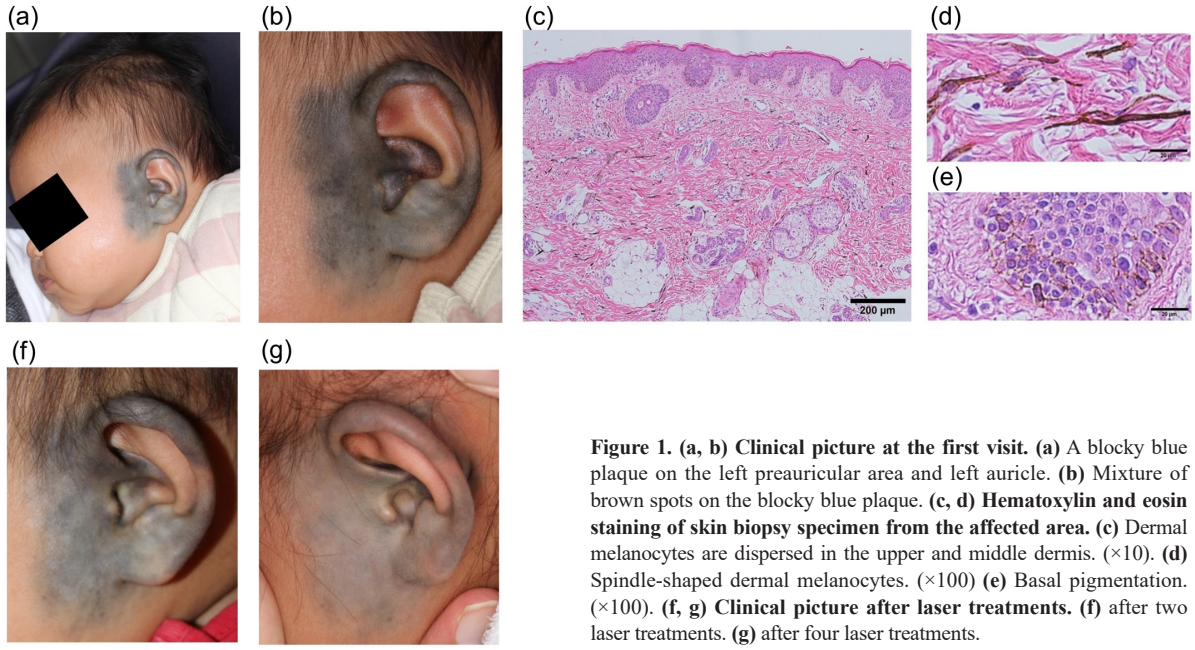

Figure 1. (a, b) Clinical picture at the first visit. (a) A blocky blue plaque on the left preauricular area and left auricle. **(b)** Mixture of brown spots on the blocky blue plaque. **(c, d) Hematoxylin and eosin staining of skin biopsy specimen from the affected area. (c)** Dermal melanocytes are dispersed in the upper and middle dermis. (×10). **(d)** Spindle-shaped dermal melanocytes. (×100) **(e)** Basal pigmentation. (×100). **(f, g) Clinical picture after laser treatments. (f)** after two laser treatments. **(g)** after four laser treatments.

laser therapy for nevus of Ota on dark skin type.

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Letter to the Editor

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A Japanese case of melanoma of unknown origin with a rare *BRAF*^{V600R} mutation was successfully treated with BRAF/MEK inhibitors

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SUMMARY Combination therapy with BRAF and MEK inhibitors (BRAF/MEKi) has shown significantly prolonged progression-free survival (PFS) and overall survival (OS) for *BRAF* mutated melanoma. Over 90% of the activating mutations are *BRAF*^{V600E} or *BRAF*^{V600K} changes. There are no reports of *BRAF*^{V600R} in Japanese patients with melanoma. The third most common *BRAF* mutation is *BRAF*^{V600R}. In this case, we detected the *BRAF*^{V600R} mutation with FoundationOne CDx in a Japanese patient with melanoma.. The patient was treated with BRAF/MEKi and maintained stable disease status for 1 year.

Keywords BRAF, V600R, melanoma, FoundationOne CDx

Letter to the Editor,

Combination therapy with BRAF and MEK inhibitors (BRAF/MEKi) has shown significantly prolonged progression-free survival (PFS) and overall survival (OS) for *BRAF*^{V600E}- and *BRAF*^{V600K}-mutated melanoma (*1*). Mutations in amino acid 600 of the *BRAF* gene account for approximately 50% and 30% of cases of melanomas in Caucasian and Japanese patients, respectively (*2*). Over 90% of the activating mutations are valine (V) to glutamic acid (E) (*BRAF*^{V600E}) or valine (V) to lysine acid (K) (*BRAF*^{V600K}) changes (*3*). The third most common *BRAF* mutation is from valine (V) to arginine (R) (*BRAF*^{V600R}), which accounts for 1-4% of cases (*3*). There are no reports of *BRAF*^{V600R} in Japanese patients with melanoma. Here, we report a case of melanoma with *BRAF*^{V600R} that was successfully treated with BRAF/MEKi.

A 67-year-old Japanese man presented to our hospital with axial lymph node swelling and subcutaneous nodules. Fluorodeoxyglucose-positron emission tomography/computed tomography (FDG-PET/CT) revealed metastatic lesions in the lungs, liver, duodenum, bone, and lymph nodes (Figure 1A). As no primary melanoma lesion was identified, a diagnosis of melanoma of unknown primary origin was made based on a biopsy of the subcutaneous nodule. Companion diagnostic tests (Cobas 4800 BRAF V600 Mutation Test; Roche Molecular Diagnostics, Pleasanton, CA, USA) did not show *BRAF*^{V600E} or ^{V600K} mutations. Therefore, combination therapy with anti-cytotoxic-T-lymphocyte-associated antigen 4 (anti-CTLA-4) and anti-programmed cell death-1 (anti-PD-1) antibodies was initiated. After the second course, hepatitis was diagnosed as an immune-related adverse event (irAE). The patient was treated with prednisolone (2 mg/kg/day), and hepatitis improved after 4 weeks. On a CT scan after irAE resolution, disease progression (PD; RECIST 1.1) was observed. Then, a cancer genome profiling test (FoundationOne CDx, which detects base substitutions, insertions, deletions, copy number abnormalities, and rearrangements in 324 genes) was performed. As shown in Figure 1B, the *BRAF*^{V600R} mutation was evident. The patient was treated with BRAF/MEKi and maintained stable disease status for 1 year (Figure 1C).

BRAF^{V600R} has been reported to increase the ability to activate MEK by increasing ERK phosphorylation as well as BRAF^{V600E} and BRAF^{V600K} (*4*). Some patients in Australia responded to BRAF/MEKi (*5*). However, clinical practice has shown that non-V600E/K BRAF mutations cannot be detected with the current companion diagnostic tests. Therefore, some patients with melanoma may have missed treatment opportunities, although the probability is low.

In this case, we detected the *BRAF*^{V600R} mutation with

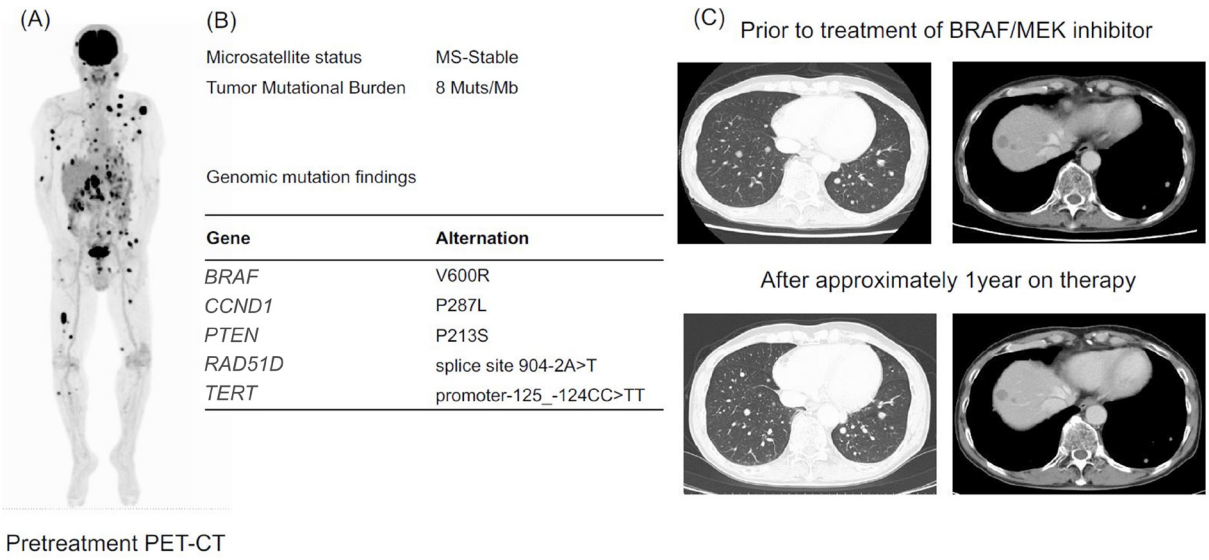

Figure 1. (a) FDG-PET/CT performed before treatment. (b) Gene alterations detected with the Foundation One CDx, a next-generation sequencing test. (c) CT performed before (above) and after (below) BRAF/MEK inhibitor therapy.

FoundationOne CDx and treated the patient with BRAF/MEKi. Multi-gene panel testing, such as FoundationOne CDx, is useful for detecting rare gene mutations. However, this method is expensive and time-consuming. A cheaper and faster method should be developed that can detect non-*BRAF*^{V600E} and -*BRAF*^{V600K} mutations such as *BRAF*^{V600R} by utilizing a melanoma-specific gene panel.

Funding: None.

Conflict of Interest: The authors have no conflicts of interest to disclose.

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