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Study of the compatibility of oral magnesium oxide preparations sold in Japan with the ICH-Q3D guideline for elemental impurities

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SUMMARY Magnesium oxide has been widely used as an antacid and constipation remedy. Currently in Japan, magnesium oxide preparations manufactured by five medical companies are marketed as prescribed generic drugs. In this study, we focused on metal elemental impurities present in 330 mg magnesium oxide tablets manufactured by each of these companies. The content of such impurities was determined by atomic absorption spectrometry and inductively coupled plasma mass spectrometry. We confirmed whether the content conformed to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Guideline for Elemental Impurities (ICH-Q3D) based on the 30% control threshold. The content of these impurities varied among the five products (preparations A-E), but in all cases met the oral permitted daily exposure (PDE) criteria stipulated in ICH-Q3D. In 5 lots of preparation C and all lots of preparation D, the equivalent cadmium (Cd) intake for a daily maximum dosage of 2 g was higher than the 30% control threshold of 1.5 μg/day. By cluster analysis, preparations A-E were classified into preparations A + B and C + D + E and/or preparations A + B, C + D and E. The present study showed that all 5 preparations sold in Japan meet the PDE value standard of ICH-Q3D, and that preparations A and B meet the 30% control threshold. It is important that for preparations failing to meet the criteria, further improvements need to be sought, and impurities in magnesium oxide preparations need to be monitored to ensure their safety.

Keywords Magnesium oxide, metal impurity, ICH-Q3D, control threshold, cluster analysis

1. Introduction

In Japan, magnesium oxide preparations are widely used as low-priced, non-addictive laxatives, and can be used relatively safely by pregnant women and children. With long-term administration, however, it is necessary to pay attention to cumulative effects of any impurities, particularly metal impurities, in the preparation. In addition, for use by pregnant women and children, it is important to comply with the stipulated standards for impurities, as in such cases higher-purity preparations are required.

In recent years, due to globalization of the pharmaceutical market, the need for international risk assessment of metal impurities has been discussed at the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). Therefore, the ICH Guideline for Elemental Impurities (ICH-Q3D) for oral preparations has been studied. Table 1 shows the permitted daily exposure (PDE) and control threshold for elemental metal impurities regulated by the ICH-Q3D guidelines in pharmaceutical products. They are grouped into 4 classes based on the toxicity of each element and the likelihood of them appearing as contaminants in pharmaceutical products (*1*). Elemental metal impurities in preparations can originate from not only the drug substance itself and additives, but also several factors such as the materials and containers used in the manufacturing process and the abundance of the elements themselves in nature (*1*). Class 1 cadmium (Cd), lead (Pb), arsenic (As) and mercury (Hg) are highly toxic elements and are subject

Table 1. Oral PDE and 30% control threshold for metals in ICH-Q3D

Class (Toxicity)	Element	Oral PDE (μg/day)	Oral concentration (μg/g) [Option 1]	Maximum daily intake in 2 g/day (μg/g) [Option 3]	30% Control threshold in 2 g/day (μg/g)
1	Cd	5	0.5	2.5	0.75
(Strong)	Pd	5	0.5	2.5	0.75
	As	15	1.5	7.5	2.25
	Hg	30	3	15	4.5
2A	Co	50	5	25	7.5
(Moderate)	V	100	10	50	15
	Ni	200	20	100	30
2B	Tl	8	0.8	4	1.2
(Moderate)	Au	100	10	50	15
	Pd	100	10	50	15
	Ir	100	10	50	15
	Os	100	10	50	15
	Rh	100	10	50	15
	Ru	100	10	50	15
	Pt	100	10	50	15
	Se	150	15	75	22.5
	Ag	150	15	75	22.5
3	Li	550	55	275	82.5
(Moderate)	Sb	1,200	120	600	180
	Ba	1,400	140	700	210
	Mo	3,000	300	1,500	450
	Cu	3,000	300	1,500	450
	Sn	6,000	600	3,000	900
	Cr	11,000	1,100	5,500	1,650

PDE: permitted daily exposure.

to the strictest regulatory values. Class 2 elements are those that exhibit toxicity depending on the route of administration. Class 2A cobalt (Co), vanadium (V), and nickel (Ni) are considered highly likely to be present in pharmaceutical products, and therefore risk assessment is essential. It is necessary to prove that Class 2B and Class 3 elements, which are considered unlikely to be present in pharmaceutical products, are below the regulated values when used in catalysts, *etc*. during the manufacture of pharmaceutical products (*1*). ICH-Q3D stipulates four options - 1, 2a, 2b, and 3 - as risk evaluation methods. Option 1 involves setting an allowable concentration limit for a common element among the components of a drug with a daily intake of 10 grams or less. Option 2a involves setting the permissible concentration limit for an element among the components of a drug for which the daily intake is specified. Option 2b involves setting the permissible concentration limit of elements among the individual components of a product for which the daily intake is specified. Option 3 involves confirming that the elements present in the final product are below the permissible concentration limit (*1*).

As shown in Table 1, Cd and Pb have a PDE of 5 μg/day for a body weight of 50 kg. In Japan, a maximum of 2 g of magnesium oxide is taken daily for constipation (*2*). Therefore, when 2.5 μg of Cd and Pb is mixed in a preparation containing 1 g of magnesium oxide, the standard is exceeded. Cd causes Itai-itai disease at high concentrations, but it accumulates in the kidneys and liver stores eve at even low concentrations and is highly toxic (*3*). Pb has a half-life of about 28 - 36 days in blood and soft tissues, but disappears when taken up by bone and has a long half-life in the order of years (*4*). In addition, the *in vivo* stagnation rate of Pb is higher in children than in adults. A concentration of 10 μg/dL causes acute poisoning of the blood and nervous system, and long-term exposure also results in chronic poisoning symptoms such as angiopathy, neuropathy and cerebral edema, as well as carcinogenicity and reproductive and developmental toxicity (*5*).

We have previously reported the results of a pilot survey of the content of heavy metal impurities in magnesium oxide preparations, pointing out differences between the preparations (*6*). In the present study, the actual condition of metal impurities contained in multiple magnesium oxide preparations sold in Japan was investigated in detail by analyzing the final products. Then, in consideration of the control threshold according to option 3 recommended by ICH-Q3D, the suitability of applying the ICH-Q3D standard to the preparations sold in Japan was examined. Furthermore, we used cluster analysis to examine the risk classification of the drugs.

2. Materials and Methods

2.1. Survey preparation

In April 2020, various dosage forms of magnesium

Table 2. The magnesium oxide formulations available on the Japanese market chosen for the study

Preparation	Formulation						
	200 mg Tablet	250 mg Tablet	300 mg Tablet	330 mg Tablet	400 mg Tablet	500 mg Tablet	Fine Granules 83%
A	○	○		○		○	○
B		○		○		○	
C	○	○	○	○	○	○	○
D		○		○		○	○
E		○		○			

○: Commercially available.

oxide preparations commercially available from 5 pharmaceutical companies (Kenei Pharmaceutical Co. Ltd., Osaka, Japan; Kyowa Chemical Industry Co. Ltd., Kagawa, Japan; Mochida Pharmaceutical Co. Ltd., Tokyo, Japan; Mylan, Tokyo, Japan; Yoshida Pharmaceutical Co. Ltd., Tokyo, Japan) were obtained (Table 2). Among those preparations, 330 mg tablet formulations of magnesium oxide were targeted for the research. Six different lots marketed between 2018 and 2020 were used.

2.2. Tablet weight measurement

Sixty tablets from each lot of each company's preparation were collected and weighed.

2.3. Quantitative analysis of Cd and Pb

Tablets of each preparation were crushed, and after acid treatment of 1 g of the powder, the sample was heated in a muffle furnace at 600°C for 3 hours. The incinerated sample was then dissolved in acid to make a 30 mL solution. The supernatant was separated and the pH adjusted to 5 with 20% aqueous ammonia and 70% nitric acid. The liquid was passed through a chelate resin Novia Skilate column (CHELATE-PA1, Hitachi High-Tech Science Corp., Tokyo, Japan), and Cd and Pb were adsorbed and separated from the matrix metal Mg. Cd and Pb on the chelate resin were then eluted off with 3N nitric acid solution to prepare a measurement solution. Inductively coupled plasma mass spectrometry (ICP-MS) (ELAN DRC II, PerkinElmer Japan Co. Ltd., Kanagawa, Japan) was used to quantify Cd and Pb by the ICP-MS method employing absolute calibration.

2.4. Quantitative analysis of As

Tablets of each preparation were crushed and 0.5 g of the powder was collected, followed by addition of 5 mL of sulfuric acid : nitric acid (1:1) and heat treatment. Then 4 mL of 20% potassium iodide solution was added, the volume was adjusted to 50 mL, and the mixture was left at room temperature for 1 hour. After being allowing to cool, As was quantified using the absolute calibration curve obtained by hydride generation atomic absorption spectrophotometry (AA-7000, Shimadzu Corp., Kyoto, Japan).

2.5. Quantitative analysis of Hg

Tablets of each preparation were crushed and 1 g of the powder was subjected to acid treatment, followed by addition of a solution of sulfuric acid, nitric acid and potassium permanganate and heat treatment. After being allowing to cool, Hg was quantified using the absolute calibration curve obtained by the reduced vaporization atomic absorption method using a mercury measurement device (HG-400, HIRANUMA Co. Ltd., Ibaraki, Japan).

2.6. Quantitative analysis of Co, Ni, V, Sn and Cr

Tablets of each preparation were crushed and 1 g of the powder was subjected to acid treatment, followed by heating in a muffle furnace at 600°C for 3 hours. The incinerated sample was then dissolved in acid to make 30 mL of solution. The supernatant was separated and ICP-MS (ELAN DRC II, PerkinElmer Japan Co. Ltd.) was used to quantify Co, Ni, V, Sn and Cr by the ICP-MS method using absolute calibration.

2.7. Reagents

ICP-MS analysis standard solution, Cd standard solution, Pb standard solution, As standard solution, Hg standard solution, and Sn standard solution were obtained from FUJIFILM Wako Chemical Corp. (Miyazaki, Japan). For the Co, Ni, V, Sn, and Cr mixed standard solution, a mixed standard solution for ICP-MS analysis was obtained from SPEX (New Jersey, USA). Sulfuric acid, nitric acid, potassium permanganate solution, potassium iodide solution and other reagents (FUJIFILM Wako Chemical Corp.) used were analytical grade. The water used was ultrapure.

2.8. Calculation of acceptable daily intake (ADI)

For Cd, Pb, and As, ADI was calculated using the following formula based on the quantitative results for each formulation.

$$\text{ADI (g/day)} = \text{Oral PDE (µg/day)} / \text{Impurity (µg/g)}$$

2.9. Characterization of heavy metals present in magnesium oxide preparations using cluster analysis

Cluster analysis was performed using JMP Pro13.2.0 (SAS Institute Inc. NC, USA). The heavy metal contents were used to interpret the characteristics of magnesium oxide preparations. Hierarchical cluster analysis was used to classify the magnesium oxide preparations objectively. This analysis used the Ward method based on Euclidean distance from the heavy metal contents. The hierarchical cluster analysis established two clusters.

2.10. Statistics

The experimental data are shown as mean ± standard error (SE). One-way ANOVA and Tukey's multiple comparison method were used to test the differences in means between each group using JMP Pro13.2.0 (SAS Institute Inc. NC, USA). The significance level was set at $p < 0.05$.

3. Results

3.1. Comparison of weight of each tablet and magnesium oxide content per gram of preparation

The measured weights of the tablets for the 6 lots of magnesium oxide preparations manufactured by drug companies A, B, C, D and E, respectively, are shown in Table 3. There was no significant difference in mass between the preparations, and the differences were all within 10%.

Table 3. Tablet weights of magnesium oxide preparations and their magnesium oxide contents

Preparation	Weight of tablet (g/tablet)	Contents of magnesium oxide (g/1 g)
A	0.378 [0.376-0.378]	0.882 [0.880-0.885]
B	0.395 [0.394-0.397]	0.843 [0.838-0.846]
C	0.396 [0.396-0.397]	0.841 [0.839-0.841]
D	0.408 [0.406-0.410]	0.816 [0.811-0.820]
E	0.402 [0.401-0.403]	0.829 [0.826-0.831]

$n = 6$, mean [range].

3.2. Comparison of metal element impurities per gram of each preparation

Table 4 shows the measured contents of elemental metal impurities contained in preparations A, B, C, D and E. The content of elemental metal impurities varied depending on the preparation, but all of them conformed to the PDE value defined by ICH-Q3D.

The PDE value for Cd in Class 1 is defined as 5 µg/day. The Cd contents of preparations A and B were less than 0.02 µg/g and 0.01 µg/g, respectively, being significantly lower than those in preparations C, D and E ($p < 0.05$). The PDE value for Pb is 5 µg/day. The Pb contents of preparations C, D and E were 0.18 µg/g, 0.13 µg/g and 0.12 µg/g, respectively, being significantly higher than those in preparations A and B ($p < 0.05$). The PDE value for As is 15 µg/day. The As contents of preparations C, D and E were 1.57 µg/g, 1.27 µg/g and 1.70 µg/g, respectively, being significantly higher than those in preparations A and B ($p < 0.05$). The PDE value for Hg is 30 µg/day. The Hg contents of all the preparations were below the measurement limit of 0.05 µg/g.

The PDE value for Class 2A Co is 50 µg/day. The Co content was below the measurement limit of 0.50 µg/g in all the preparations. The PDE value for V is 100 µg/day. The V content of preparation E was 3.12 µg/g, which was the highest. The V contents of preparations C, D and E were significantly higher than those in preparations A and B, respectively ($p < 0.05$). The PDE value for Ni is 200 µg/day. The Ni content of preparation E was the highest, at 2.55 µg/g. The Ni contents of preparations C, D and E were significantly higher than those of preparations A and B ($p < 0.05$).

The PDE value for Class 3 Sn is 6,000 µg/day. The Sn content of all preparations was less than 0.50 µg/g. The PDE value for Cr is 11,000 µg/day. The Cr content was highest in preparation E, at 6.12 µg/g.

3.3. Comparison of heavy metal element impurity contents of various magnesium oxide formulations at maximum daily dose

ICH-Q3D defines an accurate risk assessment process

Table 4. Pharmaceutical data for elemental impurities in 1 g magnesium oxide preparations

Element	Oral PDE (µg/day)	A	B	C	D	E
Cd	5	0.02 ± 0.00	0.01 ± 0.00	0.71 ± 0.05	0.83 ± 0.12	0.26 ± 0.02
Pb	5	0.02 ± 0.00	0.02 ± 0.00	0.18 ± 0.01	0.13 ± 0.01	0.12 ± 0.01
As	15	< 0.10	< 0.10	1.57 ± 0.10	1.27 ± 0.04	1.70 ± 0.16
Hg	30	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Co	50	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50
V	100	< 0.50	< 0.50	2.47 ± 0.11	2.75 ± 0.41	3.12 ± 0.51
Ni	200	0.68 ± 0.05	1.18 ± 0.05	1.97 ± 0.11	2.33 ± 0.27	2.55 ± 0.15
Sn	6,000	< 0.50	< 0.50	< 0.50	< 0.50	< 0.50
Cr	11,000	< 0.50	2.93 ± 0.48	4.25 ± 0.19	5.78 ± 0.57	6.12 ± 0.19

$n = 6$, mean ± SE (µg/g), PDE: permitted daily exposure.

by setting a 30% control threshold to ensure that the elements present in the product are below the permissible concentration PDE. In this study, the content of metal element impurities at the maximum daily dose of 2 g (Option 3) of magnesium oxide described in the attached document was compared for 6 lots of preparations A-E based on the 30% control threshold (Figure 1). In 5 lots of preparation C and all lots of preparation D, Cd present as a metal element impurity in the maximum daily dose of 2 g of magnesium oxide showed a value higher than the 30% control threshold (Figure 1a).

Table 5 shows the 30% control threshold value at the maximum daily dose of 2 g of magnesium oxide and the contents of the metal element impurities in 6 lots of preparations A to E. The underlined numbers indicate values that exceed the 30% control threshold.

3.4. Risk classification of preparations by cluster analysis

A cluster analysis was performed based on the data in

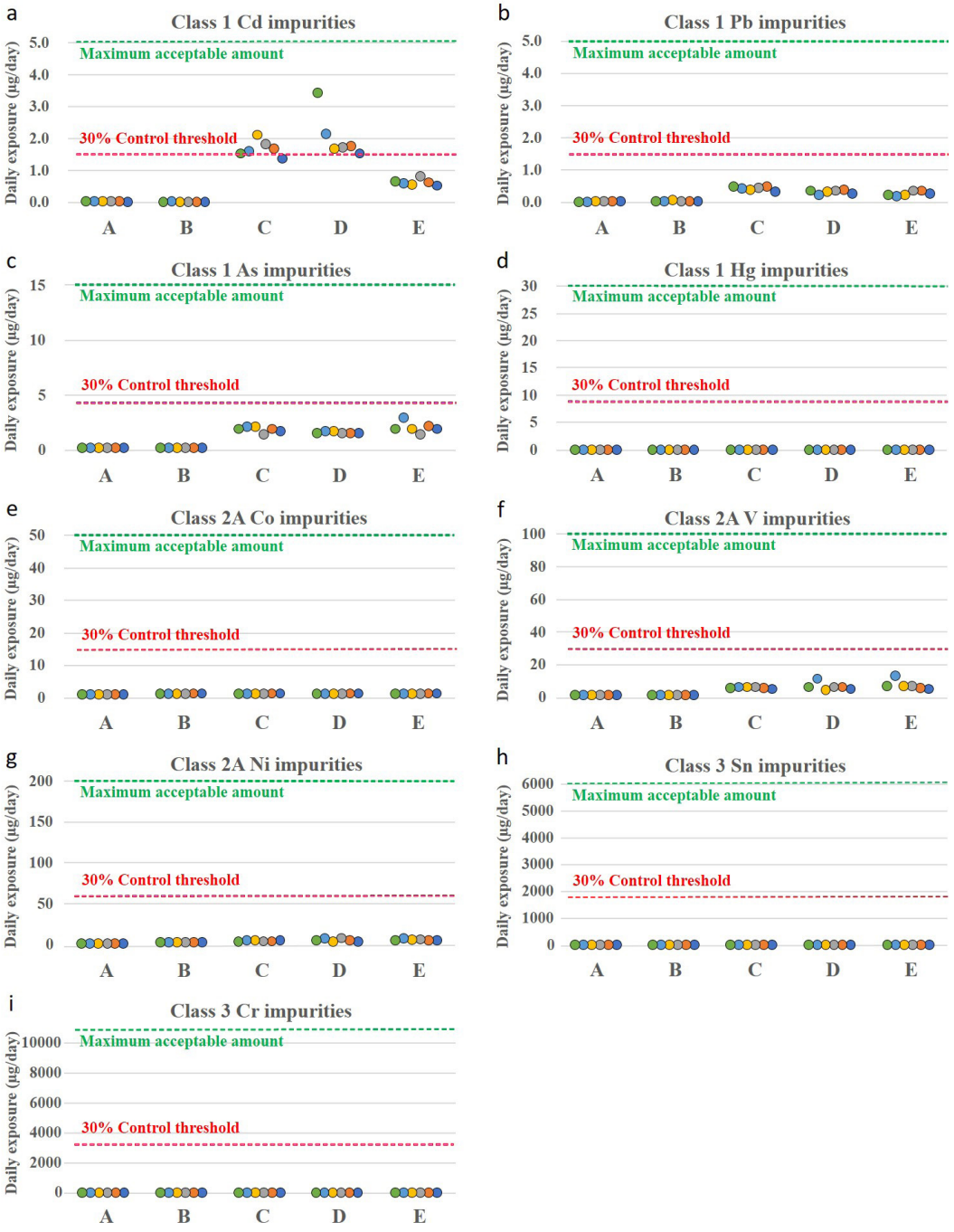

Figure 1. Heavy metal contents of the various magnesium oxide formulations for maximum daily administration. The ordinates represent daily intake exposure for each heavy metal element at the maximum daily dose of 2 g magnesium oxide. Abscissae represent preparations A-E. Each point represents one lot of each preparation. Green line indicates acceptable maximum daily intake, and red line indicates the 30% control threshold based on oral PDE at maximum daily dose of 2 g magnesium oxide.

Table 5. Pharmaceutical data for elemental impurities in magnesium oxide preparation at a maximum daily dose of 2 g

Element	30% Control threshold (μg/day)	A	B	C	D	E
Cd	1.5	0.05 ± 0.01 [0.02 - 0.05]	0.03 ± 0.01 [0.02 - 0.05]	1.68 ± 0.11 [1.35 - 2.12]	2.04 ± 0.39 [1.51 - 3.43]	0.63 ± 0.04 [0.51 - 0.82]
Pb	1.5	0.04 ± 0.01 [0.02 - 0.05]	0.05 ± 0.01 [0.05 - 0.07]	0.42 ± 0.03 [0.31 - 0.50]	0.32 ± 0.03 [0.22 - 0.39]	0.28 ± 0.02 [0.19 - 0.36]
As	4.5	< 0.20	< 0.20	1.85 ± 0.11 [1.40 - 2.10]	1.57 ± 0.05 [1.50 - 1.70]	2.03 ± 0.20 [1.40 - 2.90]
Hg	9	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10
Co	15	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
V	30	< 1.0	< 1.0	5.88 ± 0.29 [5.00 - 6.70]	6.75 ± 1.04 [4.40 - 11.60]	7.53 ± 1.25 [5.10 - 13.50]
Ni	60	1.57 ± 0.10 [1.40 - 2.00]	2.80 ± 0.15 [2.40 - 3.30]	4.67 ± 0.32 [4.00 - 5.70]	5.72 ± 0.69 [4.10 - 8.10]	6.17 ± 0.39 [5.10 - 7.00]
Sn	1,800	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
Cr	3,300	< 1.0	6.98 ± 1.22 [3.10 - 10.70]	10.12 ± 0.59 [8.10 - 11.20]	14.20 ± 1.41 [9.10 - 19.50]	14.72 ± 0.45 [14.00 - 16.80]

n = 6, mean ± SE [range] (μg/day), 30% Control thresholds are calculated from PDE, underbars represents value over 30% control threshold.

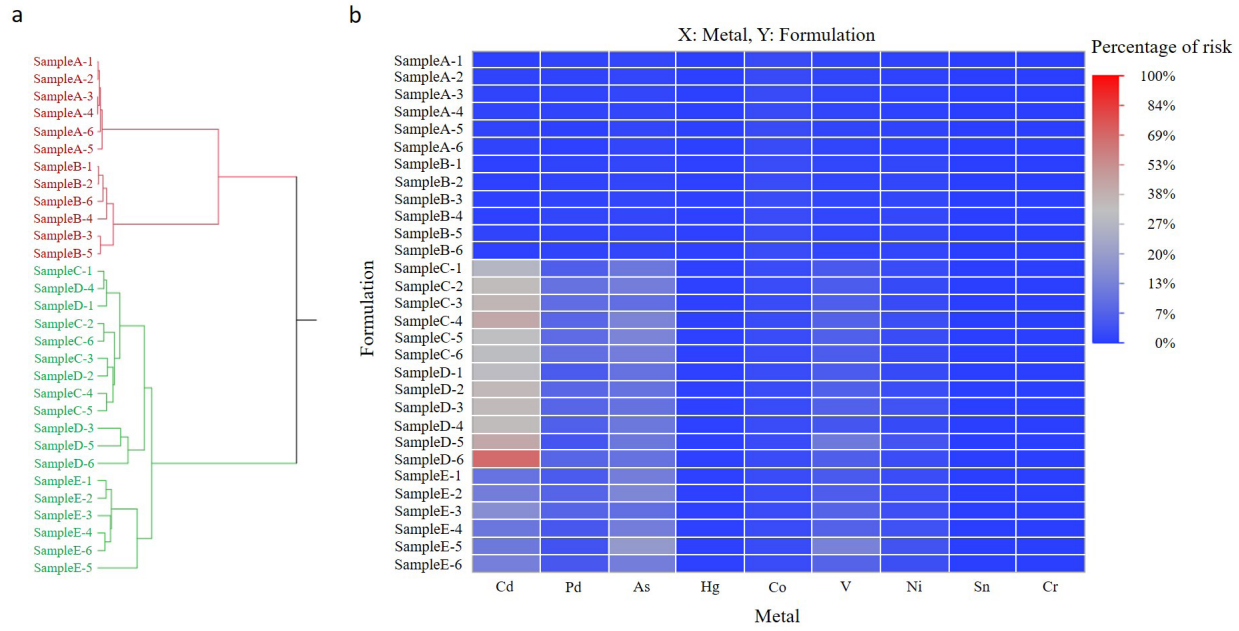

Figure 2. Cluster analysis based on the 30% control threshold for heavy metal contents of each magnesium oxide preparation. The dataset in Figure 1 was used for the cluster analysis. **(a)**: Hierarchical cluster analysis showing the presence of two major groups. **(b)**: Heat map obtained by cluster analysis. Red color shows high impurity and high risk, and blue color shows low impurity and low risk.

Figure 1 and presented as a heat map diagram (Figure 2). The color of the heat map was set to switch from blue to red at the 30% control threshold of PDE, which is the control threshold. Cluster analysis suggested that preparations A-E were classified into two clusters, preparations A + B and preparations C + D + E, or three clusters, preparations A + B, preparations C + D, and preparation E. Preparations A and B were shown to be low in metal impurities and to have a low risk of adverse events due to such impurities.

4. Discussion

Magnesium oxide preparations are pharmaceutical products containing metal elements. For their manufacture, magnesium is collected from nature as a raw material, then concentrated and purified. Therefore, to produce magnesium oxide pharmaceuticals for humans use, highly industrial technology is required, and it is important to monitor the various metal impurities mixed during both the purification of the raw magnesium and the subsequent commercial processing. In this study, we investigated whether the metal element impurities contained in the magnesium oxide preparations sold in Japan meet the standards specified in ICH-Q3D. We also conducted cluster analysis based on the control thresholds and the adverse effect risk of impurities for Class 1 (Cd, Pb, As, Hg), Class 2A (Co, V, Ni) and Class

3 (Sn, Cr) of each preparation.

It was found that the contents of Cd, Pb, As, and Hg, which have strong toxicity, in the manufactured products, did not contravene the oral PDE standard (Table 4). However, Cd (0.71 μg/g), Pb (0.18 μg/g) and As (1.57 μg/g) in preparation C, Cd (0.83 μg/g), Pb (0.13 μg/g) and As (1.27 μg/g) in preparation D, and Cd (0.26 μg/g), Pb (0.12 μg/g) and As (1.70 μg/g) in preparation E were present as elemental metal impurities. Their contents in preparations C, D and E were significantly higher than those in preparations A and B, respectively ($p < 0.05$). Data for the ADI of magnesium oxide calculated from the contents of Cd and Pb in preparations C and D showed that the ADI for Cd was 7.08 g/day (preparation C) and 6.00 g/day (preparation D) and that for Pb was 28.0 g/day (preparation C) and 38.0 g/day (preparation D). When magnesium oxide preparations are used as laxatives, the maximum daily dose for adults is 2 g/day as magnesium oxide, and therefore it can be said that none of these preparations exceeded the oral PDE value for Cd. As the PDE value for Pb was exceeded when the highest-content preparation C was taken at 28.0 g/day or more, there would be no problem with the use of a normal amount of magnesium oxide, *i.e*. 2 g/day. Furthermore, the Cd and Pb contents of the magnesium oxide preparations A and B are less than one-fifth of those in preparations C, D and E, and thus preparations A and B are considered unlikely to cause health problems.

As an index of the significance of the measured values of elemental impurities, the ICH-Q3D guideline defines the 30% level of the PDE value as a control threshold, and states that this control threshold should be used as a criterion for determining the necessity of further control of the preparation (*1*). As shown in Figure 1 and Table 5, when magnesium oxide is used clinically at a maximum daily intake of 2 g, the daily intake of Cd is 1.35-2.12 μg for preparation C and 1.51-3.43 μg for preparation D. Five lots of preparation C and all lots of preparation D, tested in this study exceeded the 30% control threshold of 1.5 μg. This suggests that for preparations C and D, it would be necessary to establish further control strategy to ensure that the PDE value is not exceeded.

In this study, based on the ICH-Q3D guidelines, risk classification based on the elemental metal impurity content of each of preparations A-E was attempted by cluster analysis. As shown in Figure 2, this demonstrated two clusters - preparations A + B and preparations C + D + E - or three clusters - preparations A + B, preparations C + D and preparation E. This also suggests that preparations A and B have low metal impurity contents and comply with the ICH-Q3D guidelines, presenting little risk to the living body. On the other hand, as shown in Figure 1 and Table 5, preparations C and D exceeded the 30% control threshold defined in the ICH-Q3D guideline, and preparations C + D were those considered to warrant additional management methods to ensure that their oral PDE value was not exceeded.

Cd exists in mineral form in nature and is obtained mainly from Cd ore for commercial use (*7*). Cd is used as a catalyst in organic synthesis, and the problem with oral exposure is nephrotoxicity (*8*). Therefore, the PDE value is set at 5 μg/day using the evaluation index for nephrotoxicity.

Pb exists in nature as both organic and inorganic forms. Organic Pb compounds are decomposed fairly quickly in the atmosphere and remain as inorganic Pb compounds in water and soil. In humans, exposure to Pb can affect neural, reproductive, developmental, immune, cardiovascular and renal function. In general, susceptibility to Pb toxicity is higher for exposure during the fetal period and childhood than during adulthood. In addition, epidemiological data suggest that blood Pb levels below 5 μg/dL may be associated with neurobehavioral disorders in children (*9*). Based on these findings, the PDE value of Pb was set at 5 μg/day. Considering the nephrotoxicity of Cd and Pb mentioned above, this point should be taken into consideration when taking magnesium oxide preparations at doses higher than those stated in the package insert or in the case of long-term administration.

As is ubiquitous in the environment and found in food, soil, drinking water and the atmosphere. Inorganic As exists as trivalent (arsenic trioxide, sodium arsenate, *etc*.) or pentavalent (sodium arsenic, arsenic pentoxide, arsenic acid, *etc*.) forms and is highly toxic in the trivalent state. The state of the element is therefore important. Since organic As is also contained in seawater, it is abundant in seaweed. Since the toxicity of organic As is lower than that of inorganic As, and the latter is related to contamination of pharmaceutical products, the standard for safety evaluation is set for inorganic As. It is reported that ingestion of drinking water containing high doses of sodium arsenate by pregnant female rats significantly increased the incidence of liver cancer in next-generation male rats (*10*). Inorganic As is known to be a carcinogen to humans (*11*). Oral intake of As is associated with skin, liver, lung, kidney and bladder cancer. The PDE value when taken orally is set at 15 μg/day based on the chronic effect of As on the skin. In this study, the As content was highest for preparation E at 1.70 μg/g, followed by preparation C at 1.57 μg/g, but both met the standard PDE value and there was only a small possibility of health hazards due to chronic toxicity.

Hg is widely distributed in the global environment and exists in three forms: metallic, inorganic and organic Hg. The form of Hg remaining in drug preparations is usually inorganic Hg. Therefore, safety assessment of Hg has been based on toxicological data for metallic Hg and inorganic Hg. The International Agency for Research on Cancer (IRC) has concluded that the carcinogenicity of inorganic Hg to humans does not fit into the previous classification (*12*). Although inorganic Hg exhibits lower bioavailability *via* oral ingestion than organic Hg,

it has various toxic effects such as neurological effects, corrosiveness, hematopoietic and renal effects, and advanced pain in skin diseases. The underlying toxicity of safety standards for inorganic Hg and its salts is nephrotoxicity. The PDE value for Hg was 30 μg/day, and the Hg content of all the preparations in this survey was less than 0.05 μg/g, which was below the detection limit.

Co, which is classified as Class 2A, is a constituent element of vitamin B12 and an essential element acting as a coenzyme in hemoglobin synthesis. However, cobalt sulfate and other water-soluble Co salts may be carcinogenic to humans. In the case of repeated oral administration, erythrocytosis is the most common problem. Co is classified as Class 2A, because it is associated with heavy metal pneumoconiosis, asthma and contact dermatitis upon inhalation exposure (*13,14*). The PDE value at the time of oral administration was set at 50 μg/day, but in this study, the content of all of the preparations was less than 0.50 μg/g, which was not problematic.

V exists in the Earth's crust in various oxidized states. V classified into Class 2A as vanadium pentoxide has a carcinogenic risk to humans (*7*). The gastrointestinal tract, cardiovascular system and blood system are the main targets of its toxicity by oral administration to humans, and the PDE value is set at 100 μg/day. In this study, the V contents of preparations C, D and E were 2.47-3.12 μg/g, being higher than those of preparations A and B at less than 0.50 μg/g. However, all of the preparations were below the PDE value, and thus considered safe.

No report has indicated Ni carcinogenicity as a result of oral administration (*15*). High oral intake can cause stomach pain, weight loss and adverse effects on the blood and kidneys. It has also been reported that oral intake of Ni in drinking water induces dermatitis in humans (*16*). The PDE value was set to 200 μg/day, but all the products measured this time were below the PDE value and there was no problem.

Since Sn contained in pharmaceutical preparations contains more inorganic Sn than organic Sn, the safety evaluation is based on inorganic Sn. The problematic adverse effect of repeated oral administration is anemia (*17*). The PDE value for Sn is 6,000 μg/day, and the Sn content of preparations A-E was less than 0.50 μg/g, which was not considered problematic.

Since Class 3 classified Cr (6+) has strong oxidizing power, Cr-induced skin disorders and carcinogenicity have been confirmed (18). Cr contained in pharmaceutical products is often in the form of Cr (0) or Cr (3+) rather than the highly toxic Cr (6+). Therefore, drug safety assessments are based on Cr (3+) toxicity information, and Cr (6+) is excluded. Since no obvious health effects of oral intake of Cr (3+) have been identified, it is classified as Class 3. According to this measurement, the concentrations of Cr in preparations B-E were relatively high, with the exception of preparation A. However, since the oral PDE value was set as high as 11,000 μg/day, the results for all the preparations were far below the PDE value, and it was considered that there was no problem.

Various potential sources of elemental impurities are: 1) Residual impurities resulting from elements added intentionally (*e.g.*, catalysts) in the production of the drug substance, excipients, or other drug product components. 2) Elemental impurities that are not added intentionally and are potentially present in the drug substance, water, or excipients used in the preparation of the drug product. 3) Elemental impurities can be potentially introduced into the drug substance and/or drug product from manufacturing equipment. 4) Elemental impurities that have the potential to be leached into the drug substance and drug product from container closure systems. Risk assessment of the drug substance should address the potential for inclusion of elemental impurities in the final drug product. The reason why the content of metal element impurities differed between the various surveyed preparations is considered to be that magnesium oxide is a compound that is affected by the conditions used for sampling of the raw material. Since the standard PDE value was not exceeded in any of the investigated preparations, there would be no problem if the daily dose was maintained within that stated in the package insert. However, for Cd, 5 lots of preparation C and all lots of preparation D exceeded the control threshold. From the viewpoint of risk management, it is necessary to carry out further controls such as changing the steps of the manufacturing process, setting standard values for additives and raw materials, or selecting an appropriate container plugging system for these preparations. In addition, the levels of metal impurities in preparations C, D and E showed were higher except for Hg, Co and Sn in preparations A and B, and the amount exceeded the dose stated in the package insert when the effect of magnesium oxide was insufficient. Therefore, an effect on the human body resulting from long-term administration, or administration to patients with renal impairment, cannot be ruled out. In this context, the present survey clarified that preparation A or B would be desirable in terms of the safety of its heavy metal content.

Currently, there are many generic drugs on the market that can reduce development costs in the pharmaceutical industry. In addition, the Ministry of Health, Labor and Welfare has a policy of promoting the transition from branded drugs to generic drugs with the aim of reducing national medical expenses. As products of the same drug diversify, even equivalence of efficacy and effect is guaranteed, the content of elemental metal impurities will vary depending on the raw materials and additives used by various pharmaceutical companies. Therefore, for long-term administration, it will be necessary to pay attention to accumulation of such impurities in the body.

In order to ensure the globalization of the pharmaceutical industry and the safety of pharmaceutical products, it is considered necessary to conduct preparation tests based on ICH-Q3D in the future. As shown in this study, there may be preparations that meet the current standards but may not meet the ICH standards in the future. For preparations that may not meet these criteria, it will be necessary to seek improvement, and at the same time it is suggested that monitoring of impurities will be required when the ICH standard is introduced.

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Original Article

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Catheter failure in the administration of hyperosmotic drugs through a peripheral vein and vascular selection: A retrospective cohort study

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SUMMARY This study aimed to determine whether the placement of a peripheral intravenous catheter (PIVC) in the cephalic vein of the forearm could prevent PIVC failure in patients receiving hyperosmotic drugs through the peripheral vein. This retrospective cohort study included patients aged ≥ 20 years who had received infusion therapy *via* a PIVC in our institution between July and November 2017. Patients were divided into groups according to PIVC insertion into the cephalic, basilic, and medial veins. PIVCs used to administer drugs with osmotic pressure ratios > 2.0 were included. The primary outcome was survival time to catheter failure. Catheter failure was defined as accidental and unplanned catheter removal. We set the cephalic vein and other veins, including the medial and basilic veins, in the forearm as cohort groups. We used the Kaplan-Meier survival curves to compare the time until catheter failure in the cohort groups. The Cox proportional hazard models were fitted, and the hazard ratios were calculated. A total of 46 catheters with hyperosmotic agents were included in the analysis. Catheter failure was observed in 25 (54.3%) cases. Time to catheter failure in patients receiving high-dose drugs *via* the cephalic vein was significantly longer than that in the other two groups ($p < 0.01$). Thus, the cephalic vein, which has a high blood flow, is the ideal site of PIVC insertion in patients receiving high drug concentrations to prevent catheter failure.

Keywords peripheral intravenous catheter, ultrasonography, blood flow, complication, adverse events

1. Introduction

Most patients require at least one peripheral vascular device when intravenous fluids and medications are being administered. The most commonly used one is the peripheral intravenous catheter (PIVC). Recent studies reported that more than 70% of patients in acute care hospitals require PIVCs (*1-3*). Notably, more than 25% of PIVCs are removed prematurely, which is known as catheter failure (*3-5*). In Japan, the prevalence of catheter removal due to catheter failure among inpatients in a university hospital was 18.8% (*6*). Catheter failure is associated with symptoms such as erythema, swelling, induration, bleeding, pain, and insufficient dripping (*7,8*). These symptoms negatively affect patients' comfort and treatment, thus preventing the continuation of infusion therapy (*1,9*). In such cases, catheters need to be replaced, resulting in an increase in labor, cost, and patient discomfort (*10*). Therefore, it is important for patients and healthcare providers to prevent PIVC failure. Risk factors of various PIVC complications are known; however, healthcare providers cannot effectively prevent these complications.

In a previous study that suggested that mechanical irritation was an important factor in catheter failure, the effectiveness of care protocols was investigated, including an ultrasonographic "pre-scan" for selecting a large-diameter vein before catheterization, a "post-scan" to confirm the position of the tip of the catheter after catheterization with ultrasonography, and the use of a flexible polyurethane catheter to reduce the mechanical irritation that contributes to the incidence of catheter failure (*11-15*). Consequently, the relative risk reduction in interventions for catheter failure was 0.60 (95% confidence interval [CI]: 0.47-0.71). In the intervention group, it was difficult to completely prevent catheter failure. Despite a reduction in the mechanical stimuli that contributed to catheter failure, complications related to

chemical irritation from pharmaceuticals could not be avoided. An effective method to prevent pharmaceutical chemical stimulation, especially from drugs administered at high concentrations, has not been established (*16-18*). High concentrations of drugs are administered *via* central veins with abundant blood flow. Moreover, the administration of drugs at an osmotic pressure more than twice as that of physiological saline increases the risk of catheter failure and ulceration, even in the dosage range permitted for administration into the peripheral veins (*19*). However, the volume of the main blood flow distribution in the forearm is unknown as previous studies limited their investigation to the veins in the upper arms (*20,21*). Therefore, it remains unclear whether catheterization in the peripheral vein with an abundant blood flow will prevent catheter failure. A previous study suggested that blood flow in the peripheral veins in the forearm is more abundant in the cephalic veins than in the other forearm veins (*22*) and indicated that the cephalic vein might be a better choice for preventing catheter failure when patients are administered drugs at a high osmotic pressure.

This study aimed to determine whether the placement of the PIVC in the cephalic vein in the forearm could reduce the probability of PIVC failure in patients administered high osmotic pressure drugs through the peripheral veins.

2. Materials and Methods

2.1. Design and setting

This retrospective cohort study included patients aged 20 years who were hospitalized and had received infusion therapy *via* a PIVC placed by the nurses at our institution between July and November 2017. The detailed study protocol has been published elsewhere (*19*). Patients were recruited from a medical department with high PIVC usage based on previous studies (*12,23*). PIVCs used to administer drugs with osmotic pressure ratios > 2.0 were included in the study. Patients receiving chemotherapy and those with poor cognitive ability were excluded. Study procedures were explained to the physicians and nurses working at the ward at the beginning of the study period. Upon admission, patients who were scheduled to receive a PIVC as part of their treatment were provided a written briefing of the study. Moreover, permission for patient intervention was obtained from the attending physician. Written informed consent was obtained from all residents or their representations. The study was performed in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of our institution (#2019009NI).

2.2. Data collection

2.2.1. Outcome

The outcome measure was survival time to catheter failure. Catheter failure was defined as the accidental or unplanned removal of catheters based on standard nursing practice for using PIVCs in our hospital in accordance with the Centers for Disease Control and Prevention guidelines (*24*). Information regarding catheter failure was obtained from a medical chart and interviews. Data on catheter insertion and removal and time were collected from the patients' medical charts. The researcher made direct observations at least twice a day and interviewed nurses and patients (*25*). The researcher also collected data on the dwelling time.

2.2.2. Catheterized vein

The researchers photographed and recorded the site where the catheter was inserted. They identified the major veins based on the anatomical location of the insertion. This study excluded cases of dorsal hand veins and upper arm implantation. The veins of the forearm were divided into three categories: cephalic, basilic, and medial veins.

2.2.3. Other variables

The following data were collected by reviewing the patients' medical charts: patient characteristics such as age, sex, and body mass index (BMI); comorbidities (presence of organ tumors); medical history (diabetes); previous treatments (history of steroid use, chemotherapeutic solutions, immunosuppressive solutions, anticoagulants, and radiation therapy); blood examination results including C-reactive protein (CRP), albumin, and platelet levels; antibiotic medications administered at baseline; and the total time of locking. Nurse-related information such as the experience of catheterization was also collected (*26*). The following data were collected through macroscopic observation: characteristics of the site for catheterization (anatomical insertion site, dominant vein, success of first catheterization attempt, times for catheterization), PIVC type (catheter material and size), and the characteristics of the target vein (diameter and depth) (*12*).

2.3. Data analysis

We compared time to PIVC failure based on catheter insertion in the cephalic, medial, and basilic veins in the forearm. We used the Kaplan-Meier survival curves to compare the time until catheter failure between the cohort groups. The log-rank test was used to compare the catheter survival rates among the groups. Cox proportional hazard models were fitted, and the hazard ratios (HRs) were calculated. Age (reference: 1 year), sex (reference: male), BMI (unit = 0.1), blood vessel diameter (unit = 0.1 mm), blood vessel depth (unit = 0.1 mm), experience of the

nurse, Alb (unit = 0.1 g/dL) and CRP (unit = 0.1 mg/dL) levels, and the use of antibacterial agents were added to the model as control variables. The problem of the Hosmer-Lemeshow test used in logistic regression analysis to evaluate the fitness of the model was clarified in this study. Data were analyzed using Stata version 14 (StataCorp, USA).

3. Results

A total of 46 catheters used to administer hyperosmotic agents were included in the analysis. Catheter failure was observed in 25 (54.3%) cases. Patient characteristics are shown in Table 1. The median age of the patients was 72 years (57-82 years), and 62.5% of the patients were male.

Figure 1 shows the Kaplan-Meier survival curves to compare the time until catheter failure among the three cohort groups. Multivariate Cox proportional hazards models were fitted to identify independent predictors of complications, including the type of the catheterized vein, catheter size, and first insertion attempts (Table 2).

Four additional cases of concomitant use of fat emulsion were excluded from the analysis. The results were similarly significant for the vascular sites ($p < 0.05$), indicating the robustness of the results.

4. Discussion

4.1. Short summary

PIVC insertion into the cephalic vein significantly reduced the hazard of catheter failure in case of high-concentration drugs. Catheter placement in the cephalic vein was associated with a longer survival time to catheter failure when using highly osmotic agents, even when morphological features of blood vessels (vessel diameter and depth) were introduced as covariates.

4.2. Outcome interpretation

The use of high-concentration drugs may cause ulceration and catheter failure (*27-29*). To prevent this, comprehensive care, such as the care-bundle

Table 1. Baseline characteristics of subjects

Items	Cephalic vein	*n* = 26	Basilic vein	*n* = 10	Medial vein	*n* = 10	Total	*n* = 46
Age, years	64.9	(17.2)	64.7	(19.6)	73.6	(16.9)	66.7	(17.6)
Sex, male	15	(57.7%)	5	(50.0%)	4	(40.0%)	24	(52.2)
Body mass index	23.5	(3.8)	24.7	(3.7)	24.4	(4.8)	23.9	(4.0)
Diameter of vein (mm)	2.8	(0.8)	2.7	(0.8)	2.4	(0.6)	2.7	(0.8)
Depth of vein (mm)	2.5	(0.9)	2.2	(0.7)	2.3	(1.1)	2.41	(0.9)
Using oversized catheters	16	(61.5)	8	(80.0)	8	(80.0)	32	(69.6)
Level of nurse's experience in PIVC insertion								
Beginner	7	(26.9%)	5	(50.0%)	7	(70.0%)	11	(23.9)
Intermediate	13	(50.0%)	2	(20.0%)	1	(10.0%)	26	(56.5)
Expert	4	(15.4%)	3	(30.0%)	2	(20.0%)	9	(19.6)
Albumin (g/dL)	3.3	(0.7)	3.5	(0.5)	0	(0.0%)	3.3	(0.7)
C-reactive protein (mg/dL)	2.4	(2.8)	3.6	(4.2)	3.0	(0.7)	3.0	(3.8)
Antibacterial drugs	18	(69.2%)	6	(60.0%)	3.6	(5.5)	27	(58.7)
Multiple puncture attempts	19	(73.1%)	2	(20.0%)	8	(80.0%)	29	(63.0)
Catheter failure	11	(42.3%)	4	(40.0%)	7	(70.0%)	22	(47.8)

The variables are presented as the mean (standard deviation) or *n* (%). Level of nurse's experience: beginner, 0-100 PIVCs; intermediate, 101-800 PIVCs; expert, > 801 PIVCs. Abbreviation: PIVC, peripheral intravenous catheter.

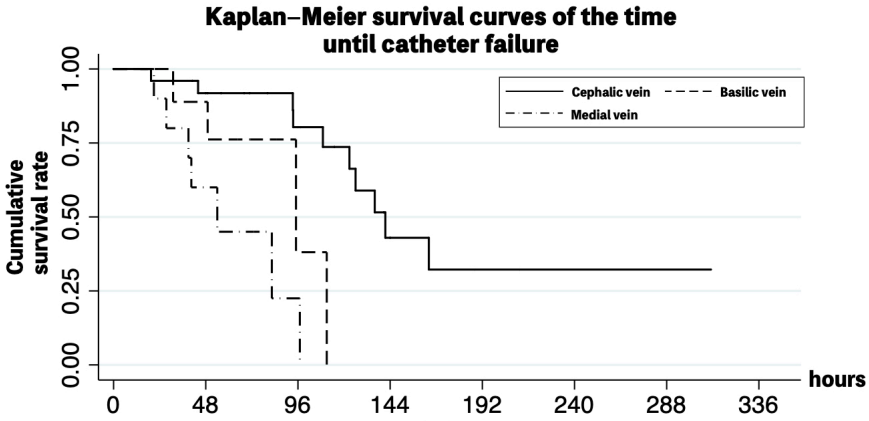

Figure 1. Kaplan-Meier survival curves of the time until catheter failure.

Table 2. Multivariate Cox proportional hazard model

n = 46	Adjusted HR	95% CI	*p*-value
Catheterized vein (compared with the cephalic vein)			
Basilic vein	3.15	0.82-11.4	0.09
Medial vein	7.73	2.16-28.1	0.01
Using oversized catheters	1.09	0.35-3.43	0.88
First punctuator attempts	1.46	0.51-4.17	0.48

Note: Hosmer-Lemeshow test; $p = 0.603$. Abbreviations: HR, hazard ratio; CI, confidence interval.

approach has been proposed (*19,30*). Furthermore, many interventions were limited to those that prevent catheter deviation and those that aimed to reduce mechanical stimulation (*19,31-33*). Although the properties of the chemical solutions have been a related factor, there are some aspects that the nurse cannot change in clinical practice, such as the drug composition (*34*). Moreover, the method of drug use is different across countries and clinical settings, and it is difficult to propose an improvement plan that can be applied universally. However, the action of the drug solution and its chemical properties are less likely to cause local tissue damage if sufficient dilution is performed. Blood dilution is important for any drug, and this study shows the importance of selecting blood flow-rich sites as a possible preventive strategy of catheter failure.

4.3. Internal validity

The concern of this study was the outcome evaluation challenges. In the present study, the assessment of catheter failure was performed by nurses in one ward, and the nurse's ability may have affected the incident rate of catheter failure. However, all cases of the catheter and non-catheter failure extractions were photographed and symptomatically identified by the researchers. Therefore, certain criteria were met, and the variability was considered minimal.

4.4. External validity and clinical implementation

This study has some limitations. There was an external validity issue with the target population. The patients were from urban areas hospitalized in an acute care university hospital with an average age of approximately 65 years. Therefore, the results of this study may not be applicable to more elderly patients (*i.e.*, > 80 years old) or those with vascular-related diseases because many patients had gastroenterology as their primary disease and did not have any vascular-related conditions.

4.5. Further research

It is necessary to examine the functional factors of the blood vessels related to catheter failure and ulceration, especially when using a high concentration of drugs. Finally, it is possible to find a sound basis for evaluating the peripheral vein blood flow and catheter management.

In conclusion, catheter placement in the cephalic vein was associated with a longer survival time to catheter failure when using highly osmotic agents, even when morphological features of blood vessels (vessel diameter and depth) were introduced as covariates. Therefore, it is necessary to consider not only the morphological but also the functional characteristics of the vessel when selecting a site for catheter placement.

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Original Article

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Physicochemical properties of brand and generic infusion fluid preparations (Part 3): Investigation of type 1 hypotonic infusion fluids

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SUMMARY In Japan, the increasing use of generic drugs has led to a reduction in drug prices, which affect the steady supply of drugs. A "basic drug" system was introduced to rescue these drugs by eliminating gaps in drug prices among preparations with the same constituents. "Type 1" hypotonic infusion fluids, which are potassium-free and commonly used to treat dehydration, meet the definition of a "basic drug" in Japan, and there are no drug price gaps. However, there is a lack of information on the physicochemical properties of "type 1" hypotonic infusion fluids, making it difficult to identify differences among them. Extracellular fluid-replacement solutions and "type 3" hypotonic infusion fluids have different pH and titratable acidity. Here, we measured the pH, titratable acidity, and osmolality of six different "type 1" hypotonic infusion fluids and compared the results with respect to risk avoidance considering metabolic acidosis, changes upon mixing, and vascular pain. There was a significant difference, or trend toward significance, in titratable acidity, which is a risk factor for metabolic acidosis in patients with impaired renal function, and pH, which is a risk factor for change upon mixing, among all combinations except one of the infusion fluids. Thus, the selection of "type 1" hypotonic infusion fluids for children with immature renal function, elderly patients with impaired renal function, and patients with unknown pathophysiology, considering titratable acidity and pH, is an effective strategy for risk avoidance.

Keywords Titratable acidity, pH, risk avoidance, impaired renal function, elderly patients, metabolic acidosis

1. Introduction

In Japan, the use of generic drugs is encouraged as a part of fiscal reforms of the national medical insurance system (*1*). As of March 2021, generic drug use by volume was 79.6% in Japan, rapidly closing in on the 80% target for 2020 (*2*). Owing to frequent reductions in drug prices, introduced by national health insurance price revisions, an increasing number of drugs have become unprofitable despite a high demand for their use as a part of insured medical care, their long-term widespread use in clinical settings, and their established safety and efficacy. Thus, there is a need to ensure a continuous and stable supply of these drugs that have become unprofitable. A provisional system was introduced with the 2016 drug pricing reforms to designate these as "basic drugs," and to offer support before they are subject to "repricing of unprofitable products" or become "minimum-priced products" (*3*). Currently, because "type 1" hypotonic infusion fluids in Japan meet the definition of an unprofitable product, all such generic and Brand versions of "type 1" hypotonic infusion fluids are designated "basic drugs" (*4*), and there is no drug price gap between them (*5*). However, as generic preparations intended for intravenous administration undergo fewer tests during development than Brand versions, they are subjected to fewer submission requirements when applying for approval (*6*).

Previously, we showed that pH, titratable acidity, osmolality, and insoluble microparticle levels differ between Brand and generic versions of extracellular fluid replacement solutions (*7*) and "type 3" hypotonic infusion fluids (*8*). Additionally, these differences contribute to metabolic acidosis, changes upon mixing, and vascular pain (*7,8*). Information on titratable acidity, osmolality, and insoluble microparticles need not be included in drug package inserts or pharmaceutical interview forms. Additionally, information on these items is not available

in the Information Package of Quality of Prescription Drugs (the so-called Blue Book) (*9*). However, besides pH, titratable acidity influences the acid-base equilibrium of blood and changes upon mixing, osmolality can cause vascular pain, and insoluble microparticles can induce adverse events (*e.g.*, phlebitis and pulmonary fiber embolism) (*10,11*). Even among preparations with the same constituents, examining the differences in these parameters is an effective strategy for risk avoidance when deciding whether a preparation is indicated (*7,8*).

In this study, we evaluated pH and other physicochemical properties (titratable acidity and osmolality) that need not be included in drug package inserts or pharmaceutical interview forms. We aimed to ascertain information that should be evaluated from the perspective of risk avoidance when deciding if a "type 1" hypotonic infusion fluid is indicated.

2. Materials and Methods

2.1. Experimental materials

Experiments were performed using two brand-equivalent drugs (labeled "Brand 1" and "Brand 2"), three brand-equivalent versions of brand 1 (labeled "Generic 1-1," "Generic 1-2," and "Generic 1-3"), and one brand-equivalent version of brand 2 (labeled "Generic 2"). Note that preparations classified as "Brand drugs" before the introduction of the "basic drugs" system are herein referred to as "brand-equivalent drugs." Similarly, preparations classified as "generic drugs" before the introduction of the "basic drugs" system are referred to as "generic-equivalent drugs."

Generic 1-2 contains the same constituents, raw drug materials, and additives as Brand 1; it is produced using the same method under license from the manufacturer of Brand 1 and approved for national health insurance coverage. Table 1 indicates whether each preparation is a brand-equivalent or a generic-equivalent drug, and includes the indicated name, constituents, and manufacturer.

2.2. Measurement of pH and physicochemical properties

Titratable acidity was determined using a TUA-701 automatic analyzer (DKK-TOA Corporation, Tokyo, Japan). Neutralization of titratable acidity was measured using 0.1 N NaOH (Hayashi Pure Chemical, Osaka, Japan) with the endpoint set to pH 7.4. pH was determined using a TUA-701 automatic analyzer (DKK-TOA Corporation). Osmolality was determined using the freezing point depression method (*12*) with supercooling using Osmostat™ OM-6040 (Arkray Factory, Inc., Shiga, Japan). Each sample was analyzed five times for each measured parameter.

2.3. Statistical analysis

Normality was confirmed using Shapiro-Wilk's W test. Two-group comparisons were made using the two-sided Student t-test or Kruskal-Wallis test. Either Tukey-Kramer test or Steel-Dwass test was used for multiple-group comparisons. All statistical analyses were performed using JMP® 14 (SAS Institute Inc., Cary, NC, USA), and results with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Titratable acidity

3.1.1. Comparison among Brand 1, Generic 1-1, Generic 1-2, and Generic 1-3

There was a significant difference in titratable acidity between Generic 1-1 and Generic 1-2 (median [IQR]: 0.77 [0.77-0.78] *vs*. 0.39 [0.38-0.39], respectively, $p = 0.043$), Generic 1-1 and Generic 1-3 (median [IQR]: 0.77 [0.77-0.78] *vs*. 0.11 [0.11-0.14], respectively, $p = 0.049$), and Generic 1-2 and Generic 1-3 (median [IQR]: 0.39 [0.38-0.39] *vs*. 0.11 [0.11-0.14], respectively, $p = 0.044$). A significant difference was also observed between Generic 1-1 and Brand 1 (median [IQR]: 0.77 [0.77-0.78] *vs*. 0.41 [0.39-0.45], respectively, $p = 0.052$), and between Brand 1 and Generic 1-3 (median [IQR]: 0.41 [0.39-0.45] *vs*. 0.11 [0.11-0.14], respectively, $p = 0.053$). There was no significant difference in titratable activity

Table 1. Brand- and Generic-equivalent drugs evaluated in the current study

Classification	Labeled name	Constituents**			
		Na^+ (mEq/L)	Cl^- (mEq/L)	Lactate$^-$ (mEq/L)	Glucose (%)
Brand-equivalent drugs*	Brand 1	90	70	20	2.6
Generic-equivalent drugs*	Generic 1-1	90	70	20	2.6
Generic-equivalent drugs*	Generic 1-2	90	70	20	2.6
Generic-equivalent drugs*	Generic1-3	90	70	20	2.6
Brand-equivalent drugs*	Brand 2	77	77	–	2.5
Generic-equivalent drugs*	Generic 2	77	77	–	3.5

*Although designated a "basic drug" in 2020, preparations classified as "Brand drugs" before the introduction of the "basic drugs" system are referred to as "brand-equivalent drugs" and "generic drugs" are referred to as "generic-equivalent drugs." **Data on constituents were obtained from the drug package insert of each preparation.

between Brand 1 and Generic 1-2 (median [IQR]: 0.41 [0.39-0.45] *vs*. 0.39 [0.38-0.39], respectively, $p = 0.235$).

The titratable acidity of Generic 1-1 was ~1.8-fold higher than that of Generic 1-2, ~7-fold higher than that of Generic 1-3, and ~1.9-fold higher than that of Brand 1. The titratable acidity of Brand 1 was ~3.8-fold higher than that of Generic 1-3 and that of Generic 1-2 was ~3.5-fold higher than that of Generic 1-3 (Figure 1A).

3.1.2. Comparison between Brand 2 and Generic 2

There was a significant difference in titratable acidity between Brand 2 and Generic 2 (mean ± SD: 0.04 ± 0 *vs*. 0.11 ± 0.01, respectively, $p < 0.0001$). The titratable acidity of Generic 2 was ~2.8-fold higher than that of Brand 2 (Figure 1B).

3.1.3. Comparison between Brand 1 and Brand 2

There was a significant difference in titratable acidity between Brand 1 and Brand 2 (mean ± SD: 0.42 ± 0.03 *vs*. 0.04 ± 0, respectively, $p < 0.0001$). The titratable acidity of Brand 1 was ~10.5-fold higher than that of Brand 2 (Figure 1C).

3.2. pH

3.2.1. Comparison among Brand 1, Generic 1-1, Generic 1-2, and Generic 1-3

There was a significant difference in pH between Generic 1-3 and Generic 1-2 (median [IQR]: 6.06 [6.01-6.06] *vs*. 5.47 [5.47-5.48], respectively, $p = 0.043$), Generic 1-3 and Brand 1 (median [IQR]: 6.06 [6.01-6.06] *vs*. 5.46 [5.46-5.47], respectively, $p = 0.049$), Generic 1-3 and Generic 1-1 (median [IQR]: 6.06 [6.01-6.06] *vs*. 5.18 [5.18-5.19], respectively, $p = 0.043$), Generic 1-2 and Generic 1-1 (median [IQR]: 5.47 [5.47-5.48] *vs*. 5.18 [5.18-5.19], respectively, $p = 0.038$), and Brand 1 and Generic 1-1 (median [IQR]: 5.46 [5.46-5.47] *vs*. 5.18 [5.18-5.19], respectively, $p = 0.044$). There was no significant difference between Brand 1 and Generic 1-2 (median [IQR]: 5.46 [5.46-5.47] *vs*. 5.47 [5.47-5.48], respectively, $p = 0.108$). The pH of Generic 1-3 was ~0.59-fold higher than that of Generic 1-2, ~0.60-fold higher than that of Brand 1, and ~0.88 higher than that of Generic 1-1. The pH of Generic 1-2 was ~0.29-fold higher than that of Generic 1-1 and the pH of Brand 1 was ~0.28-fold higher than that of Generic 1-1 (Figure 2A).

3.2.2. Comparison between Brand 2 and Generic 2

There was a significant difference in pH between Brand 2 and Generic 2 (mean ± SD: 4.98 ± 0.02 *vs*. 4.63 ± 0.02, respectively, $p < 0.0001$). The pH of Brand 2 was ~0.35-fold higher than that of Generic 2 (Figure 2B).

3.2.3. Comparison between Brand 1 and Brand 2

There was a significant difference in pH between Brand

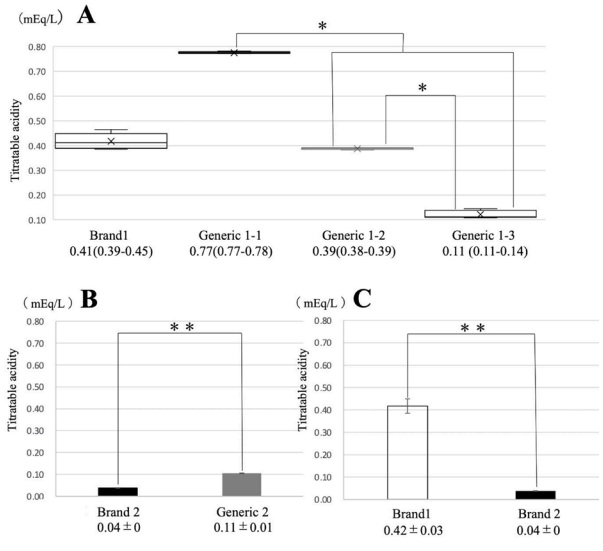

Figure 1. Comparison of titratable acidity between brand-equivalent drugs and generic-equivalent drugs. (A) Comparisons among Brand 1, Generic 1-1, Generic 1-2, and Generic 1-3. Data are presented as median, and the upper and lower ends of the box represent the third and first quartiles, respectively. **(B)** Two-group comparisons between Brand 2 and Generic 2 and **(C)** between Brand 1 and Brand 2. For parts **(B)** and **(C)**, data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$.

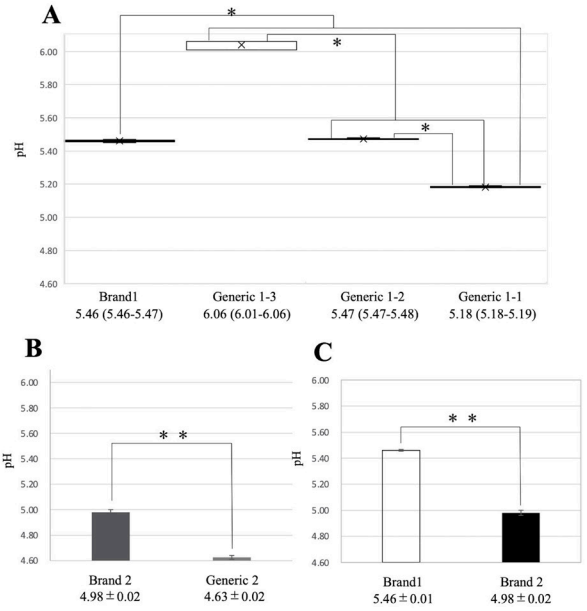

Figure 2. Comparison of pH between brand-equivalent drugs and generic-equivalent drugs. (A) Multiple comparisons among Brand 1, Generic 1-3, Generic 1-2, and Generic 1-1. Data are presented as median, and the upper and lower ends of the box represent the third and first quartiles, respectively. **(B)** Two-group comparisons between Brand 2 and Generic 2 and **(C)** between Brand 1 and Brand 2. For parts **(B)** and **(C)**, data are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$.

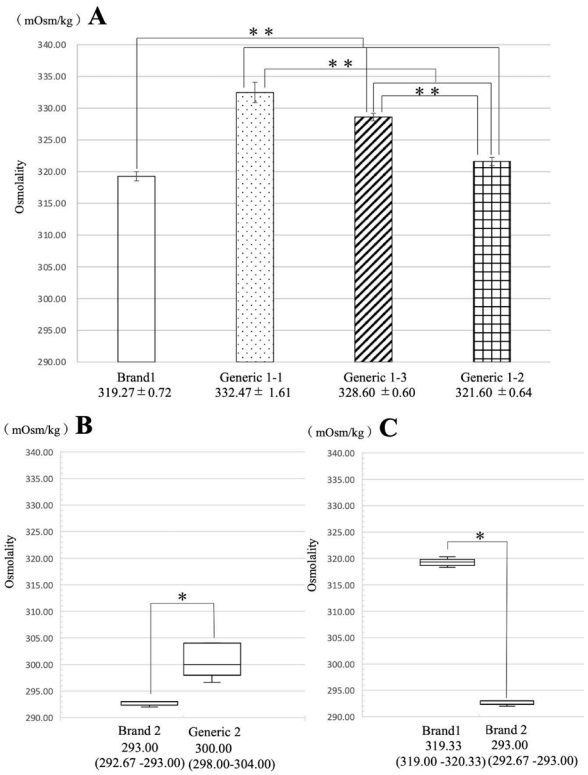

Figure 3. Comparison of osmolality between brand-equivalent drugs and generic-equivalent drugs. (A) Comparisons among Brand 1, Generic 1-1, Generic 1-3, and Generic 1-2. Data are presented as mean ± SD. **(B)** Two-group comparisons between Brand 2 and Generic 2 and **(C)** between Brand 1 and Brand 2. For parts **(B)** and **(C)**, data are presented as median, and the upper and lower ends of the box represent the third and first quartiles, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$.

1 and Brand 2 (mean ± SD: 5.46 ± 0.01 *vs*. 4.98 ± 0.02, respectively, $p < 0.0001$). The pH of Brand 1 was ~0.48-fold higher than that of Brand 2 (Figure 2C).

3.3. Osmolality

3.3.1. Comparison of Brand 1, Generic 1-1, Generic 1-2, and Generic 1-3

There was a significant difference in osmolality between Generic 1-1 and Generic 1-3 (mean ± SD: 332.47 ± 1.61 *vs*. 328.60 ± 0.60, respectively, $p < 0.0001$), Generic 1-1 and Generic 1-2 (mean ± SD: 332.47 ± 1.61 *vs*. 321.60 ± 0.64, respectively, $p < 0.0001$), Generic 1-1 and Brand 1 (mean ± SD: 332.47 ± 161 *vs*. 319.27 ± 0.72, respectively, $p < 0.0001$), Generic 1-3 and Generic 1-2 (mean ± SD: 328.6 ± 0.60 *vs*. 321.60 ± 0.64, respectively, $p < 0.0001$), Generic 1-3 and Brand 1 (mean ± SD: 328.6 ± 0.60 *vs*. 319.27 ± 0.72, respectively, $p < 0.0001$), and Generic 1-2 and Brand 1 (mean ± SD: 321.60 ± 0.64 *vs*. 319.27 ± 0.72, respectively, $p = 0.009$).

The osmolality of Generic 1-1 was ~3.87 mOsm/kg higher than that of Generic 1-3, ~10.87 mOsm/kg higher than that of Generic 1-2, and ~13.20 mOsm/kg higher than that of Brand 1. The osmolality of Generic 1-3 was ~7.00 mOsm/kg higher than that of Generic 1-2 and ~9.33 mOsm/kg higher than that of Brand 1. The osmolality of Generic 1-2 was ~2.33 mOsm/kg higher than that of Brand 1 (Figure 3A).

3.3.2. Comparison of Brand 2 and Generic 2

There was a significant difference in osmolality between Brand 2 and Generic 2 (median [IQR]: 293.00 [292.67-293.00] *vs*. 300.00 [298.00-304.00], respectively, $p = 0.011$). The osmolality of Generic 2 was ~7.00 mOsm/kg higher than that of Brand 2 (Figure 3B).

3.3.3. Comparison of Brand 1 and Brand 2

There was a significant difference in osmolality between Brand 1 and Brand 2 (median [IQR]: 319.33 [319.00-320.33] *vs*. 293.00 [292.67-293.00], respectively, $p = 0.011$). The osmolality of Brand 1 was ~26.33 mOsm/kg higher than that of Brand 2 (Figure 3B).

4. Discussion

In Japan, drug package inserts, pharmaceutical interview forms, and pharmaceutical product information outlines are common sources of information when using pharmaceuticals (*13,14*). In 2018, the Generic Pharmaceutical Quality Information Study Committee published the Information Package of Quality of Prescription Drugs (Blue Book) (*9*) to provide information on and ensure the quality of generic pharmaceuticals. Since then, this "Blue Book" has become a useful source of information. The Tokyo Metropolitan Government also reported that, based on the results of a 2019 questionnaire concerning generic pharmaceuticals, Pharmaceutical and Medical Device Delivery Services (PMDA Medi-Navi) and pharmaceutical company websites are common sources of information on generic drugs (*15*). However, it is difficult to obtain information on titratable acidity, osmolality, and insoluble microparticles from these information sources. This information need not be included in drug package inserts or pharmaceutical interview forms.

Titratable acidity is calculated by titrating the acidity of a material with a standard base; in clinical terms, it can be described as the amount of base (0.1 mol/L NaOH) needed to titrate the infusion fluid preparation to the pH of human blood (7.4) (*16*). Although fixed acids affect the titratable acidity of these preparations, information on some fixed acids such as acetic acid need not be included in the drug package insert (*17*). Therefore, for example, adding acetic acid to an infusion fluid preparation will have a limited effect on pH, but it will increase titratable acidity. For this reason, adding fixed acids that need not be mandatorily included in drug package inserts results in different titratable acidities among preparations with the same constituents. Furthermore, because fixed

acids are processed by the kidneys, administering preparations with high titratable acidities increases the risk of metabolic acidosis in patients with impaired renal function, elderly patients, and children with immature renal function (*18*).

The results of the present study revealed a significant difference, or trend toward significance, in titratable acidity among all combinations of "type 1" hypotonic infusion fluids with the same constituents, except between Brand 1 and Generic 1-2. Humans produce fixed acids at a rate of 1 mEq/kg/d (*19*). If 1,000 mL of Generic 1-1, which presented the highest titratable acidity, is administered over 1 d, the kidneys of a person weighing 50 kg will process 50.78 mEq of fixed acids or 1.02-times their normal acid-processing capacity. This would be considered a low risk in patients with a normal renal function. However, in Japan, such "type 1" hypotonic infusion fluids do not contain potassium and can be administered to patients with unknown pathophysiology. Therefore, assuming "type 1" hypotonic infusion fluids will be administered to patients with impaired renal function, the indications of a patient for these preparations must be determined by evaluating the risk posed by different titratable acidities among preparations with the same constituents.

Differences in pH may affect the occurrence of pH-dependent changes upon mixing. The results of this study revealed a significant difference in pH among all combinations of "type 1" hypotonic infusion fluids with the same constituents, except between Generic 1-2 and Brand 1. This is illustrated by examining the likelihood of a pH change upon mixing when Omepral® injection 20 (omeprazole sodium hydrate) is administered from a side tube of Brand 1 or Generic 1-1, both of which have significantly different pH. The pH variability of Omepral® injection 20 (*20*) and data gathered in the present study indicate that because the pH change point of Omepral® injection 20 is 5.29 (*20*), it can be administered from a side tube of Brand 1 (pH 5.46), but not from a side tube of Generic 1-1 (pH 5.18), owing to the likelihood of pH change upon mixing (Figure 4). This is also illustrated by examining the likelihood of pH change upon mixing when Dormicum® injection 10 mg (change point pH of 4.72) (*21*) is administered from a side tube of Brand 2 (pH 4.98) or Generic 2 (pH 4.63). Based on pH variability testing of Dormicum® injection 10 mg (*21*) and the results of the present study, Dormicum® Injection 10 mg cannot be administered from a side tube of Brand 2 owing to the likelihood of pH change upon mixing. However, it can be administered from a side tube of Generic 2 (Figure 5).

Whether the risk posed by a difference in pH, as

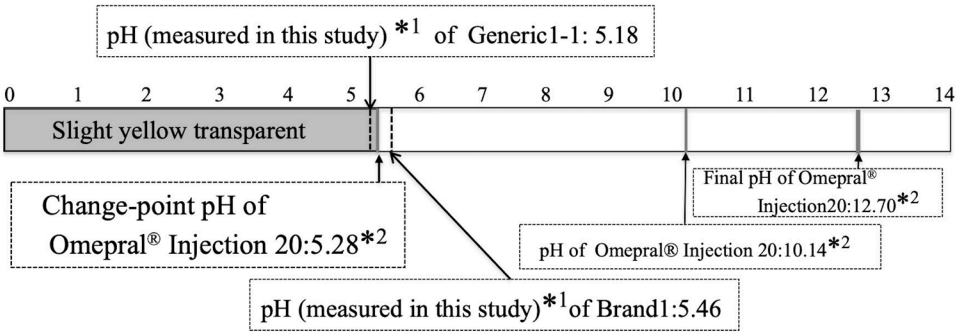

Figure 4. Predicting whether Omepral® Injection 20 (omeprazole sodium hydrate) can be mixed with Brand 1 and Generic 1-1. *1 pH values measured in this study were used as the pH values for Brand 1 and Generic 1-1. *2 The pH, final pH, and change-point pH of the Omepral® Injection 20 were obtained from pH variability test results in the Omepral® Injection 20 pharmaceutical interview form. ® represents trademark.

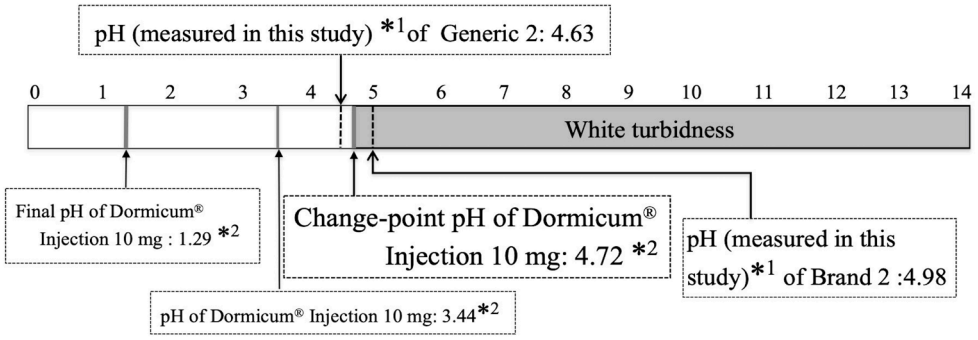

Figure 5. Predicting whether Dormicum® Injection 10 mg (midazolam) can be mixed with a Brand 2 and Generic 2. *1 pH values measured in this study were used as the pH values for Brand 2 and Generic 2. *2 The pH, final pH, and change point pH of Dormicum® Injection 10 mg were obtained from pH variability test results in the Dormicum® Injection 10 mg pharmaceutical interview form. ® represents trademark.

illustrated above, can be attributed to titratable acidity, as previously discussed, is not clear. Consequently, the indications of a patient for "type 1" hypotonic infusion fluids with the same constituents must be determined by evaluating the risk posed by the differences in pH, in addition to the differences in titratable acidity. Note that pH variability is tested by adding small amounts of 0.1 M hydrochloric acid or 0.1 M sodium hydroxide into 10 mL of infusion fluid to identify pH-dependent changes in appearance. The change-point pH is defined as the pH at which a change in appearance occurs, and the final pH is defined as the pH measured after adding 10 mL of 0.1 M hydrochloric acid or 0.1 M sodium hydroxide when there is no change in appearance (*22*).

Vascular pain is commonly reported at osmolalities of approximately 600 mOsm/kg (*23*). Although a significant difference in osmolality was observed in the present study among all combinations of "type 1" hypotonic infusion fluids with the same constituents, the measured osmolalities ranged between 300 and 333 mOsm/kg, suggesting no clinically important risk of vascular pain.
There are two forms of "type 1" hypotonic infusion fluid, and the results of the present study revealed a significant difference in titratable acidity, pH, and osmolality among these Brand-equivalent drugs. Recognizing the differences in titratable acidity, pH, and osmolality even among preparations of the same type (in this case, "type 1" hypotonic infusion fluids) is an effective strategy for risk avoidance when considering patient indications.

"Type 1" hypotonic infusion fluids do not contain potassium and can, therefore, be administered to children with immature renal function, elderly patients with impaired renal function, and patients with unknown pathophysiology. Furthermore, utilizing the findings of this study when considering prescription questions and other evaluations of appropriate usage will aid the safe and effective provision of medical care.

Previously, we reported that differences in insoluble microparticles between Brand and generic pharmaceuticals are risk factors associated with extracellular fluid replacement solutions (*7*) and "type 3" hypotonic infusion fluids (*8*). However, in this study, we did not investigate insoluble microparticles. Insoluble microparticles must be removed from these preparations based on multiple reports showing that they accumulate in the body following intravenous administration (*24,25*). In addition, glass fragments and other foreign substances generated during administration and mixing operations need to be removed (*26*). Such particles can be effectively removed with a filter during administration (*27-29*). When administering a preparation that is absorbed by or interacts with the filter, changing the filter diameter, filter material, or method of administration is an effective strategy for risk avoidance (*30,31*). We considered that it was necessary to use a filter when administering the injection, regardless of the presence or absence of existing insoluble microparticles in the preparation. For this reason, we did not examine the insoluble fine particles in this study.

In conclusion, we revealed that differences in pH and titratable acidity are risk factors associated with "type 1" hypotonic infusion fluids. Because the physicochemical properties that pose such risks differ by infusion fluid type, the same tests should be performed by strictly adhering to a unified procedure for other hypotonic infusion fluids (types 2 and 4), "type 3" hypotonic infusion fluids with added glucose, and nutritional infusion fluids. Findings from such studies must continue to be applied in clinical settings.

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Original Article

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Electrolytic-reduction ion water induces ceramide synthesis in human skin keratinocytes

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SUMMARY Ceramides play a critical role in the skin barrier. We previously demonstrated that electrolytic-reduction ion water (ERI) improves skin integrity and enhances the protective barrier function of the epidermis. Here, we first examine the effect of ERI on the expression of ceramide synthesis–related enzymes in human skin keratinocytes. The expression of enzymes involved in the elongation of very-long-chain fatty acids protein 4 (ELOVL4) was increased after treatment with ERI-containing media. The expression of ceramide synthase 3 (CerS3), which binds ultra-long-chain fatty acids to sphingosine to produce ceramides found in the skin, was also increased. Subsequently, we examined the expression of ceramides in keratinocytes treated with ERI using thin-layer chromatography. The results showed that ERI increased the ceramide content, and these ceramides were more hydrophobic than those extracted from untreated keratinocytes. These results suggest that ERI enhances the expression of enzymes involved in the synthesis of ceramides containing ultra-long-chain fatty acid residues, which have a protective function in the skin.

Keywords elongation of very-long-chain fatty acids protein 4, ceramide synthase 3, skin barrier

1. Introduction

Skin is the tissue located on the external surface of the body. The epidermis, in particular, is responsible for defense against various environmental stimuli. For this reason, the skin has a physically resistant structure. One of the elements of the skin's physical barrier is a lamellar structure composed of ceramides (*1*). This structure is also known as the intercellular lipids and consists of repeating oil and water phases, constituting a functional barrier that is responsible for maintaining the moisture of the skin (*2*). In addition to ceramides, this structure is also composed of other lipids, such as free cholesterol and free fatty acids. The molecular species of the ceramides constituting the lamellar structure in the epidermis greatly influence the barrier function and moisture retention capacity of the skin.

There are more than one thousand molecular species of ceramides, which are distinguished by differences in the length of the carbon chain between the fatty acid and sphingosine (*3*). Ceramides have a structure in which the sphingosine and fatty acid residue are ester-bonded and are biosynthesized from serine with palmitic acid as a precursor through the pathway shown in Figure 1.

Ceramides in the skin are biosynthesized primarily by keratinocytes and, unlike in other organs, contain very long chain fatty acids specific to skin (*4*). These are bound to sphingosine by ceramide synthase 3 (CerS3) to produce the ceramides (*5*).

Mutations in CerS3 are detrimental to the barrier function of the skin and maintenance of moisture. CerS3-deficient mice die due to epidermal water loss immediately after birth (*6*). In humans, ichthyosis is an inherited skin condition characterized by deterioration of the barrier function of the skin caused by mutations in the enzymes involved in the biosynthesis of ceramides with very long chain fatty acids (*7,8*). In addition, the stratum corneum of patients with atopic dermatitis contains fewer ceramides of high molecular weight and more ceramides of low molecular weight than the proportions that are found in healthy individuals (*9,10*).

Our research group has been investigating the effects of electrolytic-reduction ion water (ERI) on the skin. ERI is an aqueous solution with surfactant activity and skin moisturizing properties that has been used as a base component for cosmetics. We previously reported that ERI promotes wound healing in a mouse model of burns (*11*), promotes healing of scalds in children while preventing keloid scarring (*12,13*), and

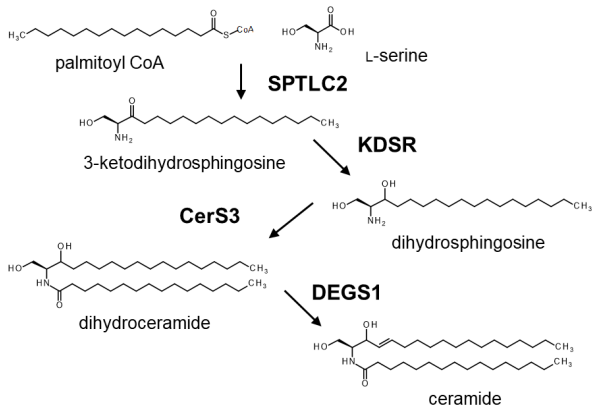

Figure 1. Ceramide synthesis pathway. SPTLC2: serine palmitoyltransferase long-chain base subunit 2; KDSR: 3-ketodihydrosphingosine reductase; CerS3: ceramide synthase 3; DEGS1: delta 4-desaturase, sphingolipid 1.

leads to improvement of atopic dermatitis (*14*). In the present study, we aimed to clarify whether the effect of ERI on the skin is through improvement in ceramide synthesis or biosynthesis of very long chain fatty acids and associated improvement of the barrier function. For this purpose, we investigated changes in the expression of enzymes related to the synthesis of very long chain fatty acids and ceramides in keratinocytes treated with ERI.

2. Materials and Methods

2.1. Materials

ERI was obtain from AI system products (Aichi, Japan). Normal human epidermal keratinocyte (NHEK) cells were purchased from Promo Cell (Heidelberg, Germany). All other chemicals were of reagent grade.

2.2. Cellular activity

NHEK were cultured in Keratinocyte Growth Medium 2 (Promo Cell, Germany) under humidified air containing 5% CO_2 at 37°C. The cells were seeded into a 96-well plate (10^4 cells/well) and cultured overnight at 37°C in a humidified atmosphere with 5% CO_2. Next, various concentrations of ERI were added to the culture medium, and the cells were incubated for a further 24 h. Cellular activity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Dojindo, Kumamoto, Japan) assay. Briefly, the cells were treated with 110 µL of Dulbecco's modified Eagle's medium containing MTT (0.5 mg/mL) and incubated at 37°C for 4 h. Subsequently, 100 µL of 10% sodium dodecyl sulfate in 10 mM hydrochloric acid was added to dissolve the formazan crystals that formed in metabolically active cells. Spectrophotometric absorbance was determined at 560 nm using a microplate reader (VersaMAX, Molecular device, San Jose, CA, USA). The absorbance was calculated and expressed as percentage of cellular activity.

2.3. Expression of ceramide synthesis–related genes

The effects of ERI on the expression of ceramide synthesis–related genes were assessed using real time-polymerase chain reaction (RT-PCR). NHEK cells were seeded in a six-well tissue culture plate (10^5 cells/well) and incubated overnight. Subsequently, the cells were treated with various concentrations of ERI and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. Total RNA was extracted from the NHEK cells using TriPure Isolation Reagent (Roche Life Science, Indianapolis, IN, USA), and cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) according to the instructions provided by the manufacturer. Specific primers were designed to amplify human serine palmitoyltransferase long chain base subunit 2 (*SPTLC2*) (5'-CCAGACTGTCAGGAGCAACCATTA-3' and 5'-CGTGTCCGAGGCTGACCATA-3'), human 3-ketodihydrosphingosine reductase (*KDSR*) (5'-GTGGTGGTTACAGGAGGTTC-3' and 5'-AATTTACCAGCATGTCCACT-3'), human *CerS3* (5'-ACATTCCACAAGGCAACCATTG-3' and 5'-CTCTTGATTCCGCCGACTCC-3'), human delta 4-desaturase, sphingolipid 1 (*DEGS*1) (5'- GCGTTTGGCAGTTGCATTAA -3' and 5'-CATTGTGGGCAATCTCATGAA -3'), human elongation of very long chain fatty acids protein (*ELOVL*) 1 (5'-ATTCTCCTGACCTACGTGTACTT-3' and 5'-TTCCGATTAGCCATGATGCGA-3'), human *ELOVL4* (5'-CATGTGTATCATCACTGTACG-3' and 5'-AAAGGAATTCAACTGGGCTC-3'), human *ELOVL7* (5'-TTCCATCATACCATCATGCC-3' and 5'-CCCAATGCAGAAAGTCCATA-3'), and human *β-actin* (5'-AGTCCTGTGGCATCCACGAAAC-3' and 5'-GCAGTGATCTCCTTCTGCATCC-3'). RT-PCR was performed using StepOne (Applied Biosystems, Foster City, CA, USA). Thermocycling was performed under the following conditions: one cycle at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. The cycle threshold (C_t) values for each sample were normalized to that of *β-actin*, and the relative expression was calculated using the comparative C_t method.

2.4. Western blotting analysis

Samples (10 µg) of the cell lysates were separated on a 12% (w/v) polyacrylamide gel (*15*). Proteins were blotted onto nitrocellulose membranes (Protran BA85; GE Healthcare, Chicago, IL, USA) in a semi-dry blotting system (NA-1513; Nihon Eidoh Co., Tokyo, Japan) (*16*). Nitrocellulose membranes were blocked with 2% (w/v) skim milk in phosphate-buffered saline. Blocked

membranes were incubated with anti-CerS3 rabbit antibody (1:2,000; Cusabio, Houston, TX, USA), with anti-ELOVL4 rabbit antibody (1:1,000; Cusabio, USA), or with anti-β-actin mouse monoclonal antibody (1:5,000, Wako Chemical, Tokyo, Japan). This was followed by incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) goat antibody (1:5,000; BioSource, Camarillo, CA, USA) for CerS3 or ELOVL4, or horseradish peroxidase-conjugated anti-mouse IgG goat antibody (1:5,000; Wako Chemical) for β-actin. The blots were subsequently developed using an Immunostar LD (Wako Chemical, Japan) or Imobiron (Merck Millipore, Billerica, MA, USA) using LuminoGraph (Atto Corporation, Tokyo, Japan). The bands were densitometrically analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.5. Ceramide analysis

The NHEK treated with ERI were washed with phosphate-buffered saline; lipids were extracted with chloroform/methanol (1:2, v/v) and maintained at room temperature for 1 h. Next, the liquids were collected, and potassium chloride aqueous solution was added up to a concentration of 0.13% potassium chloride. The samples were centrifuged at 1,000 × g at room temperature for 5 min. The lower phase was transferred to a new tube, while the upper layer was re-extracted with chloroform; this was followed by centrifugation at 1,000 × g at room temperature for 5 min. The extract was dried at 50°C under a stream of nitrogen gas. The residue was dissolved in 100 μL of chloroform/methanol and stored at −20°C until use.

The lipid extracts were analyzed using normal-phase thin-layer chromatography plates (TLC, Silica gel 60; Merck Millipore, Darmstadt, Germany) through the following three solvent systems: (1) chloroform/methanol/water (40:10:1, v/v), developed to 2 cm from the bottom, dried, and developed again to 5 cm from the bottom; (2) chloroform/methanol/acetic acid (47:2:0.5, v/v), developed to 1.5 cm from the top; and (3) hexane/diethylether/acetic acid (65:35:1, v/v) developed to the top. The separated lipids were detected using a copper phosphate reagent (3% $CuSO_4$ in 8% [v/v] phosphoric acid solution) and heating at 180°C.

3. Results

3.1. Confirmation of optimal concentration for cell testing in ERI-containing media

Cellular activity was assessed to allow a correct evaluation of this test using ERI at concentrations ranging from 5.0×10^{-6}% to 50% (v/v). ERI did not influence the activation of NHEK cells at concentrations ≤ 5% (Figure 2). In a study of burn wounds in mice (*11*), atopic dermatitis (*12*) and burn wounds (*13,14)* in humans, the healing effect of ERI was observed at 100% concentration when applied to skin tissue, due to its very low alkali reserve of 0.1 g/100 mL. However, since the direct application of ERI to NHEK cells is affected by pH and surface-active effects, the concentration at which ceramide synthesis can be accurately evaluated in this study was 5% or lower.

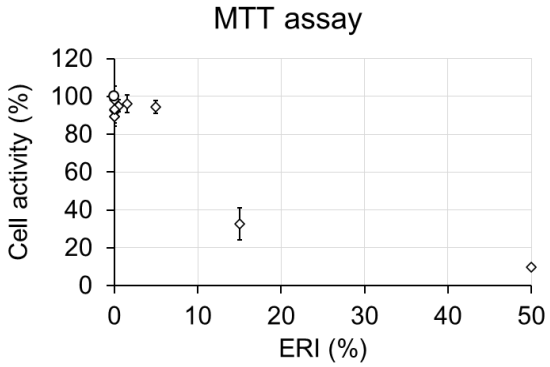

Figure 2. Activity of cells treated with ERI. ERI was added to the cell culture media. Cell activity was measured by MTT assay. Data are shown as the mean ± SD of eight biological replicates. The circle indicates un-treated control. ERI: electrolytic-reduction ion water; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

3.2. Expression levels of ceramide synthesis–related enzyme genes

To examine the expression of ceramide synthesis–related enzymes in keratinocytes treated with ERI, mRNA was extracted from NHEK cells and RT-PCR was performed (Figures 3A-3G). Treatment with ERI increased the expression of *CerS3* and *ELOVL4* mRNA in a dose-dependent manner. In particular, the expression levels of *CerS3* and *ELOVL4* were significantly increased after treatment with 0.5% (v/v) ERI. In contrast, the expression of *SPTLC2*, *KDSR*, *DEGS1*, *ELOVL1*, and *ELOVL7* remained unchanged.

3.3. Levels of CerS3 and ELOVL4 proteins

The levels of CerS3 and ELOVL4 proteins were assessed by western blotting analysis (Figure 4A). CerS3 protein levels were increased after treatment with ERI in a dose-dependent manner (Figure 4B). CerS3 expression in the NHEK by 5% ERI treatment increased more than 3.5-fold. In addition, the expression of ELOVL4 protein was slightly increased after treatment with ERI at concentrations ranging 0.5-5% (v/v) and then ELOVL4 expression induced more than 4.5-fold by 5% ERI treatment (Figure 4C). β-actin levels did not differ among these cells.

3.4. Expression of ceramides in keratinocytes

The ceramide analysis performed using thin-layer

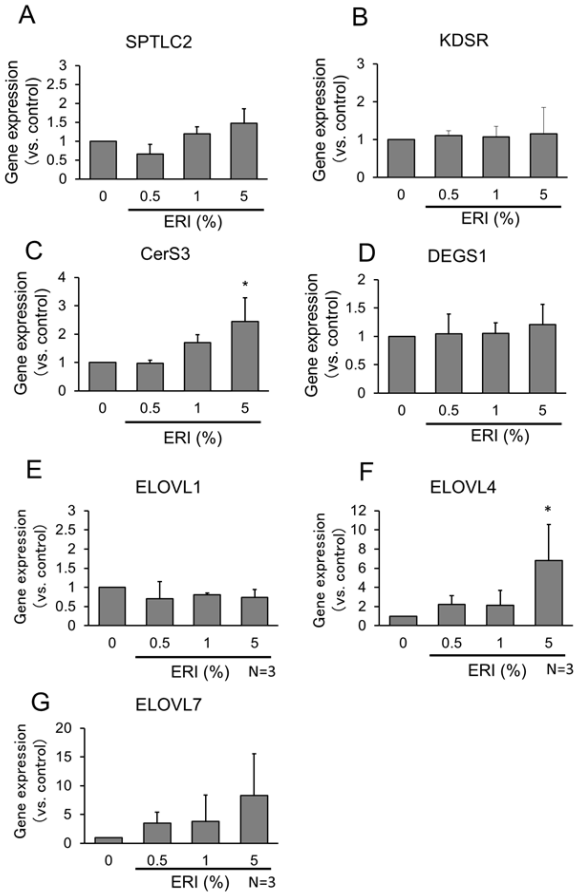

Figure 3. RT-PCR quantification of the expression levels of ceramide synthesis–related enzyme genes. Expression levels of *SPTLC2* **(A)**, *KDS*R **(B)**, *CerS3* **(C)**, *DEGS1* **(D)**, *ELOVL1* **(E)**, *ELOVL4* **(F)**, and *ELOVL7* **(G)**. The mRNA was extracted from NHEK cells treated with 0.5%, 1%, or 5% ERI added to the cell culture medium for 72 h. The expression of mRNA was determined by real time PCR. The expression levels of each enzyme were normalized to those of β-actin. Data are shown as the mean ± SE of three biological replicates. Asterisks: $p < 0.05$ compared with untreated controls. CerS3: ceramide synthase 3; DEGS1: delta 4-desaturase, sphingolipid 1; ELOVL1: elongation of very-long-chain fatty acids protein 1; ERI: electrolytic-reduction ion water; KDSR: 3-ketodihydrosphingosine reductase; NHEK: normal human epidermal keratinocytes; SPTLC2: serine palmitoyltransferase long-chain base subunit 2.

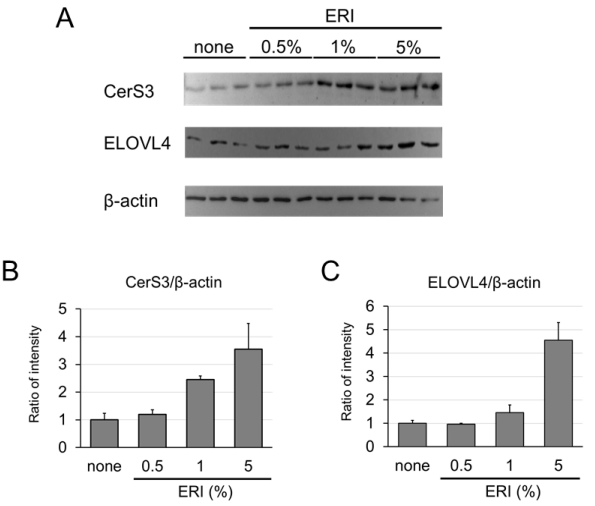

Figure 4. Western blotting analysis of CerS3 and ELOVL4. The protein levels of CerS3, ELOVL4, and β-actin were determined using lysates prepared from NHEK cells treated with 0.5%, 1%, or 5% ERI added to the cell culture medium for 72 h. The expression of CerS3 and ELOVL4 proteins was detected by western blotting analysis **(A)**. The protein content was normalized to the level of β-actin. The intensity of CerS3 **(B)** and ELOVL4 **(C)** in were measured by densitometry. Data represent means ± SD ($n = 3$ *vs*. None). CerS3: ceramide synthase 3; DEGS1: delta 4-desaturase, sphingolipid 1; ELOVL4: elongation of very-long-chain fatty acids protein 4; ERI: electrolytic-reduction ion water; KDSR: 3-ketodihydrosphingosine reductase; NHEK: normal human epidermal keratinocytes; SPTLC2: serine palmitoyltransferase long-chain base subunit 2.

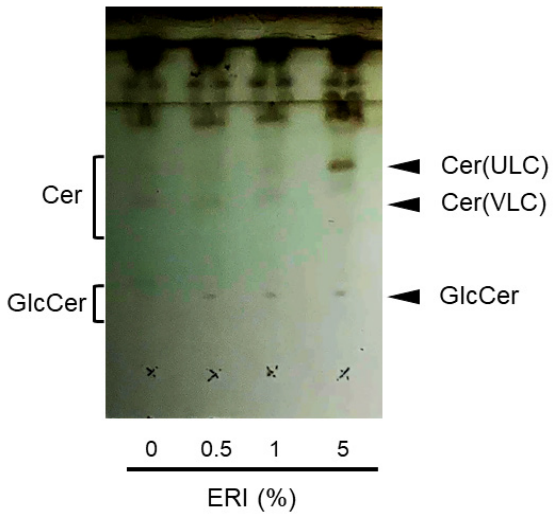

Figure 5. Ceramide-related lipids in the keratinocyte after ERI treatment. The lipids were extracted from NHEK cells treated with 0.5%, 1%, or 5% ERI added to the cell culture medium for 72 h. The extracted lipids were separated by normal-phase TLC and detected by copper sulfate/phosphoric acid solution. Cer: ceramide; GlcCer: glucosylceramide; ERI: electrolytic-reduction ion water; ULC: ultra-long-chain; VLC: very-long-chain.

chromatography (Figure 5) confirmed that higher concentrations of ERI were associated with larger sizes of spots in the glucosylceramide (GlcCer) and ceramide regions and darker color. This result indicated that ERI promoted the synthesis of GlcCer and ceramides in a concentration-dependent manner and increased the intracellular content of GlcCer and ceramides. It was confirmed that higher concentrations of ERI were linked to longer development distances of the spots in the GlcCer and ceramide regions (particularly the ultra long chain ceramide region). These results demonstrated that treatment with higher concentrations of ERI promotes the synthesis of fatty acids with longer carbon chain lengths.

4. Discussion

Studies have shown that ERI of 100% concentration improves thermal wounds in mice (*11*), atopic dermatitis (*12*) and promotes skin healing processes after burns

(*13,14*) in humans. In this study, we hypothesized that ERI may also be involved in the regulation of ceramide synthesis. The epidermis produces ceramides with a characteristic fatty acid structure. Fatty acids with alkyl chain lengths of 11-20, 21-25, and ≥ 26 carbons are considered long-, very-long-, and ultra-long-chain fatty acids, respectively (*16*). In most tissues, the fatty acid residues of ceramides have alkyl chains containing 16-24 carbons (*17,18*), while ceramides of the epidermis additionally include 26-36-carbon ultra-long-chain fatty acid residues. Ceramides synthesized in keratinocytes have fatty acids residues with longer carbon chains than those synthesized in other tissues.

Biosynthesis of ultra-long-chain fatty acid residues is necessary to allow the synthesis of ceramides that incorporate them. Therefore, we evaluated the effects of ERI on the expression of enzymes involved in the synthesis of ultra-long-chain fatty acids in keratinocytes. For this purpose, we evaluated the expression of *ELOVL1*, *ELOVL4*, and *ELOVL7* genes. Addition of ERI to the cell culture medium increased the expression of *ELOVL4*. ELOVL4 is essential for the synthesis of fatty acids with alkyl chain lengths of ≥ 28 carbons, which are characteristically abundant in the epidermis; moreover, recessive mutation of the *ELOVL4* gene is known to cause ichthyosis (*19*). *ELOVL4* knockout in mice is lethal shortly after birth due to failure of skin barrier formation, owing to the inability to synthesize very long chain fatty acids used for the synthesis of ceramides in the epidermis (*20*).

Treatment of keratinocytes with ERI increased the expression of *CerS3*. The CerS family is involved in the amide binding reaction between sphingosine and fatty acids (*21*). CerS enzymes are categorized into molecular types 1-6, and each CerS type is involved in the binding of fatty acids of particular chain lengths to sphingosine. Among the CerS types, only CerS3 is capable of carrying out this action on the ultra-long-chain fatty acids synthesized in the skin. Therefore, similar to ELOVL4-knockout mice, CerS3-knockout mice die shortly after birth due to failure of skin barrier formation (*8*).

While we were unable to determine the ingredients contained in ERI, the liver X receptor alpha (LXRα) pathway is a known regulatory mechanism that alters enzymes involved in ceramide synthesis (*22*). LXR is a transcription factor expressed in tissues (*e.g.*, liver) and regulates the expression of cholesterol and fatty acid metabolic enzymes (*22*). LXRα increases the amount of ceramides in the epidermis of the skin by promoting fatty acid synthesis, thereby enhancing the barrier mechanism of the skin (*22*). It was recently reported that treatment with an LXRα ligand induces the expression of *ELOVL4*, *ELOVL7*, and *CERS3* mRNA in keratinocytes (*23*). In this study, similar to LXRα, treatment with ERI enhanced the expression of *ELOVL4* and *CerS3*. Furthermore, LXRα activation resulted in a non-statistically significant trend towards increased expression of *ELOVL7*, as also observed following treatment with ERI. These results indicate that the mechanism involved in the role of ERI in CerS expression may be related to LXRα.

In summary, this study demonstrated that treatment with ERI induced *ELOVL4* and *CerS3* expression in keratinocytes. ELOVL4 and CerS3 are key enzymes for the synthesis of ceramides containing ultra-long-chain fatty acids. As this type of ceramide is essential in the assembly of the skin barrier, this finding presents a pathway through ceramide synthesis as one of the mechanisms involved in the protective effects of ERI on the skin.

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Original Article

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Post COVID-19 sequelae: A prospective observational study from Northern India

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SUMMARY Post COVID-19 sequelae are a constellation of symptoms often reported after recovering from COVID-19. There is a need to better understand the clinical spectrum and long-term course of this clinical entity. The aim of this study is to describe the clinical features and risk factors of post COVID-19 sequelae in the North Indian population. This prospective observational study was conducted at a tertiary healthcare centre in Northern India between October 2020 and February 2021. Patients aged >18 years with laboratory-confirmed COVID-19 were recruited after at least two weeks of diagnosis, and details were captured. A total of 1234 patients were recruited and followed up for a median duration of 91 days (IQR: 45-181 days). Among them, 495 (40.1%) had persistent symptoms post-discharge or recovery. In 223 (18.1%) patients, the symptoms resolved within four weeks; 150 (12.1%) patients had symptoms till 12 weeks, and 122 (9.9%) patients had symptoms beyond 12 weeks of diagnosis/symptom-onset of COVID-19. Most common symptoms included myalgia (10.9%), fatigue (5.5%), shortness of breath (6.1%), cough (2.1%), insomnia (1.4%), mood disturbances (0.48%) and anxiety (0.6%). Patients who were hospitalized were more likely to report fatigue as a feature of long COVID. Hypothyroidism (OR: 4.13, 95% CI: 2.2-7.6, *p*-value < 0.001) and hypoxia ($SpO_2 \leq 93\%$) (OR: 1.7, 95% CI: 1.1-2.4, *p*-value 0.012) were identified as risk factors for long COVID sequelae. In conclusion, long COVID symptoms were common (22%), and 9.9% had the post COVID-19 syndrome. Myalgias, fatigue and dyspnoea were common symptoms. Patients with hypothyroidism and hypoxia during acute illness were at higher risk of long COVID.

Keywords COVID-19, post COVID-19 sequelae, long COVID, post COVID-19 syndrome

1. Introduction

Coronavirus disease 2019 (COVID-19) is a multisystem disease caused by the severe acute respiratory syndrome corona virus-2 (SARS-CoV-2). While the respiratory system bears the maximal brunt of the disease, the virus may affect all major systems in the body. Even after recovery from the disease, widespread respiratory, circulatory, neurological, and musculoskeletal complaints may persist. The epidemio-pathological basis of these 'Post COVID-19' complaints is not completely understood. Apart from direct viral damage, these post COVID-19 sequelae may also be attributed to the immune response, cytokine storm, as a side effect of the therapy, underlying comorbidities or due to a combination of any of these. Additionally, the role of psychosocial factors like prolonged isolation, fear of infecting family members, socio-economic disturbances, and the stigma associated with the infection may have far-reaching consequences on mental health and well-being (*1*).

As per NICE (National Institute for Health and Care Excellence) guidelines, post COVID-19 sequelae have been divided into acute COVID-19, ongoing symptomatic COVID-19 and post COVID-19 syndrome (*2*). The term 'long COVID' is commonly being used to describe signs and symptoms that continue or develop after acute COVID 19. It includes both ongoing

symptomatic COVID 19 and post COVID 19 syndrome.

The epidemiology of post COVID-19 sequelae is not well described (*3*). We conducted this prospective observational study to describe the incidence and risk factors of post COVID-19 sequelae among the Northern Indian population.

2. Materials and Methods

2.1. Study design and settings

This prospective observational study was conducted in a tertiary care centre in Delhi, India, between October 2020 and February 2021.

2.2. Participants

The study included patients of age more than 18 years who had a confirmed COVID-19 infection (as per WHO definition) at least two weeks prior to the enrolment, had been discharged from the institute after treatment for COVID-19 and were willing to participate (*4*).

2.3. Data collection

Participants were followed-up either at the institute's post COVID-19 clinic or telephonically. Interviews were conducted by residents trained in administering the questionnaire and able to communicate with the patients in their local languages. Verbal consent was taken before the enrolment. Confidentiality of subjects was ensured throughout the study. The study protocol was approved by the Institute Ethics Committee (IECPG/162/4/2020).

The questionnaire included details related to demographics, symptomatology, hospitalization, and oxygen use during the acute disease as well as any symptoms after recovery from acute COVID-19. Patients were systematically asked about a list of post COVID-19 symptoms (dyspnea, myalgia, fatigue, anosmia, ageusia, chest pain, cough, mood disturbances *etc*.), but they were also free to report any other symptoms that they considered relevant. A follow-up interview was conducted after one month and three months to look for the resolution of symptoms or any new symptoms. Patients could visit the clinic at any time if they desired.

The severity of acute COVID-19 was defined as mild, moderate or severe with reference to national guidelines for COVID-19 (*5*). The patients with COVID-19 without evidence of breathlessness or hypoxia (defined as room air oxygen saturation ≤ 94%) during the course of acute illness were categorized as mild COVID-19 disease. Those who had breathlessness and a room air oxygen saturation (SpO_2) of ≥ 90% and ≤ 93% were catagorised as moderate disease and those with room air SpO_2 of < 90% were categorized as severe COVID-19 disease. The post COVID-19 symptoms of the patients were classified as per NICE guidelines (*2*). Acute COVID-19 has been defined as patients with symptoms and signs of COVID-19 for up to 4 weeks. Ongoing symptomatic COVID-19 includes patients with symptoms and signs from 4 to 12 weeks. Patients with symptoms and signs that develop during or after an infection consistent with COVID-19, which continued for more than 12 weeks and are not explained by an alternative diagnosis, are said to have Post COVID-19 syndrome. Long COVID is defined as signs and symptoms that persist or develop after acute COVID-19 and included both "ongoing symptomatic COVID-19" and "post COVID-19 syndrome" (*2*).

2.4. Statistical analysis

The data were analyzed using STATA 16.0 (StataCorp, College Station, TX) software. Categorical variables are presented as *n* (%), continuous variables are presented as mean (standard deviation [SD]) or median (interquartile range [IQR]) as applicable. For the comparison of variables, two groups were considered. Group 1 included patients who developed the long COVID, and Group 2 included patients who did not develop long COVID. Categorical variables were compared using the Fisher exact test or chi-square test, wherever applicable, and continuous variables were compared using an independent sample Student's *t*-test. In addition, a binary logistic regression model was developed to assess the impact of different variables on the likelihood of developing post COVID-19 syndrome with the forward conditional method. Independent variables which had a *p*-value of < 0.2 were included for binary logistic regression. Statistical significance was set at a *p*-value of < 0.05.

3. Results

3.1. Clinical characteristics of the patients in our study

A total of 2,243 patients were contacted, of whom 1,645 patients responded. Of these, 147 were excluded as they were less than 18 years old, and 264 patients did not consent to the study. Thus, a total of 1,234 patients were recruited for the study. We followed up 276 patients (26%) for more than six months, 264 (24%) for 3-6 months, 398 (37%) for up to three months, and 140 (13%) for less than 1-month post-discharge/post-end of isolation. The median duration of follow-up was 91 days (IQR: 45-181 days).

Our cohort had a mean age of 41.4 ± 14.2 years and included 856 (69.4%) males. Of these 1,234 patients, 1,059 (85.8%) patients had mild COVID-19 disease, 135 (10.9%) had moderate, and 40 (3.3%) patients had severe COVID-19 disease. In addition, there were 352 (28.5%) patients who had at least one comorbidity. The most common comorbidities were diabetes mellitus (13.4%), hypertension (8.1%) and hypothyroidism

Table 1. Demographic profile of the patients recruited for the study (*n* = 1,234)

Variables	Observations *n* (%)
Age in years (mean ± SD)	41.6 ± 14.2
Range	18-97 years
Gender	
Males	856 (69.4)
Females	378 (30.6)
Disease severity	
Mild ($SpO_2 \geq 94\%$ on room air)	1,059 (85.8)
Moderate ($SpO_2 \geq 90\%$ & $\leq 93\%$ on room air)	135 (10.9)
Severe ($SpO_2 < 90\%$ on room air)	40 (3.3)
Comorbidities	352 (28.5)
Diabetes mellitus	165 (13.4)
Hypertension	100 (8.1)
Hypothyroidism	71 (5.8)
Chronic lung disease	24 (1.9)
Connective tissue diseases	20 (1.6)
Tuberculosis	28 (2.3)
Coronary artery disease	30 (2.4)
Chronic kidney disease	27 (2.2)
Chronic liver disease	8 (0.6)
HIV	5 (0.4)
Malignancy	21 (1.7)
Post-transplant	3 (0.2)
Patients requiring admission	711 (56.6)
Admission details	
Duration of hospital stay (*n* = 711)	
Mean ± SD	11.4 ± 5.6 days
Median (IQR)	10 (7-14) days
Oxygen requirement	175 (14.2)
Ventilator requirement	15 (1.2)

(5.8%) (Table 1).

A total of 711 (56.6%) patients were hospitalized for COVID-19, for a mean duration of 11.4 ± 5.6 days, with a range of 1-36 days. Oxygen support was required by 175 patients (14.2%), of whom 15 patients (1.2%) required ventilatory support (Table 1).

3.2. The post COVID-19 symptomatology in the patients

Of these 1234 patients, 495 (40.11%) patients had symptoms after their discharge or end of quarantine (Figure 1). In 223 (18.1%) patients, the symptoms resolved within four weeks. The remaining 272 (22.0%) patients had long COVID. Of all patients with long COVID, 150 (12.1%) patients had symptoms till twelve weeks, and 122 (9.9%) patients had symptoms beyond twelve weeks. The most common long COVID symptoms included myalgia (10.9%), fatigue (5.5%), shortness of breath (6.1%), dry cough (2.1%) and chest pain (1.2%). Other symptoms included insomnia (1.4%), mood disturbances (0.48%) and anxiety (0.6%) (Table 2; Figure 2). The proportion of patients reporting persistent symptoms diminished with a longer time to follow-up (Table 2).

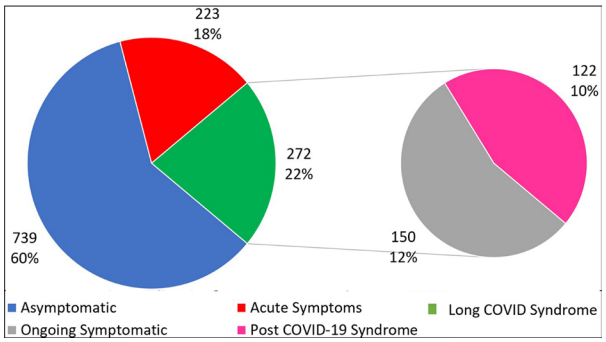

Figure 1. Post COVID-19 symptoms in the study group (*n* = 1,234). The blue portion represents patients who did not have any symptoms. The red portion represents the patients with acute COVID-19. The green portion represents patients with long COVID symptoms, which included patients with ongoing symptoms, represented as the grey portion, and post COVID-19 syndrome, represented as the pink portion.

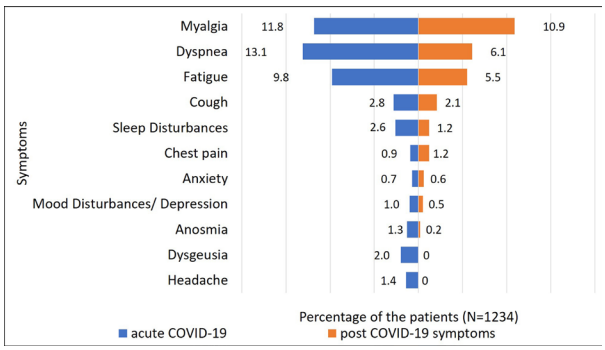

Figure 2. Spectrum of post COVID-19 symptoms (*n* = 1,234). The blue graph represents the patients (in percentage) with symptoms less than four weeks from diagnosis, and the orange graph represents the patients (in percentage) with symptoms beyond four weeks from diagnosis.

Table 2. Post COVID-19 symptomatology among the recruited patients (*n* = 1234)

Symptoms	Long COVID Symptoms		Duration of symptoms in days Median (IQR)
	Ongoing symptoms *n* (%)	Post COVID-19 syndrome *n* (%)	
Loss of taste	0	0	-
Loss of smell	2 (0.16)	0	-
Anxiety	4 (0.3)	4 (0.3)	
Low mood/depression	4 (0.32)	2 (0.16)	67 (40-177)
Chest pain	8 (0.7)	6 (0.5)	60 (41-112)
Insomnia	13 (1.1)	4 (0.3)	45 (40-70)
Cough	14 (1.1)	12 (1)	60 (45-118)
Dyspnea	33 (2.7)	42 (3.4)	90 (45-124)
Fatigue	45 (3.7)	22 (1.8)	60 (45-135)
Myalgia	80 (6.5)	54 (4.4)	60 (45-150)
Total*	150 (12.1)	122 (9.9)	

*The total numbers do not add up as many patients had more than 1 symptom.

3.3. Clinical characteristics of the patients hospitalized

The characteristics of patients who were admitted are compared with patients who were not hospitalized for the management of COVID-19 in Table 3. Patients with increasing age, comorbidities like diabetes mellitus, hypertension, coronary artery disease and hypothyroidism were more likely to be hospitalized. Patients who were hospitalized were also more likely to report fatigue as a feature of long COVID.

3.4. Risk factors for developing long COVID symptoms

The risk of developing long COVID symptoms was higher in patients with hypothyroidism (OR: 4.13, 95% CI: 2.2-7.6, *p*-value < 0.001) and disease severity. Compared to mild disease, the patients who had either moderate or severe COVID-19 disease, *i.e.* the patients having hypoxia or SpO_2 on room air of ≤ 93%, was associated with a higher chance of developing long COVID symptoms (OR: 1.7, 95% CI: 1.1-2.4, *p*-value 0.012) (Table 4).

4. Discussion

The COVID-19 pandemic has affected hundreds of millions of people. Even after recovery, prolonged symptoms have been noted. These post COVID-19

Table 3. Comparison of characteristics between hospitalized and non-hospitalized patients

Variable	Non- Hospitalised, *n* = 523	Hospitalised, *n* = 711	*p* value	OR (95% CI)	*p* value
Age in years (Mean + SD)	38.1 ± 11.9	44.2 ± 15.3	< 0.001	1.01-1.03	< 0.001
Comorbidity	61 (11.7)	291 (40.9)	< 0.001		
Diabetes Mellitus	42 (8)	123 (17.3)	< 0.001	1.5 (1-2.2)	0.05
Hypertension	14 (2.7)	86 (12.1)	< 0.001	3.1 (1.7-5.7)	< 0.001
Coronary Artery Disease	2 (0.4)	28 (41.9)	< 0.001	4.6 (1.1-20.1)	0.04
Chronic Kidney Disease	4 (0.7)	23 (3.2)	0.003		0.07
Chronic Lung disease	4 (0.8)	20 (4.9)	0.01		0.15
Chronic Liver Disease	0	8 (1.1)	< 0.001	1	
Malignancy	0	21 (3)	< 0.001	1	
Hypothyroidism	3 (0.6)	68 (9.6)	< 0.001	15.8 (4.9-51)	< 0.001
Long COVID	103 (19.7)	169 (23.8)	0.09		0.15
1.Cough	9 (1.7)	17 (2.4)	0.6		
2.Dyspnea	18 (3.4)	57 (8)	0.001	2.9 (1.4-6.3)	0.006
3.Fatigue	17 (3.3)	58 (8.2)	< 0.001		
4.Myalgia	64 (12.2)	70 (9.8)	0.2		
Post COVID-19 syndrome	50 (9.6)	72 (10.1)	0.8		
1.Cough	8 (1.5)	4 (0.6)	0.14		0.8
2.Dyspnea	15 (2.9)	27 (3.8)	0.4		
3.Fatigue	5 (1)	17 (2.4)	0.08		
4.Myalgia	19 (3.6)	35 (4.9)	0.3		

Table 4. Multi-variate analysis for risk factors of developing Long COVID symptoms

Variable	No long COVID *n* = 962	long COVID *n* = 272	*p*-value (univariate)	Multi-variate analysis	
				OR (95% CI)	*p* value
Age	40.8 ± 14.6	44.3 ± 13	< 0.001	1.01 (0.99-1.02)	0.056
Gender					
Males	665 (69.1)	191 (70.2)	0.8		
Females	297 (30.9)	81 (29.8)			
Comorbidities	256 (26.6)	96 (35.3)	0.006		0.24
Diabetes Mellitus	120 (12.5)	45 (16.5)	0.09		0.18
Hypertension	75 (7.8)	25 (9.2)	0.45		
Hypothyroidism	38 (4)	33 (12.1)	< 0.001	4.13 (2.2-7.6)	< 0.001
Chronic Lung disease	16 (1.7)	8 (2.9)	0.21		
Connective Tissue Disorder	14 (1.5)	6 (2.2)	0.4		
Coronary Artery Disease	26 (2.7)	4 (1.5)	0.4		
Chronic Kidney Disease	23 (2.4)	4 (1.5)	0.5		
Need of ventilator	10 (1)	5 (1.8)	0.34		
Mild disease	839 (87.2)	220 (80.9)	0.01	Taken as 1	
Moderate disease/Severe disease*	123 (12.8)	52 (19.1)	0.01	1.7 (1.1-2.4)	0.012
Admission	542 (56.3)	169 (62.1)	0.09		0.7

*Moderate disease are patients with COVID-19 disease who had a room air saturation of ≥ 90% & ≤ 93% and Severe disease are the patients with COVID-19 disease who had a room air saturation of ≤ 90%.

symptoms significantly affect the quality of life in patients (*6*). Long COVID, or post COVID-19 sequelae, is being seen in a growing number of patients reporting a constellation of symptoms, both pulmonary and extrapulmonary, with known or undeciphered mechanisms.

In our cohort of 1,234 patients, 272 (22%) patients had symptoms that persisted beyond four weeks or 'long COVID' (*2*). This is lower than previous estimates of prevalence which range from 50-80% (*3,7-13*). Multiple reasons may have caused this; the proportions of mild and asymptomatic patients recruited in previous studies were lower; many of the studies included had a risk of selection bias, with symptomatic patients more likely to seek medical attention.

Persistent musculoskeletal complaints, including fatigue and myalgias, have been reported in 37-62% of patients who recovered from COVID-19 (*3,7-9,11,13,14*). The most common post COVID-19 symptoms in our cohort were myalgia (134, 10.9%) and fatigue (67, 5.5%). Studies have demonstrated frontal and cerebellar hypometabolism in patients with post COVID-19 fatigue (*15*). Decreased levels of neurotransmitters, reduced neuronal excitability, inflammation, and inhibition in the firing of motor neuron units have all been hypothesized as central factors contributing to post COVID-19 fatigue and myalgias (*16*). Metabolic factors like vitamin D deficiency, anaemia, hypothyroidism, and underlying chronic diseases also contribute to prolonged fatigue (*17*). Importantly, the role of psychological influences cannot be ruled out.

The massive socio-economic upheaval caused by the pandemic has had a significant impact on the psyche of the population. Isolation, mobility restrictions, fear of infection, financial losses, and stigma have led to mood disorders (*18,19*). In our study, 49 (4%) patients reported difficulty sleeping while 17 (1.4%) patients each had depression and anxiety that occurred after recovery from the infection. However, these complaints were resolved within three weeks in most patients.

Apart from psychosomatic complaints, many patients suffered from prolonged respiratory complaints, including shortness of breath (75, 6.1%) and dry cough (26, 2.1%). This is lesser than the previously estimated prevalence of 24-40% and 11-14% of dyspnoea and cough, respectively (*3,8,10,14*). In addition to the causes discussed above, a higher proportion of patients with underlying respiratory conditions may have contributed to the higher prevalence in previous studies. Dyspnea was also the most persistent complaint, with a median duration of 90 days in our cohort.

Pulmonary parenchymal injury and acute respiratory distress syndrome (ARDS) are hallmarks of acute COVID-19. Direct viral injury, cell and cytokine and cell-mediated injury, activation of profibrotic pathways, and trauma due to positive pressure ventilation can cause permanent scarring of lung parenchyma (*20*). Follow-up studies have shown that in up to 98% of patients, abnormalities like ground-glass opacities, crazy paving patterns and bands of fibrosis persist in chest imaging even after 28 days from symptom onset (*21*). Additionally, COVID-19 induced thromboembolic microangiopathy, and the resulting immune-inflammatory cascade causes sizable damage to the pulmonary vasculature (*22*). This may explain the high prevalence of chest pain in patients with dyspnea post COVID-19.

Neurotropism by the SARS-CoV-2 virus, neuronal inflammation mediated by direct invasion and bystander injury by the immune response to the virus, and neuroimmunomodulation through the vagus nerve has been implicated in cough hypersensitivity causing persistent cough in post COVID-19 (*23*). This is like the neurogenic sensitization that has been proposed for chronic fatigue syndrome. Thus, this may explain the co-existence of other symptoms like fatigue, myalgia and neurocognitive symptoms in post COVID-19 patients (*24*).

The factors associated with long COVID symptoms in our study were the severity of the COVID-19 infection (mild *vs*. severe/moderate) and hypothyroidism. More severe illness is associated with a prolonged course of the disease and more significant damage. In patients with comorbidities like hypothyroidism, musculoskeletal and respiratory complaints are common. Additionally, non-mild COVID-19 may induce a sick euthyroid state, recovery from which may be delayed in patients with hypothyroidism (*25*). Thyroid disorder may adversely affect the disease outcome in several ways like the effect of the virus on the tissue distribution of ACE2 receptor, increased burden of cardiovascular and psychiatric comorbidities in turn affecting the metabolic stress (*26*). Hypothyroidism (with or without levothyroxine supplementation) has shown to negatively affect the long-term outcomes in COVID-19 survivors in our study, and more prospective studies can throw more light on this association.

Our study is a large prospective study recruiting 1,234 patients of all severity of COVID-19. It is also the first study to recruit many patients with an asymptomatic and mild infection and thus may provide a more accurate estimate of the prevalence of post COVID-19 symptoms in a real-life scenario. Furthermore, since patients were actively asked about various symptoms at pre-defined intervals, recall bias was reduced. To our knowledge, our study is the first to show that hypothyroid patients are at higher risk of developing long COVID symptoms. However, more robust studies are required to draw further conclusions.

The study has a few limitations. There is an inherent selection bias since the centre is a tertiary care hospital, and more patients requiring admission were tested in the facility. However, the admission policy had varied over the period, which was based on the caseload in the community and the availability of beds. Many patients

with mild disease were admitted for management of co-existing systemic illness. Loss to follow-up is inherent in all cohort studies, and this was exacerbated in our study due to significant social upheaval and mobility restrictions during the pandemic. Due to mobility restrictions and limited services during the pandemic surge, data on laboratory workup and imaging of patients could not be collected systematically.

In conclusion, long COVID symptoms were common and seen in 22% of the recovered patients whereas 9.9% had symptoms that persisted beyond three months from symptom onset/diagnosis of COVID-19. The most common among the spectrum of symptoms include myalgias, fatigue and dyspnoea. In addition, patients with hypothyroidism and hypoxia (room air $SpO_2 \leq 93\%$) during acute illness were at higher risk for developing long COVID.

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Brief Report

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Association of bleeding symptoms during influenza infection and administered drugs

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SUMMARY On March 1, 2019, the Ministry of Health, Labour and Welfare added bleeding symptoms to adverse reaction package inserts as a possible adverse event for a new anti-influenza drug, baloxavir marboxil, because 13 patients with bleeding symptoms were identified among influenza patients taking the drug. Nevertheless, aspects of the epidemiology of bleeding symptoms among influenza patients remain unclear. This study elucidated bleeding symptoms among influenza patients and hospitalized patients as severe cases. A survey was administered to all physicians in Japan during the 2019-2020 season for reporting of bleeding symptoms in influenza patients. The survey elicited information about outcomes, assuming associated underlying diseases and drugs in addition to administered drugs including acetaminophen and anti-influenza (antiviral) drugs. We received reports of 63 cases with bleeding symptoms, including 5 cases of hospitalized patients. Among all patients, 54% had been administered oseltamivir; 10% had been administered baloxavir marboxil. Among hospitalized patients, all had been administered acetaminophen; 40% of them had been administered oseltamivir, and one patient had been administered baloxavir marboxil. Accumulation of bleeding symptom cases is expected to be necessary to evaluate the association.

Keywords Acetaminophen, baloxavir marboxil, bleeding symptom, influenza, oseltamivir

1. Introduction

Since 13 cases of bleeding symptoms were reported to the Ministry of Health, Labour and Welfare (MHLW) from influenza patients using the newly launched anti-influenza virus drug endonuclease inhibitor, baloxavir marboxil, bleeding symptoms were added to instructions for drug package inserts on March 1, 2019. However, the epidemiology of bleeding symptoms among influenza patients, especially their association with anti-influenza virus drugs, was not well known then. The present study was conducted using a survey and a brief report of its results to elucidate epidemiological aspects of bleeding symptoms.

2. Methods

Under cooperation with MHLW, we surveyed all physicians throughout Japan about influenza patients with bleeding symptoms from November 1, 2019 through March 31, 2020. In addition to eliciting demographic information of patients such as gender and age, influenza-related information including the highest body temperature, vaccination history, rapid test results, onset date, administered drugs including acetaminophen, and anti-influenza virus drugs, we also asked about details of bleeding symptoms such as the following. (*1*) Clinical features: intracranial, conjunctiva, nose, intraoral, petechia, ecchymosis, hemarthrosis, hematemesis (upper gastrointestinal tract), sputum, hemoptysis, melena, stool, macroscopic hematuria, abnormal genital bleeding, and oozing. (*2*) Outcome: hemostasis (or arrest of bleeding symptoms) by pressure with no treatment, hemostatic by some treatment, hospitalization, and blood transfusion or fluid infusion. (*3*) Assumed associated underlying diseases: hemophilia, leukemia, thrombocytopenia, gastric ulcer and ulcerative colitis. (*4*) Assumed associated drugs: anticancer drugs (*1*), immunosuppressants, non-steroidal anti-inflammatory drugs such as antiplatelet agents (*2,3*), and

coagulation factor inhibitors such as anticoagulants (*3-6*).

After summarizing basic characteristics such as gender, age and the highest body temperature, we assessed bleeding sites and outcomes, with assumed associated and underlying diseases and drugs. Finally, we examined data related to administered anti-influenza virus drugs and acetaminophen before bleeding symptoms. Case groups of two types were analyzed: all reported bleeding symptoms cases and cases limited to those of hospitalized patients.

This study was approved by the Committee for Ethical Consideration, National Institution of Infectious Diseases, Japan: approval numbers were 261, 312, 375, and 462. Approval by the Kawasaki City Institution for Health and Safety, Committee for Ethical Consideration was 01-3.

3. Results and Discussion

We received 63 reports of bleeding symptoms associated with influenza, among which 34 cases (55%) were of female patients. Distributions of age and highest body temperature are presented respectively in Figures 1 and 2. These patients' average age was 18 years old, but the median was 8.5 years. The age distribution was skewed to a younger age, but patients of all age classes were

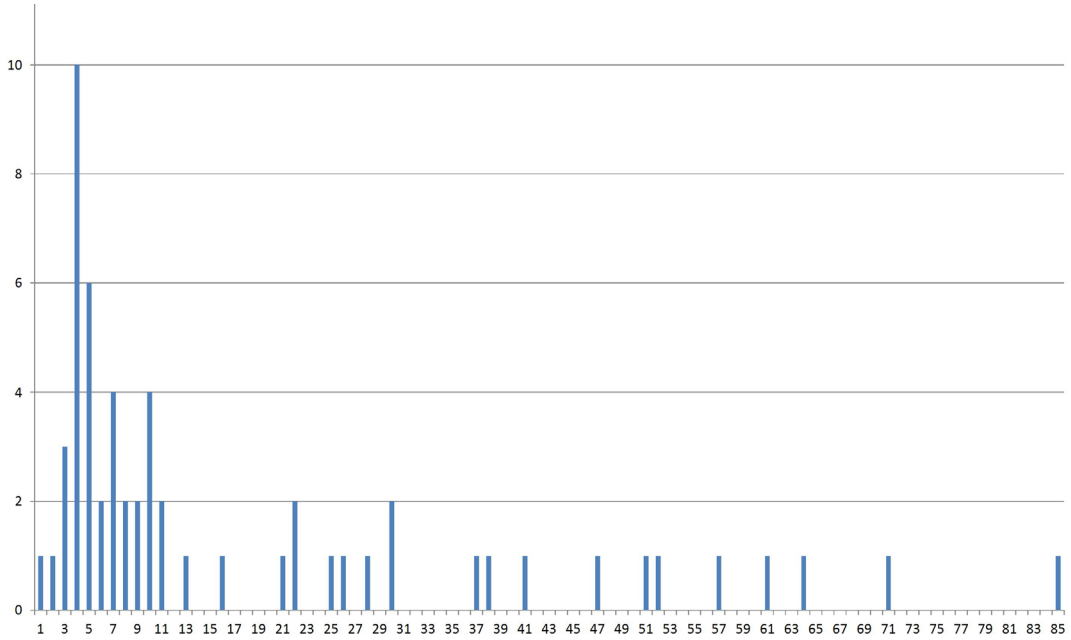

Figure 1. Age distribution in all bleeding symptom cases (*n* = 63). *Note*: Average age was 18 years old; median age was 8.5 years old.

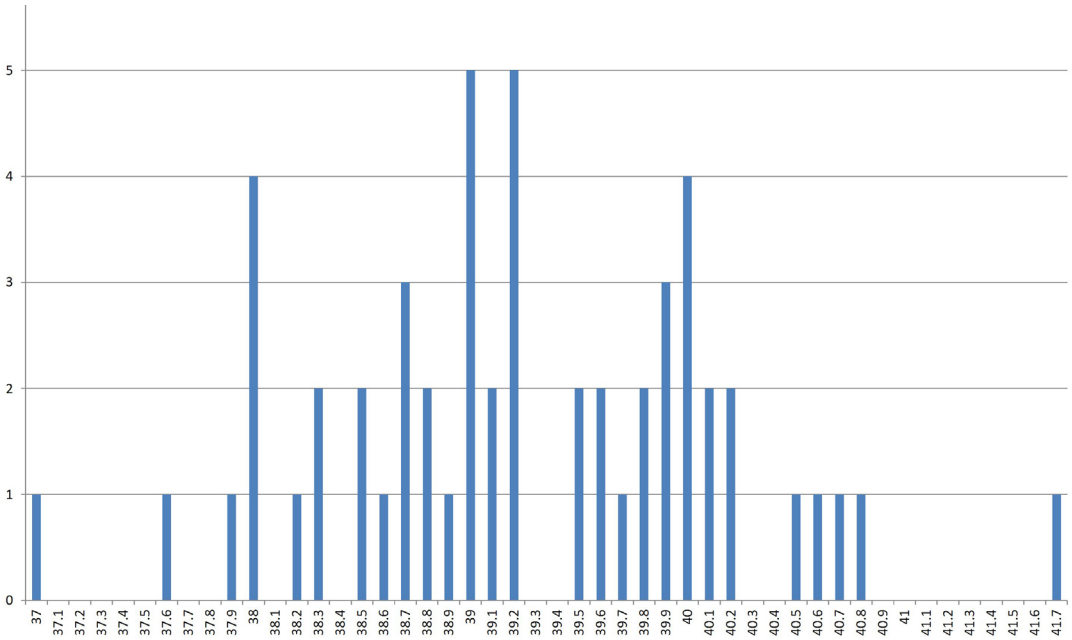

Figure 2. Distribution of the highest body temperature (*n* = 63). Note: Average and median of body temperature were 39.2°C.

reported. Both the average and median body temperature were 39.2°C. Regarding the vaccine history, 26 (58%) patients had received no influenza vaccination: those who had received one dose were 8 patients (19%); those with two doses were 11 patients (24%).

Bleeding sites in all cases are shown in Figure 3, for which multiple answers were allowed. The highest frequency site was the nose (29 cases), followed by melena (7 cases). Outcomes in all cases are presented in Figure 4. Multiple answers were accepted. Almost half of

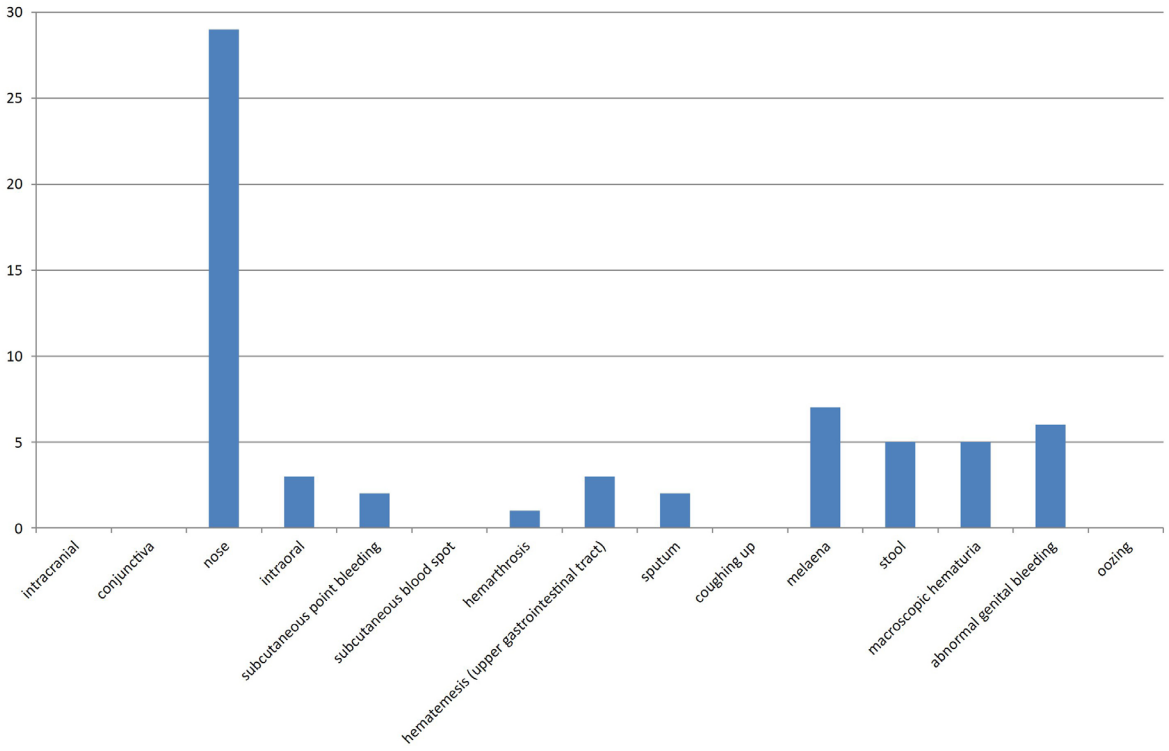

Figure 3. Bleeding site (*n* = 63). *Note*: Multiple answers were accepted.

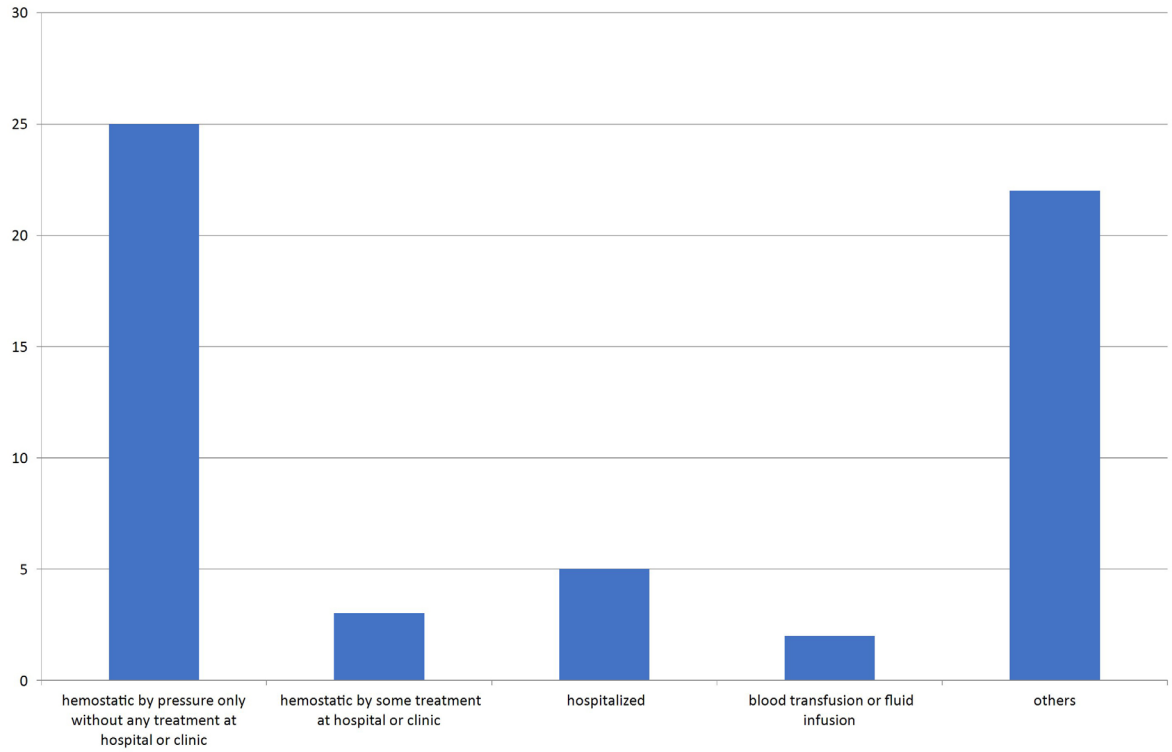

Figure 4. Outcome (*n* = 63). *Note*: Multiple answers were accepted.

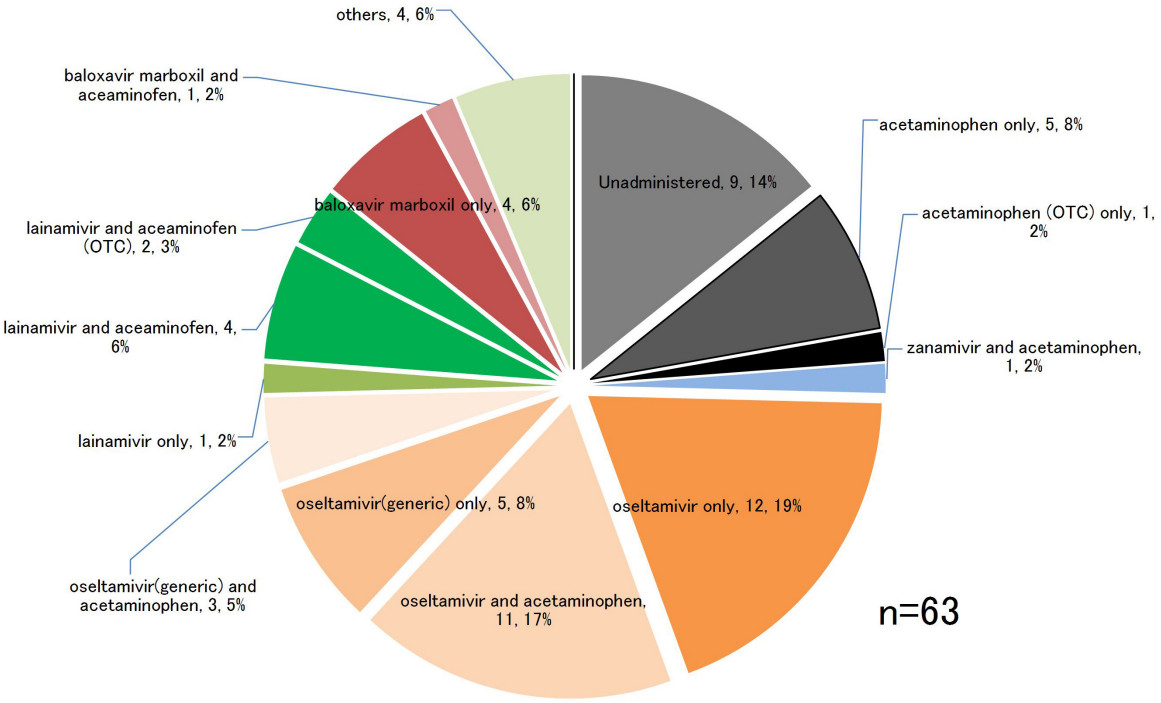

Figure 5. Combinations of administered acetaminophen and anti-influenza virus drugs in all cases (*n* = 63). *Note*: Others included four cases with oseltamivir + acetaminophen + peramivir, oseltamivir (generic) + peramivir, oseltamivir (generic) + acetaminophen + peramivir and baloxavir marboxil + peramivir + acetaminophen.

the cases, 25, were resolved with no treatment other than pressure. However, others were 22 cases. Stomach ulcers were reported for two cases, presumably associated with underlying diseases. For 52 cases, no drug among the assumed associated drugs was reported. They were recorded as lacking drug treatment.

Figure 5 shows combinations of administered acetaminophen and anti-influenza virus drugs for all cases. The proportion of no considered drug administered was 14%. That of acetaminophen including its over-the-counter (unprescribed) formulations was 10%. Oseltamivir accounted for 54%. Baloxavir marboxil accounted for only approximately 10%.

We administered all of the hospitalized patients reported at our site. One patient among them (case C in Tables 1 and 2) was not hospitalized for bleeding symptoms. Actually, she had asthma and had received steroid treatment before bleeding symptoms. Subsequently, she showed upper gastrointestinal tract bleeding during the hospital visit.

Table 1 presents age, gender, blood transfusion or fluid infusion, vaccine history, and the highest body temperature, assuming associated underlying diseases, drugs, and bleeding sites for hospitalized patients. No particular pattern was apparent for age or gender. No patient had been vaccinated. Two patients had received blood transfusion or fluid infusion. No assumption was made of associated underlying diseases and drugs, although they were not reported in four cases.

Table 2 shows findings for administration of acetaminophen and anti-influenza virus drugs among hospitalized patients. All patients had been administered acetaminophen. All patients were administered acetaminophen before bleeding symptoms. Three patients were administered oseltamivir. One patient took peramivir and baloxavir marboxil. However, no patient used zanamivir or laninamivir.

Even though 8.5 years old was the median age, people older than 80 years old were reported to have experienced bleeding. Therefore, patients were not only younger people. Moreover, bleeding symptoms were severer for female patients than for male patients. This finding differs from those related to abnormal behavior among influenza patients, for whom the incidence of abnormal behavior among younger male patients was higher than among others. Even for hospitalized patients, no particular pattern was found for age or gender. Most patients reported no associated underlying disease or related drug. That was true even among hospitalized patients.

Regarding the combinations of administered acetaminophen and anti-influenza virus drugs, the remarkable proportion of patients who had not been administered these drugs or who had been administered acetaminophen without anti-influenza virus drugs was similar to that of patients exhibiting abnormal behavior (*7-9*). The share of patients receiving oseltamivir might be higher than reported from an earlier study examining abnormal behavior among influenza patients. Even among hospitalized patients, 2 of 5 patients used

Table 1. Fundamental characteristics assuming associated underlying diseases and drug and bleeding site in hospitalized cases (n = 5)

Case	Age (years old)	Blood transfusion or fluid infusion	Gender	Vaccine history	Highest body temperature (°C)	Assuming associated underlying diseases	Assuming associated drug	Bleeding site			
								hematemesis (upper gastrointestinal tract)	melena	sputum	stool
A	52	-	N.A.	–	N.A.	N.A.	N.A.	-	-	+	-
B	1	-	Male	N.A.	40.0	–	N.A.	+	-	-	-
C	4	+	Male	–	40.8	N.A.	–	+	+	-	-
D	85	+	Female	N.A.	39.7	N.A.	N.A.	-	+	-	-
E	16	-	Female	N.A.	38.7	N.A.	N.A.	-	-	-	+

Note: "+" denotes yes; "-" represents no. No leading site other than hematemesis (upper gastrointestinal tract), melena, sputum, and stool was reported. Assumed associated underlying diseases were hemophilia, leukemia, thrombocytopenia, stomach ulcer and ulcerative colitis. Assumed associated drugs were anticancer drugs, immunosuppressants, non-steroidal anti-inflammatory drugs such as antiplatelet agents and coagulation factor inhibitors such as anticoagulants. Case C was administered steroids for asthma treatment before bleeding symptoms.

Table 2. Administered acetaminophen and anti-influenza virus drug in hospitalized cases (n = 5)

cases	acetaminophen	oseltamivir	baloxavir marboxil	peramivir
A	+	-	-	-
B	+	+	-	-
C	+	+	-	-
D	+	-	+	+
E	+	+	-	-

Note: "+" represents administered and "-" denotes drug not-administered before bleeding symptoms. No patient was administered zanamivir or laninamivir.

oseltamivir.

The proportions of influenza patients administered acetaminophen or anti-influenza virus drug or not administered acetaminophen and anti-influenza virus drugs is not known precisely. However, accompanying research investigating abnormal behavior administered a questionnaire survey contemporaneously with the report described herein: 352 non-life-threatening abnormal behavior cases were identified (*10*). In those cases, 99 (28%) patients had been administered acetaminophen including its over-the-counter formulations; 129 cases (37%) had been administered oseltamivir. In addition, 72 cases (6%) were not administered acetaminophen or any anti-influenza virus drug. These proportions do not resemble those of the entire population of all influenza patients. Especially, most influenza patients with abnormal behavior were male and younger than 19 years old. Their characteristics might bias the proportions of administered acetaminophen and/or anti-influenza virus drugs. However, their magnitude cannot be evaluated precisely. At least, the proportions of acetaminophen administered among bleeding and hospitalized cases and of oseltamivir administered among all bleeding cases were higher than those of cases of reported abnormality. For several reasons, these differences cannot be evaluated statistically.

These findings indicate that acetaminophen and oseltamivir might be associated with higher likelihood of bleeding symptoms than other anti-influenza virus drugs. To evaluate these associations, adequate data of bleeding symptoms among influenza patients must first be accumulated. Then the total amounts of prescriptions for these drugs must be controlled. The incidence rate among people using these drugs must also be considered.

During the study period, the influenza patients nationwide were estimated as approximately nine million based on Prescription Surveillance (*11-13*) (*http://prescription.orca.med.or.jp/syndromic/kanjyasuikei/*). Therefore, approximately 7.5% of all residents in Japan were infected by influenza. When this number was used for influenza patients, the total number of bleeding symptoms cases, 63, represents 7 cases per million influenza patients, with hospitalization bleeding symptom cases as 0.56 per million influenza patients. The sample

size might be too small to analyze differences in the incidence of bleeding symptom cases according to the administered drug.

Influenza itself might be associated with thrombocytopenia or aberrant coagulation, especially in severe cases with systemic inflammatory response syndrome, including avian influenza (*14-17*). Unfortunately, these were not considered in any association with administered drugs, especially anti-influenza virus drugs. Results of this study demonstrated that even patients with mild seasonal influenza might show bleeding symptoms, even though most incidents were self-limited. In fact, 14% of all reported patients were not administered acetaminophen or any anti-influenza virus drug. Influenza can cause mucosal inflammation and thrombocytopenia. Therefore, influenza with mild symptoms might play some role in the occurrence of bleeding symptoms. Results of the present study indicate that bleeding symptoms can be associated with influenza.

This study has some limitations. First, although we identified 63 cases in all and 5 cases among hospitalized cases, the sample is expected to be very small. Findings related to their characteristics might differ when sufficient data are accumulated. Therefore, the results obtained from the present study must be understand as merely interim findings. Secondly, although we summarized bleeding symptom epidemiology descriptively, the symptoms must be analyzed statistically with an incidence ratio, as in an abnormal behavior study (*6-9*). Application of that methodology remains as a challenging subject for future research. Thirdly, even though we combined questions about blood transfusion and fluid infusion in our questionnaire, great differences were found among treatments of blood transfusion and fluid infusion. A revised questionnaire must be administered in a later study.

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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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Antiviral effect of electrolyzed reduced water on SARS-CoV-2

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SUMMARY The inhibitory activity of electrolyzed reduced water (ERW) against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which is the etiological agent responsible for coronavirus disease 2019 (COVID-19), was tested *in vitro* on Vero E6 cells using a plaque assay. Infectious virus titers of cells treated with ERW 100%, 50% and 33.3% solutions and phosphate buffered saline (PBS, negative control) and exposed to the virus suspension for 60 seconds were 2.25, 2.65, 3.21 and 7.38, respectively. ERW has a high pH and low surface tension. It is considered that the alkaline property of ERW breaks down phospholipids and proteins of envelopes. The role of pH and reducibility on the virucidal effect of ERW should be further evaluated. This study provides a foundation for utilizing ERW as an effective antiviral aqueous solution in a variety of applications.

Keywords Electrolyzed reduced water, alkaline electrolyzed water, reducibility, SARS-CoV-2, antiviral activity

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiological agent responsible for Coronavirus disease 2019 (COVID-19). The global pandemic of COVID-19 has led to an urgent need to develop antiviral agents. Disinfectants, such as benzalkonium chloride and ethyl alcohol-based formulations, have sufficient antiviral activity but also cytotoxicity (*1*). In terms of safety, low cytotoxicity of disinfectants is important.

Electrolyzed water is generated through water electrolysis containing sodium chloride or potassium chloride. At the anode, acidic electrolyzed water is obtained and this has a low pH, high oxidation-reduction potential (ORP) and high concentrations of dissolved oxygen and free chlorine. At the cathode, alkaline electrolyzed water is obtained and this has a high pH, low ORP and a high concentration of ionized alkali metals such as sodium and potassium. It is known that acidic electrolyzed water has marked bactericidal activity and is regarded as a non-environmental hazard; thus, it is widely used as a disinfectant for foods (*2*). Several studies have shown that acidic electrolyzed water has antiviral effects against some kinds of viruses including SARS-CoV-2 (*3-6*). However, hypochlorous acid produced as acidic electrolyzed water (effective chlorine concentration: 100 ppm) has been reported to be insufficiently effective against SARS-CoV-2 under protein loading conditions (*7*). In addition, it is known that acidic electrolyzed water is cytotoxic (*8*), whereas alkaline electrolyzed water has low cytotoxicity compared to acidic water (*9*) and is safe to drink (*10*). Several studies have shown that alkaline electrolyzed water has antibacterial activity against some bacteria (*11,12*) and antiviral effects against some viruses (*13*). However, there have been no reports on the anti-SARS-CoV-2 effect of alkaline electrolyzed water. Therefore, this study aimed to evaluate the virucidal effects of alkaline electrolyzed water against SARS-CoV-2. Okajima *et al.* performed bactericidal tests against periodontopathic bacteria (*14*) and found anti-inflammatory and healing effects in thermal wounds (*15*) using electrolyzed reduced water (ERW, product name "S-100™", supplied by A. I. System Products Co.). Although ERW is characterized by alkalinity, its composition differs from that of the general alkaline electrolytic water described above. ERW is produced by electrolysis of water containing natural salt at high voltage, resulting in a weakly basic liquid containing 0.3% inorganic salts, such as sodium, potassium, calcium, magnesium, chlorine, silicon and phosphorus (*16*), with a pending alkali reserve value equivalent to 0.1 g/100 mL. In this study, the antiviral effect of ERW (classified as one of the alkaline electrolytic waters) on SARS-CoV-2 was evaluated.

2. Materials and Methods

2.1. Materials

The JPN/TY/WK-521 strain of SARS-CoV-2 was provided by National Institute of Inflectional Disease, Japan. Vero E6 cells (VeroE6/TMPRSS2; #JCRB1819) were purchased from the Japan collection of Research Bioresources: JCRB1819. Dulbecco's modified Eagle's medium (DMEM, low-glucose; SIGMA, #D6046), Minimum Essential Medium Eagle (EMEM; SIGMA, #M4655), Fetal Bovine Serum (FBS; SIGMA, #173012) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Soya Casein Digest Lecithin Polysorbate Broth (SCDLP; Bouillon medium, #E-MC72), was purchased from Eiken Chemical Co., Ltd. (Tokyo, Japan). Costar® 6 Well Clear TC-Treated Multiple Well Plates, Bulk Packed, Sterile (Product #3506), were purchased from Corning (NY, USA). Minimum Essential Medium Eagle (modified) with Earle's salt (EMEM; MP Biomedicals Catalog #1010122), was purchased from MP Biomedicals (CA, USA). ERW for evaluation of this study was provided by A. I. System Products Corp. (Aichi, Japan). In this study, the antiviral activity of 100%, 50% and 33.3% ERW was evaluated. The physical properties of ERW, purified water and sodium hydroxide solution at each dilution rate are shown in Table 1. The pH and biological antioxidant potential (BAP) values in the table were measured with a pH meter (HI9916N; Hanna Instruments Japan Co., Ltd., Chiba, Japan) and Hand spectrophotometer (FREE carrio duo; Wismerll Co., Ltd., Tokyo, Japan), respectively. BAP is a method for quantifying the reducing power of a reaction from an oxidized state (Fe^{3+}) to a reduced state (Fe^{2+}) (*17*).

2.2. Methods

2.2.1. Preparation of virus suspension

SARS-CoV-2 was added to the Vero E6 cells and incubated with EMEM at 37°C. The cell suspension was transferred to a conical tube and centrifuged at 1,000 × g for 15 min at 4°C. The supernatant was used as a test virus suspension.

Table 1. Characteristics of purified water, sodium hydroxide solution and ERW.

Items	pH at 25°C	BAP/μmol/L
Purified Water	5.7	258
NaOH aq.	12.1	267
ERW 100%	12.1	4,905
ERW 50%	11.8	2,469
ERW 33.3%	11.7	1,724

2.2.2. Cytotoxicity test

Cytotoxicity test was performed to confirm that ERW had no cytotoxic effect on Vero E6 cells. EMEM (0.1 mL) was added to 1.9 mL of ERW; 0.1 mL of this mixture was then added to 0.9 mL of inactivating agent (SCDLP: DMEM including 2% FBS = 1:9) and then stirred well. This was repeated to prepare a 10-fold dilution of the series from 10^{-1}-10^{-4}. Cytotoxicity was determined using a plaque assay (see 2.2.5. for details).

2.2.3. Susceptibility of cells to the virus

This test was performed to confirm the susceptibility of cells to the virus. EMEM (0.1 mL) was added to 1.9 mL of ERW and then stirred well; 0.5 mL of this was then mixed well with 4.5 mL of inactivating agent (SCDLP: DMEM including 2% FBS = 1:9) at room temperature. This was repeated to prepare a 10-fold dilution of the series from 10^{-1}-10^{-4}. Virus suspension, prepared at 4-6 × 10^4 PFU/mL, was added at a rate of 1% to each dilution and incubated at room temperature for 10 min. As a negative control, cells were treated with phosphate buffered saline (PBS). Susceptibility of cells to virus was determined by a plaque assay.

2.2.4. Anti-SARS-CoV-2 effect of ERW

Virus suspension (0.1 mL) was added to 1.9 mL of ERW 100%, 50% or 33.3% ERW solutions (diluted with purified water), and then mixtures were stirred sufficiently. After being kept at 25°C for 20 seconds, 60 seconds or 5 minutes, 0.5 mL of each mixture was added to 4.5 mL of inactivating agent (SCDLP: DMEM including 2% FBS = 1:9) and stirred well. The prepared solution was used as the test solution. Using this test solution, various 10-fold diluted solutions of the series from 10^{-1}-10^{-4} were prepared as shown in Figure 1. As a negative control, cells were treated with PBS. The anti-SARS-CoV-2 effect of ERW was determined using a plaque assay.

2.2.5. Plaque assay

Monolayer Vero E6 cells were cultured in a six-well plate and growth medium was removed from the plate. A serial dilution of the virus-containing supernatants (test solution) was inoculated into the Vero E6 cell monolayer. The cells were then incubated for viral adsorption in a 37°C 5% CO_2 incubator and the test solutions were spread over cells every 15 minutes. After cleaning the cell surface with EMEM, cells were overlaid with 3 mL/well of EMEM including 2% FBS. Cells were then incubated for a further 40-48 h. After fixing the cells, plaque phenotypes were visualized by staining with methylene blue. Plaque numbers were counted as plaque-forming units per 0.1 mL (PFU/0.1 mL).

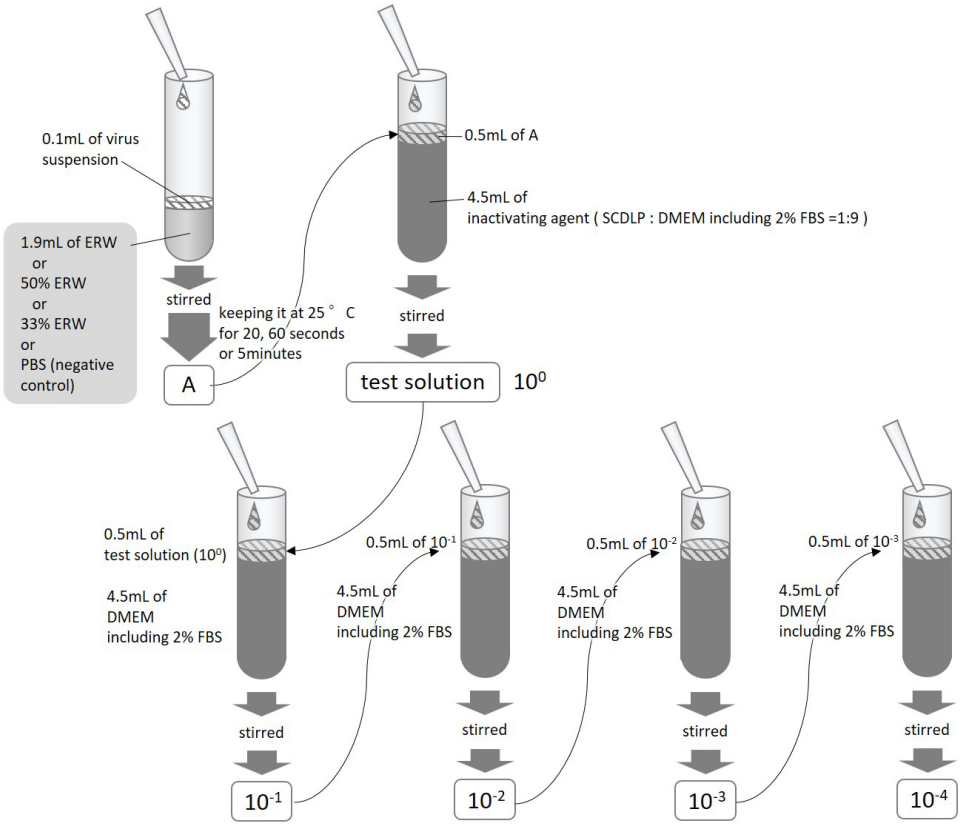

Figure 1. Schematic diagram of dilution method.

2.2.6. Statistical analysis

For each experimental result, significant differences were analyzed using the paired *t*-test. The results are expressed as the mean ± standard deviation. When $p \leq 0.05$ and $p \leq 0.01$, there was a difference at a significance level of 0.05 and 0.01, respectively.

3. Results and Discussion

In the experiment to investigate if ERW has cytotoxic effects on Vero E6 cells (experiment 2.2.1), results showed that ERW 100%, 50% and 33.3% solutions had no cytotoxicity in test conditions. In addition, we confirmed the susceptibility of cells to the virus (experiment 2.2.2). Infectious virus titers in cells exposed to ERW 100%, 50% and 33.3% solutions and PBS were 2.59, 2.60, 2.62 and 2.62 PFU/mL (Log10), respectively. The virus infectivity titer was successfully measured without influence of the sample by diluting the test solution 10-fold with an inactivating agent (SCDLP: DMEM including 2% FBS = 1:9).

To investigate whether ERW has a potential as an anti-SARS-CoV-2 agent, SARS-CoV-2-infected Vero E6 cells were treated for 20 seconds, 60 seconds or 5 minutes with various dilutions of ERW. The results demonstrated that ERW inhibited SARS-CoV-2 replication, whereas virus replication was observed in the negative control (Figure 2). Infectious virus titers in cells treated with ERW 100%, 50% and 33.3% solutions and PBS and exposed to the virus suspension for 60 seconds were 2.25, 2.65, 3.21 and 7.38 PFU/mL (Log10), respectively (Figure 3).

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus. The virus envelope is typically derived from portions of the host cell membranes (phospholipids and proteins), but include some viral glycoproteins. It is considered that the inoculation of ERW breaks down phospholipids and proteins of envelopes. ERW not only has high pH, but also high reducibility, which is not found in NaOH aqueous solutions. Previously, the antiviral activity of NaOH solutions has been reported (*18*), and in the case of SARS-CoV, it took 1-2 hours to reduce more than 5 log10 at pH > 12 (*19,20*). In addition, some reducing agents have been reported to inactivate Sindbis virus and Mayaro virus within 3 hours by cleaving the disulfide bonds of the viral membrane proteins (*21*). In such studies, high pH and high reducibility have antiviral effects but do not lead to inactivation in a short time. On the other hand, ERW, which has both properties, showed antiviral activity of more than 4 log10 in 20 seconds at 100% concentration of pH = 12.1, BAP = 4,905 μmol/L and more than 3 log10 in 60 seconds at 33.3% concentration of pH = 11.3, BAP = 1,724 μmol/L. From these results, it is considered

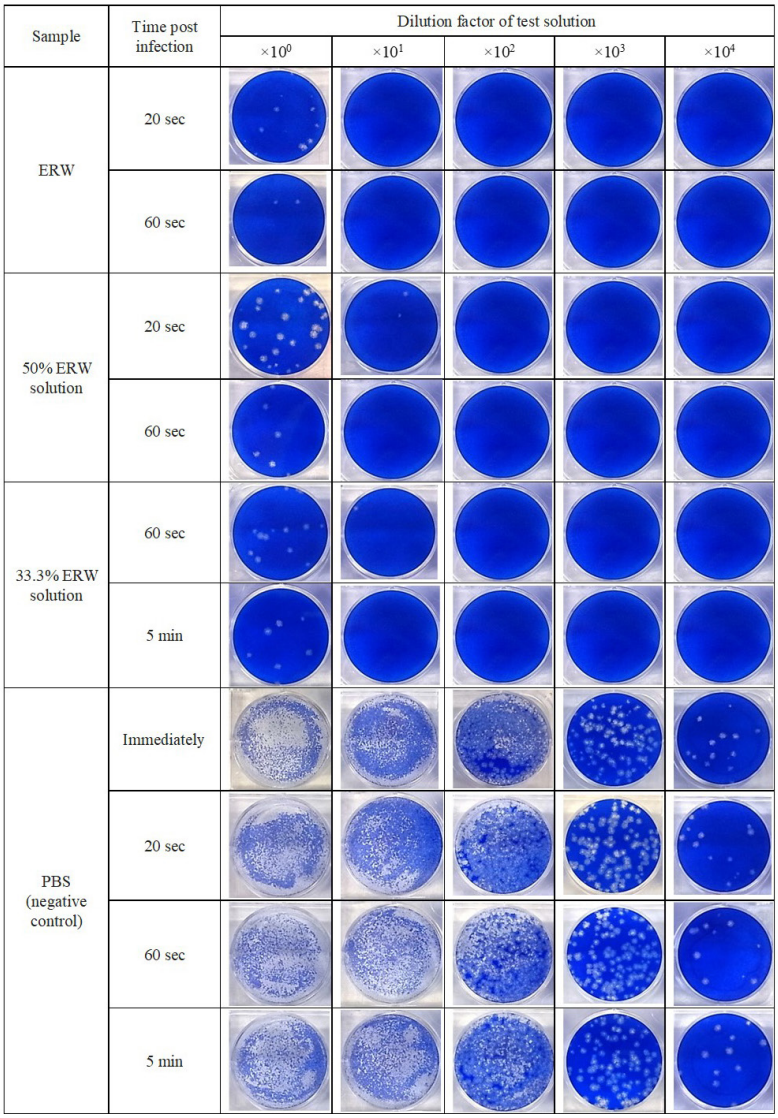

Figure 2. Photographs of the plaque assay for counting SARS-CoV-2. These are the results of photographing a petri dish from above after culturing the virus in each condition. The white spots in the petri dish are called "plaques" and represent the state of cytopathic caused by the virus infection. If there is a large amount of virus infection, the plaques will overlap. In order to avoid it, the test solution is diluted after stopping the drug reaction until the plaques can be counted correctly. The viral titer is calculated from the number of plaques counted, the dilution factor and the virus concentration in the test solution (plaque assay).

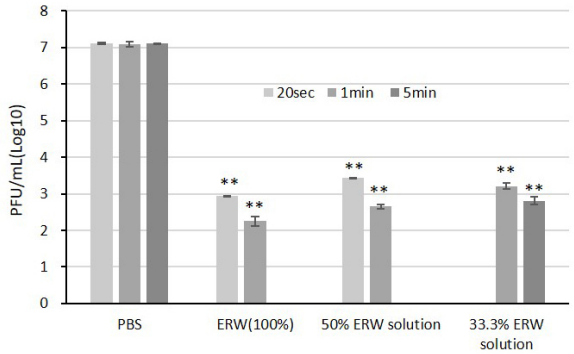

Figure 3. Infectious virus titers in cells treated with various concentration of ERW solutions and PBS. Infectious virus titers (PFU/mL (Log10)) of SARS-CoV-2 of cells mixed with ERW (100%), 50% ERW solution, 33.3% ERW solution and PBS as negative control and exposed to virus suspension for 20 seconds, 1 minute or 5 minutes (mean ± S.D., $n = 3$). $^{**}p < 0.01$ (*t*-test *vs.* PBS).

that the antiviral mechanism of ERW is attributed to the synergistic effect of pH and reducibility properties, which resulted in instantaneous antiviral action. The role of pH and reducibility on the antiviral effect of ERW should be further evaluated.

In conclusion, this study demonstrated the anti-SARS-CoV-2 activity of ERW using a plaque assay. Potent anti-SARS-CoV-2 activities, together with the lack of cytotoxic effects, support the further development of ERW as a monotherapy or in combination with other effective agents against SARS-CoV-2 infection.

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Conflict of Interest: The electrolyzed reduced water (ERW, product name S-100) used in this study was manufactured by A. I. System products, Corp. Yoshinao Okajima, Masahiro Okajima and Mitsuo Ikeda are employees of A. I. System products, Corp.

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Brief Report

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Role of systemic corticosteroids in preventing hypoxia among patients with mild COVID-19: An observational study

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SUMMARY Use of systemic corticosteroids is well-established in COVID-19 patients with hypoxia; however, there is scant data on its role in patients with mild disease and prolonged symptoms as a measure to prevent disease progression. The aim of this study is to evaluate the role of systemic corticosteroids in preventing hypoxia ($SpO_2 \leq 93\%$ on room-air) among mild COVID-19 patients. An observational study was conducted among symptomatic COVID-19 patients taking oral corticosteroids and attending institute teleconsultation facility between 10th-30th June 2021. Patients who were already on corticosteroids for other indication or required oxygen supplementation before or within 24-hours of initiation of corticosteroids were excluded. A total of 140 consecutive symptomatic COVID-19 patients were included. Higher baseline C-reactive protein (OR: 1.03, 95% CI: 1.02-1.06, $p < 0.001$) and early systemic corticosteroid (within 7 days) initiation (OR: 6.5, 95% CI: 2.1-20.1, $p = 0.001$) were independent risk factors for developing hypoxia ($SpO_2 \leq 93\%$). Progression to hypoxia was significantly higher in patients who received corticosteroids before day 7 of illness (36.7%, 95% CI, 23.4-51.7%) compared to ≥ 7 of illness (14.3%, 95% CI, 7.8-23.2%) for persistent fever. Systemic corticosteroids within 7 days from symptom-onset were harmful and increased the risk of progression to hypoxia, whereas it may decrease the risk of progression when administered on or beyond 7 days in patients with mild COVID-19 and persistent symptoms. A well-designed randomised controlled trial is required to validate the findings.

Keywords COVID-19, corticosteroids, hypoxia, prolonged fever

1. Introduction

The successive waves of the ongoing pandemic have overwhelmed even the best of healthcare infrastructure globally. While significant evidence has been generated on treatment options for admitted patients with hypoxia; not much progress has been made among non-hypoxic patients barring monoclonal antibodies, the economics of which makes it unlikely to be a game-changer.

Identifying risk factors for targeted intervention and preventing progression to hypoxia requiring hospitalization, is a much-needed lacunae in coronavirus disease-2019 (COVID-19) management which needs more attention. Some observational studies have reported prolonged fever as a risk factor for progression to severe disease (*1*). The randomized controlled trials on corticosteroids used in COVID-19 have not addressed this group of patients. In the RECOVERY trial, corticosteroids were used early in the disease course in non-hypoxic patients (median 6 days), which theoretically is likely to be harmful (*2*).

In April 2021, at the height of the second wave in India, guidelines were issued recommending low-dose corticosteroids in confirmed COVID-19 patients with prolonged fever beyond 7 days on a case-to-case basis (*3*). In this setting, this observational study was carried out to evaluate the role of systemic corticosteroids in preventing hypoxia ($SpO_2 \leq 93\%$ on room-air) among mild COVID-19 patients.

2. Methodology

This was an ambispective observational study approved by the Institute Ethics Committee and carried out between 10^{th}-30^{th} June 2021. Confirmed COVID-19 patients who had availed the teleconsultation facility at our tertiary care hospital in Delhi, India, were contacted. A confirmed case was defined as per the World Health Organization COVID-19 case definition (*4*). Consecutive patients who were symptomatic and

prescribed systemic oral corticosteroids by their treating physician for COVID-19 illness were invited to fill up a pre-designed questionnaire after informed consent. Patients who were already on corticosteroids for other indication or required oxygen supplementation before or within 24-hours of initiation of corticosteroids were excluded. Patients already on other immunosuppressive drugs and pregnant females were also excluded.

Patient characteristics, clinical details, vaccination, investigations, day, type and dose of corticosteroid at initiation, response to treatment, and progression to hypoxia ($SpO_2 \leq 93\%$ on room-air) requiring oxygen supplementation were recorded. The records and variables were analysed using Statistical Software Stata 16·0 (StataCorp, College Station, TX). Categorical data were expressed as frequency and percentage, quantitative data expressed as mean (SD) and median (IQR). Independent *t*-test was used to compare means, Mann-Whitney *U* test for medians and Chi square/ Fisher's exact test for categorical variables. A binary logistic regression model was developed to assess the impact of different variables (which had $p < 0.2$) on the likelihood of development of hypoxia with the forward conditional method. Kaplan-Meier curves were constructed for the oxygen requirement for the two groups (early corticosteroid intake and late corticosteroid intake) and the difference between the two curves was estimated using the log rank test. For the outcome of oxygen requirement, hazard ratios were computed using a Cox proportional hazards model. Univariate Cox models were initially used to determine significant effects of each covariate, following which these were incorporated into a multivariate Cox model. Statistical significance was considered at *p*-value less than 0.05.

3. Results and Discussion

A total of 145 patients were interviewed among which 5 were excluded as they required oxygen supplementation before or within 24-hours of initiation of corticosteroids. Among the 140 patients included in the study, the mean (SD) age was 45.4 (15.6) years and 87 (62.1%) were males. Patients were divided into two groups: those who received systemic corticosteroids before day 7 of illness (early) and patients who received corticosteroids on or after day 7 (late) for persistent symptoms. Age, gender distribution, presence of overall comorbidities, vaccination status and C-reactive protein (CRP) values were comparable between the groups; however, diabetes was more common in the early corticosteroids group (Table 1). The mean duration of illness at initiation of corticosteroids in the first group was 4.9 ± 1.6 days and 8.6 ± 2.0 days in the second group ($p < 0.001$). There was no significant difference in the type, initial dose or duration of corticosteroid use. Incidence of hypoxia was significantly higher in patients who received corticosteroids before day 7 of illness (36.7%, 95% CI, 23.4-51.7%) compared to patients who received corticosteroids on or after day 7 of illness (14.3%, 95%CI, 7.8-23.2%) with *p*-value < 0.005. We also analysed for risk factors of developing

Table 1. Comparison of patient profile receiving corticosteroids before day 7 of illness and receiving corticosteroids on or after 7 days of illness (*n* = 140)

Items	Corticosteroids before day 7 of illness (*n* = 49), *n* (%)	Corticosteroids on or after day 7 of illness (n = 91), *n* (%)	*p*-value
Age in years[a]	47.98 ± 16.99	43.98 ± 14.71	0.15
Male gender	30 (61.2)	57 (62.6)	1
Comorbidities			
Hypertension	8 (16.3)	17 (18.7)	0.8
Diabetes	13 (26.5)	11 (12.1)	0.04
Coronary artery disease	1 (2)	4 (4.4)	0.66
COPD/Asthma	2 (4.1)	1 (1.1)	0.28
Hypothyroidism	5 (10.2)	2 (2.2)	0.05
Vaccination status			0.3
Unvaccinated	22 (44.9)	51 (56)	
Single dose	19 (38.8)	24 (26.4)	
Both doses	8 (16.3)	16 (17.6)	
First available CRP in mg/dL[b,c]	14 (4-32)	20.5 (9.8-43)	0.07
Duration of illness in days at initiation of corticosteroids[a]	4.9 ± 1.62	8.65 ± 2.04	< 0.001
Type of corticosteroids			
Dexamethasone	22 (44.9)	45 (49.5)	0.7
Methylprednisolone	22 (44.9)	39 (42.8)	0.9
Prednisolone	5 (10.2)	7 (7.7)	0.8
Dose at initiation (Methylprednisolone equivalent in mg/day)[c]	32 (20-60)	32 (30-48)	0.71
Duration of corticosteroids treatment in days[c]	10 (7-13)	7 (5-11)	0.06
Oxygen requirement	18 (36.7)	13 (14.3)	0.005
Duration of illness at oxygen requirement in days	10.94 ± 4.66	11.1 ± 1.3	0.92

[a.] mean ± S.D.; [b.] Available for 41/49 in first group and 74/91 in the second group of patients; [c.] median (IQR).

Table 2. Comparison of patient profile based on development of hypoxia (*n* = 140)

Items	No hypoxia (*n* = 109) *n* (%)	Hypoxia (*n* = 31) *n* (%)	*p*-value	Multivariate analysis	
				95% CI	*p*-value
Age (in years)[a]	43 ± 13.9	53.9 ± 18.4	0.005		0.1
Male gender	69 (63.3)	18 (58.1)	0.68		
Comorbidities					0.5
Diabetes	17 (15.6)	7 (22.6)	0.42		
Hypertension	18 (16.5)	7 (22.6)	0.43		
Coronary Artery Disease	3 (2.8)	2 (6.5)	0.3		
COPD/Asthma	3 (2.8)	0 (0)	1		
Hypothyroidism	4 (3.7)	3 (9.7)	0.18		
COVID-19 Vaccine status			0.42		
Not vaccinated	60 (55)	13 (41.9)			
1 dose received	31 (28.5)	12 (38.7)			
2 doses received	18 (16.5)	6 (19.4)			
CRP (mg/dL)[b]	14 (5.7-32)	41 (18.9-59.5)	< 0.001	1.03 (1.02-1.06)	< 0.001
Corticosteroids administered			0.005	6.48 (2.1-20.1)	0.001
< 7 days of illness	31 (28.4)	18 (58.1)			
> 7 days of illness	78 (71.6)	13 (41.9)			

[a] mean ± S.D.; [b] median (IQR).

hypoxia by multivariate logistic regression analysis (Table 2). The risk of developing hypoxia was higher in patients with a high baseline CRP (OR: 1.03, 95% CI: 1.02-1.06, $p < 0.001$) and in patients in whom corticosteroids were initiated before day 7 of illness (OR: 6.5, 95% CI: 2.1-20.1, $p = 0.001$) (Table 2).

The Kaplan-Meier curves depicting the probability of oxygen requirement till a follow-up of 30 days between patients with early versus delayed corticosteroid intake is shown in Figure 1. The difference between the survival curves was significant using the log rank test ($\chi^2 = 11.19$, $p = 0.0008$). Oxygen requirement at 30 days was significantly higher in patients with elevated CRP (HR: 1.02, 95% CI = 1.01-1.04, $p < 0.0001$) and in patients with early corticosteroid intake during the course of illness (HR: 4.38, 95% CI = 1.84-10.4, $p = 0.001$).

Out of 140 patients, 63 (45%) patients did not report any adverse events with use of corticosteroids. Among patients who reported adverse events, deranged blood glucose was most common, reported by 33.5% of the participants, followed by gastritis (14.3%) and elevated blood pressures (4.3%).

Initiation of corticosteroids in the early phase of COVID-19 illness (before 7 days from symptom-onset) and higher CRP levels were found to be risk factors for progression to hypoxia. A trend towards decreased incidence of hypoxia was observed among patients with prolonged symptoms who were initiated on corticosteroids on or after 7 days from symptoms-onset.

The pathophysiology of COVID-19 disease comprises three phases: early infection phase, pulmonary phase and hyper-inflammatory phase. The second phase is a transition zone between the viraemic (lasts for around 5-7 days) and the inflammatory phase. End of the pulmonary phase and beginning of hyper-inflammatory

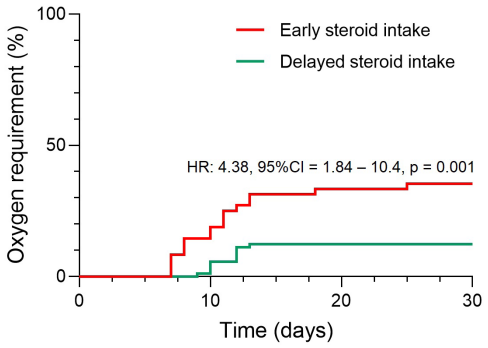

Figure 1. The Kaplan-Meier curves depicting the probability of oxygen requirement between patients with early corticosteroid intake versus delayed corticosteroid intake (*n* = 140).

phase usually marks the onset of respiratory failure. A clinical marker demarcating the onset of immune dysregulation before setting in of respiratory failure would appropriately guide the timing of corticosteroids. A recent systematic review concluded that the virus remains viable only for a short duration. While most studies successfully cultured the virus within the first week of illness, no study reported viable virus beyond day 9 (*5,6*). This may explain the harmful clinical effects of corticosteroids when given in the first week of illness.

Grade and patterns of fever at admission have been correlated with progression, severity and mortality in COVID-19 (*1,7*). While, very few studies have taken into account the duration of fever, a study from Singapore conducted on 142 patients found that both prolonged (27.8% *vs*. 0.9%; 95% CI 9.7-53.5, $p < 0.01$) and saddleback fever (14.3% *vs*. 0.9%; $p = 0.03$) were associated with hypoxia compared with controls. It also found that patients with prolonged fever were more

likely to require ICU admission (11.1% *vs*. 0.9%; p = 0.05) (*8*). In comparison, our study findings suggest a nearly 50% reduction in the incidence of hypoxia in patients receiving corticosteroids with persistent symptoms (27.8% *vs*. 14.3%) and an increase to 36.7% when given early. However, definitive conclusions about the benefit of corticosteroids cannot be drawn given the small sample size, wide confidence intervals and lack of a control group.

A recent trial compared the effect of 6 mg *vs*. 12 mg dexamethasone in COVID-19 patients with severe hypoxemia, where no statistically significant difference was seen at 28 days in the days alive without life support. However non-hypoxemic patients were not included in this study (*9*). The RECOVERY collaboration group found dexamethasone use with lower 28-day mortality than the usual care group when used in patients with invasive and non-invasive oxygenation, but not in the "not hypoxic" group (RR: 0.95, 95% CI 0.84-1.07) (*2*). Although the investigators report an overall benefit with dexamethasone when given after 7 days from onset of illness, it was not sub-analysed in non-hypoxic patients. Rather, Bahl *et al*. reported mortality benefit in patients when corticosteroids were initiated after 7 days of symptom onset (HR: 0.56, 95% CI 0.33-0.95; p = 0.03). The same was not reported for patients requiring oxygen therapy other than invasive mechanical ventilation (*10*).

A recent systematic review reaffirmed the role of corticosteroids in prevention of disease progression (RR: 0.77; 95% CI: 0.64-0.92; p = 0.005). However, progression was mostly defined as requiring mechanical ventilation/ICU transfer/death, and none of the studies looked at progression from absence of hypoxia to development of hypoxia (*11*).

Numerous studies and meta-analyses have not only shown a strong association between disease severity and mortality with CRP values but also with disease progression (*12,13*). Similarly, in our study high CRP levels at baseline was found to be a predictor of disease progression. However, being a retrospective study, due to limitation of the tests performed an ROC curve could not be generated to get a cut-off value. Diabetes being a known risk factor for COVID-19 severity, it may be a potential confounder. However, on multivariate regression analysis diabetes was not found to be a significant independent risk factor for developing hypoxia in the present study.

The study was limited by a small sample size and lack of a control group of patients with prolonged symptoms not receiving systemic corticosteroids. After the guidelines were notified, most of such patients were initiated on corticosteroids by their treating physicians. Selection bias may have occurred as ours is a tertiary care hospital, more symptomatic patients may have availed the teleconsultation facility. Since this was an interview-based study, as such it is subject to recall bias. The study design may have precluded inclusion of more severe patients and thus missed on patients with poor outcomes.

4. Conclusions

Systemic corticosteroids before day 7 from symptom-onset are harmful and increase the risk of progression to hypoxia in symptomatic patients with mild COVID-19. Corticosteroids given for prolonged symptoms on or beyond day 7 may decrease the risk of progression to hypoxia. A well-designed randomized controlled trial (RCT) is urgently required to address this issue.

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

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Communication

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Does immunosuppressive property of non-steroidal anti-inflammatory drugs (NSAIDs) reduce COVID-19 vaccine-induced systemic side effects?

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SUMMARY To help stop the coronavirus disease 2019 (COVID-19) pandemic, vaccines are currently the most critical tool. However, the COVID-19 mRNA vaccines frequently cause systemic side effects shortly after the injection, such as fever, headache and generalized fatigue. In our survey, after receiving the second dose of the COVID-19 vaccine, 80% developed fever, 62% headache and 69% generalized fatigue. Among people who required antipyretics, the average durations of fever and headache were significantly shorter in those who took non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin, loxoprofen and ibuprofen, than those who took acetaminophen. In our patch-clamp studies, NSAIDs effectively suppressed the delayed rectifier K^+-channel (Kv1.3) currents in T-lymphocytes and thus exerted immunosuppressive effects. Because of this pharmacological property, the use of NSAIDs should be more effective in reducing the vaccine-induced systemic side effects that are caused primarily by the enhanced cellular immunity.

Keywords Coronavirus disease 2019 (COVID-19), vaccine, side effects, non-steroidal anti-inflammatory drugs (NSAIDs)

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is still raging worldwide (*1*). In the absence of drugs or other therapeutics for treating COVID-19, vaccines against the virus are currently the most critical tool to help stop the pandemic (*2*). Despite the spread of new SARS-CoV-2 variants that are highly contagious, the vaccination of two doses is considered to be effective in reducing the severity of the disease and decreasing the mortality rates (*3*). However, the administration of COVID-19 mRNA vaccines frequently causes systemic side effects shortly after the injection, such as fever, headache, generalized fatigue, chill, arthralgia, muscle pain and nausea (*4*). Although they are usually self-limited, serious side effects can cause long-term health problems (*5*).

In our survey, among 231 Japanese aged 18 to 22 years who received Pfizer/BioNTech vaccines, 23% developed fever, 25% headache and 33% generalized fatigue after the first dose. These percentages markedly increased after the second dose, in which 80% developed fever, 62% headache and 69% generalized fatigue. The findings were consistent with those obtained from recent studies describing that the vaccine-induced side effects after the second dose were more intense than those after the first dose, especially in younger people (*4*). In our survey, in most people, the symptoms after the first dose subsided spontaneously within 2 days. However, after the second dose, nearly half of the people that developed fever, headache and generalized fatigue required antipyretics, such as acetaminophen and the other non-steroidal anti-inflammatory drugs (NSAIDs; aspirin, loxoprofen or ibuprofen). The average duration of these symptoms was not significantly different between people who did not take any medications and those who took acetaminophen. However, as shown in Figure 1, the average duration of fever and headache was significantly shorter in those who took NSAIDs than in those who took acetaminophen (Figure 1 A and B).

In addition to producing viral spike proteins, COVID-19 mRNA vaccines stimulate the activity of T-lymphocytes and facilitate their cytokine production (*6*). Therefore, the systemic side effects caused by the vaccine are thought to be attributable to the enhanced cellular immunity (*7*). NSAIDs are commonly used as anti-inflammatory, analgesic and antipyretic agents in daily clinical practice. Additionally, due to their immunosuppressive property (*8*), NSAIDs have also been used in the treatment of autoimmune disorders, including systemic lupus erythematosus and rheumatoid

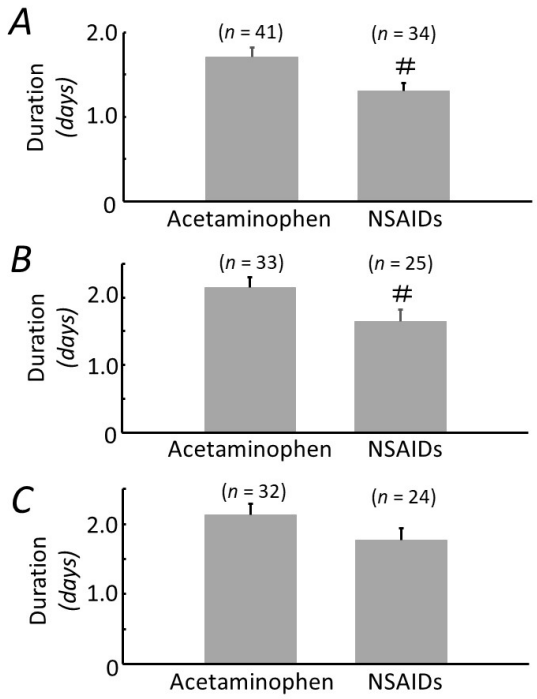

Figure 1. Effects of acetaminophen and non-steroidal anti-inflammatory drugs (NSAIDs) on the duration of systemic side effects after receiving the second dose of the COVID-19 vaccines. Durations of fever **(A)**, headache **(B)** and generalized fatigue **(C)** after receiving the second dose of the COVID-19 vaccine were compared between those who took acetaminophen and NSAIDs (aspirin, loxoprofen or ibuprofen). $^{\#}p < 0.05$ *vs*. those who took acetaminophen. Values are means ± SEM. Differences were analyzed by ANOVA followed by Dunnett's *t* test

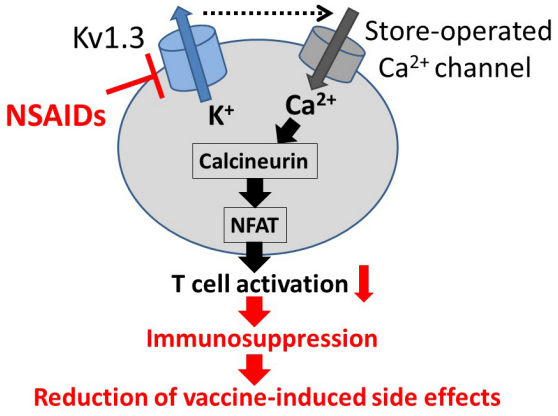

Figure 2. Mechanisms by which non-steroidal anti-inflammatory drugs (NSAIDs) reduce COVID-19 vaccine-induced systemic side effects. Kv1.3-channels promote calcium influx and trigger the proliferation and activation of T-lymphocytes (*15*). The increased cytosolic calcium concentration stimulates the phosphatase calcineurin, which de-phosphorylates the nuclear factor of activated T cells (NFAT), causing its accumulation in the nucleus and binding to the promoter region of cytokine-encoding genes. NSAIDs inhibit Kv1.3-channels and suppress the activity of T-lymphocytes. Consequently, the drugs exert an immunosuppressive property and reduce the vaccine-induced systemic side effects.

arthritis (*9*). In our previous case reports, NSAIDs were actually effective in reducing systemic symptoms triggered by the enhanced autoimmunity (*10,11*). Concerning the mechanisms by which NSAIDs exert this immunosuppressive property, NSAIDs inhibit the migration of leukocytes or directly suppress their cytokine production, either cyclooxygenase (COX) -dependently or -independently (*12,13*). Since the enhanced cellular immunity was the primary pathogenesis of the vaccine-induced side effects, the immunosuppressive effect of NSAIDs was thought to be responsible for the more rapid reduction of fever, headache and generalized fatigue in our survey (Figure 1).

In our patch-clamp studies, we have revealed that NSAIDs, such as aspirin, indomethacin and diclofenac, functionally inhibited delayed rectifier K^+-channels (Kv1.3) expressed in T-lymphocytes, and thus suppressed the activity of the cells (*14*) (Figure 2). Since the channels are highly expressed in T-lymphocytes (*15*), and since selective blockade of the channels actually repressed the immune response in lymphocytes (*16*), this mechanism was thought to be largely responsible for the immunosuppressive property of NSAIDs (Figure 2). Concerning this pharmacological property, besides the use of immunosuppressive drugs or corticosteroids, the use of selective Kv1.3-channel inhibitors may also be beneficial. The early administration of these drugs may not only shorten the duration of the vaccine-induced systemic side effects, but also prevent serious complications after the vaccination, such as myocarditis and pericarditis (*17*). Recently, we have additionally demonstrated in our patch-clamp studies that drugs such as statins (lovastatin, simvastatin), antibiotics (clarithromycin, chloroquine), anti-hypertensive drugs (nifedipine, benidipine, diltiazem, verapamil) and anti-allergic drugs (cetirizine, fexofenadine, azelastine, terfenadine), also strongly suppress the Kv1.3-channel currents in T-lymphocytes (*15,18-20*). In this context, besides NSAIDs, these drugs may also be potentially effective in reducing vaccine-induced systemic side effects.

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Nakata M, Tang W. Japan-China Joint Medical Workshop on Drug Discoveries and Therapeutics 2008: The need of Asian pharmaceutical researchers' cooperation. Drug Discov Ther. 2008; 2:262-263.

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Darby S, Hill D, Auvinen A, *et al*. Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

Example 3 (Sample book reference):

Shalev AY. Post-traumatic stress disorder: Diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

Example 4 (Sample web page reference):

World Health Organization. The World Health Report 2008 – primary health care: Now more than ever. *http://www.who.int/whr/2008/whr08_en.pdf* (accessed September 23, 2010).

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