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Urolithins and intestinal health

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SUMMARY There are trillions of microorganisms in the human intestine. They can react to the intestinal micro-environment by metabolizing food or producing small molecular compounds to affect the host's digestive ability and resist the risk of infection and autoimmune diseases. Many studies have revealed that intestinal flora and its metabolites play an important role in human physiology and the development of diseases. Urolithins are kind of intestinal microbiota metabolites of ellagitannins (ETs) and ellagic acid (EA) with potent biological activity *in vivo*. However, different individuals have different intestinal flora. According to the different metabolites from ETs and EA, it is divided into three metabo-types including UM-A, UM-B and UM-0. This paper reviews the origin of urolithins, the urolithin producing microorganisms and the effects of urolithins on regulating intestinal diseases. This review will provide a theoretical basis for the regulation of urolithins in the homeostasis of intestinal flora and a reference for the scientific utilization of urolithins and foods rich in ETs and EA.

Keywords Urolithin, intestinal microbiota, intestinal health

1. Introduction

It is estimated that the number of bacteria in human intestine is about 10 times that of human cells. The micro-ecological balance of intestinal flora affects energy absorption and immune system, thus affecting human health. Studies have reported that small molecular compounds produced by intestinal microbiota have direct correlation with human health. For example, short chain fatty acids (SCFAs), produced by *Bacteroides* and *Firmicutes* through anaerobic fermentation of dietary fiber, can enhance host immunity (*1,2*). Indole produced by intestinal flora can regulate the host's immune system and inflammatory diseases (*3*). Flavonoid metabolites of intestinal flora can reduce the prevalence of obesity (*4*). Meanwhile, specific intestinal bacteria and their products can lead to human diseases. For example, the small molecule TMAO produced by intestinal microbiota from nutrients rich in choline is related to many metabolic diseases (*5,6*) Colibactin produced by a polyketide synthase positive (PKS^+) *Escherichia coli* can promote the occurrence of colorectal cancer (CRC) (*7*). Therefore, it is of great significance to study the mechanism of the intestinal microbiota metabolites and their occurrence together with their effects on development of diseases.

Ellagitannins (ETs) and ellagic acid (EA) are natural polyphenols found in fruits and nuts, as well as some traditional Chinese medicines. The physical and chemical properties of ETs with high molecular weight and strong polarity determine their low bioavailability. The micro-ecological balance of intestinal microbiota affects energy absorption from food and immune system, thus affecting human health. As metabolites of intestinal flora, urolithins have many biological activities, such as anti-oxidation (*8*), anti-inflammation (*9*), inducing fat browning (*10*), and regulating lipid metabolism (*11*). In recent years, the regulatory effects of urolithins on intestinal microbiota and intestinal inflammatory diseases have also received widely attention (*12*). Here, we review the origin of urolithins, urolithin-producing microorganisms and the regulatory effects on intestinal related diseases.

2. The origin of urolithins

Urolithins (uros) are polyhydroxyl derivatives of diphenylpyran-6-one, which can be considered as a combination of coumarin and isocoumarin in chemical structure. Although uro-M5 has been reported to be isolated from plants *Terminalia* (*13*), *Rosa chinensis* (*14*), *Lagerstroemia speciosa* (*15*), *Punica granatum* (*16*), *Mallotus furetianus* (*17*), and uro-A from pomegranate (*18*), uros are not common in nature.

After eating food rich in ETs, most of them are first metabolized to EA in stomach and small intestine of mammals. Then, EA loses a lactone ring to obtain uro-M5 under the action of esterase and decarboxylase in intestinal microbiota, and then gradually loses hydroxyl groups under the action of dehydroxylase to form a class of internal metabolites with different hydroxyl substitutions (*19,20*). Uro-A and uro-B were first isolated from sheep kidney stones as EA metabolites (*21*), and then were found in urine, feces, bile, prostate, colon and milk of human, rat, mouse, cow, pig, beaver and other animals. Compared with ETs and EA, urolithins are more easily absorbed in colon and can be detected in blood a few hours later under the action of intestinal microbiota. After that, it is widely distributed in the cells of the body or enters the liver with the blood circulation to participate in phase II metabolism, which is gluconic acidified, sulfated or methylated to further exert biological effects (*22,23*). The concentrations of phase II metabolites in human plasma were uro-A glucuronide with 0.024-35 μM, iso-uro-A glucuronide with 0.0045-0.745 μM and uro-B glucuronide with 0.012-7.3 μM (*24*), respectively. With the application of high-throughput and high-sensitivity detection methods, more and more urolithins and their derivatives have been discovered and studied. Members of the urolithin family include uro-M5, uro-D, uro-M6, uro-E, uro-C, uro-M7, iso-uro-A, uro-B, uro-A, uro-M6R, uro-M7R, uro-CR and uro-AR (Figure 1) and their corresponding phase II metabolites (*25,26*). According to final metabolic products, it is divided into three metabo-types including UM-A (producing only uro-A conjugates), UM-B (producing uro-A, isouro-A and/or uro-B) and UM-0 (no urolithins) (*27,28*). Uro-AR exists in both UM-A and UM-B metabo-types (*25*). Studies have shown that the urolithin producing ability and the metabo-types are not closely related to food sources, age and health status (*27*), but are determined by the intestinal microorganisms that can metabolize ETs and EA. However, the analysis of the metabo-types of 839 healthy people aged from 5 to 90 showed that 70-80% of healthy young people aged 5-30 were UM-A, 10-20% were UM-B, while UM-B type increased in people aged 30-90 (*29*). In addition, individual health status such as obesity, colon cancer, hyperlipidemia, cardiovascular disease also affects metabo-types. Romo-Vaquero *et al.* analyzed the intestinal microflora of 249 healthy individuals by 16S rDNA sequencing. The results showed that bacteria *Coriobacteriaceae* may be the relationship between the level of UMs and blood cholesterol. From the current research, UM-A may be more conducive to health, while UM-B may be associated with some diseases and flora disorders (*30*).

3. Urolithin-producing strains from intestinal microorganisms

Urolithins have been found in many animals such as mice, rats, beavers, sheep, cattle and humans after eating foods rich in ETs (*31,32*). Recently, urolithin-producing microorganisms have also been reported. The transformation of ETs and EA by fecal microorganisms of 6 volunteers in anaerobic environment was studied. Uro-A was detected in the fermentation products by fecal bacteria of different volunteers, which confirmed that uro-A is the metabolite of ETs *in vitro* for the first time, but the concentrations and yields were different, indicating that the composition of individual fecal flora was different (*33*). Studies have also been carried out on the isolation of microorganisms which can convert EA into urolithins from the feces of healthy people. Selma *et al.* confirmed for the first time that the new species *Gordonibacter urolithinfaciens* DSM27213 and *G. pamelaeae* DSM19378 have the ability to convert EA to urolithins in stationary culture under anaerobic conditions *in vitro*, and HPLC-DAD-MS analysis showed that pentahydroxy uro-M5, tetrahydroxy uro-M6 and trihydroxy uro-C were produced sequentially, but uro-A and uro-B were not detected in pure culture. It is suggested that the UM-A or UM-B

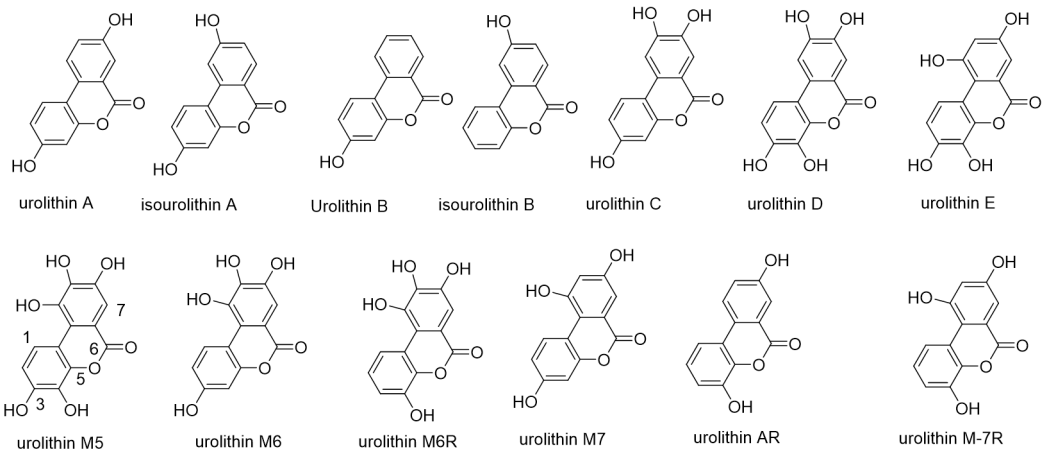

Figure 1. The presentative structures of urolithins.

metabolism may require the participation of other microorganisms or the regulation of culture conditions (*34-36*). Strains *Ellagibacteris isourolithinifaciens* DSM104140 and *G. urolithinfaciens* DSM27213 could convert EA into uro-M5, uro-M6, uro-C and isouro-A (*37*); 48 strains of *Bifidobacteria* and 1070 strains of other bacteria were isolated from the feces of healthy women and their ability to produce urolithins were tested, only strain *Bifidobacterium pseudocatenulatum* INIA P815 could convert EA into uro-A in Brain-Heart Infusion (BHI) medium (*38*).

The number of bacteria in human intestinal tract is about 10 times that of human cells, obviously, the study of microorganisms (genes or enzymes) involved in the transformation of EA *in vivo* is not deep enough. The key rate-limiting steps of urolithin production *in vivo* have not been solved (*20*). The future research should combine the traditional microbial isolation and microbial culture technology together with metagenomics and culturomics to determine the key genes or enzymes involved in the production of urolithins and carry out *in vivo* investigation, and further to explore the role of these microorganisms and enzymes in the regulation of intestinal flora and their effects on human health (*39*).

4. Urolithins and intestinal health

ETs and EA are polyphenols present in a variety of fruits, vegetables, nuts and medicinal plants, with a variety of biological activities. Most of EA is metabolized by intestinal flora to produce a series of urolithins that are more easily absorbed, and their concentrations in different tissues ranging from 0.003 to 50 μM (*40*). Therefore, urolithins may be the real bioactive substances rich in ETs in organisms. Uro-A has been reported as a potential molecule in regulating metabolic diseases such as neuro-inflammation, cardiovascular disease and obesity (*41*). Anti-oxidation, anti-inflammation, anti-cancer, anti-obesity and neuroprotective activities have been reported (*42*). Also, uro-A showed protective potential in the gastrointestinal inflammatory diseases such as CRC and inflammatory bowel disease (IBD) (*12*). Here, we mainly summarize the role of urolithins in regulating intestinal flora and intestinal health.

4.1. Urolithins inhibit bacterial infection

The virulence factor of bacteria is the guarantee for the stable existence of intestinal flora in human digestive tract and against human immunity (*43*). Inhibiting virulence is an important way to control pathogen infection. Four μM uro-A and uro-B can reduce the levels of N-hexanoyl-L-homoserinelactone (C6-HSL) and N-(3-oxohexanoyl)-L-homoserinelactone (3-oxo-C6-HSL) in *Yersinia enterocolitica*, thus inhibit the formation of quorum sensing-related biofilm and the movement ability of bacteria, and maintain the balance of intestinal flora (*44*). Uro-M5 is an inhibitor of type three secretion system of Salmonella and can protect the host by reducing virulence and inflammation (*45*). Moreover, uro-A, uro-B and uro-D have certain antibacterial activity, but their antibacterial activities are weak, coupled with their weak cytotoxicity, the researchers attributed their antibacterial activity to high intake (*46*). The above results suggested that urolithins are inhibitors of bacterial virulence without killing pathogens, and are substitute antibiotics without producing drug resistance (*47*).

4.2. Urolithins regulate intestinal flora

The intestines of human contain 100 trillion viable bacteria, including beneficial and harmful to human health. After feeding uro-A to colitis mice for 10 days, the abundance of the beneficial bacteria *Lactobacillus*, *Bifidobacterium* and *Clostridium* in fecal samples significantly increased (*48*). Also, the abundance of *Akkermansia* and *Gordonibacter* in intestinal flora of uro-A producers was higher than that of non-uro-A producers (*49*). The body weight of high-fat diet induced obese mice was greatly reduced by treatment with 2.5 mg/kg uro-A or uro-B. 16S rDNA sequencing analysis showed that the anti-obesity effects of uro-A or uro-B may play an important role in weight loss by regulating intestinal flora (*50*). Uro-A can also help restore colon tissue damage and regulate intestinal flora, thereby reducing inflammation (*51*). Food-derived metabolites can also regulate intestinal flora (*24*). Medicinal edible plants rich in ETs have different metabo-types (UM-A, UM-B and UM-0) after intestinal flora metabolism, and different metabo-types also reflect the differences of intestinal flora. Foods rich in ETs can increase the abundance of urolithin-producing *Gordonibacter* in fecal microorganisms (*52*). High-fat diet caused intestinal flora disorder in mice, in which *Ruminococcus* increased significantly. Compared with high-fat diet, foods rich in polyphenols increased the abundance of *Roseburia* and decreased the abundance of *Mogibacteriaceae,* while the polyphenols in red raspberry seeds increased the abundance of *Bifidobacterium* (*53*). Therefore, ET-containing food and urolithins can increase the beneficial bacteria such as *Akkermansia* and *Bifidobacteria*, and can restore normal intestinal balance and produce beneficial effects to maintain intestinal homeostasis.

4.3. Urolithins enhance the function of intestinal barrier

Uro-A showed anti-inflammatory activity against mouse Raw264.7 macrophages induced by lipopolysaccharide (LPS). Uro-A pretreatment and post-treatment of DSS induced colitis mice can reduce

inflammatory signals and up-regulate the expression of tumor suppressor genes, thus alleviating colonic injury and playing an important role in regulating the balance of intestinal flora in mice (*48*). Aromatic hydrocarbon receptor (AhR) plays an important regulatory role in enteritis. Recent studies on inflammatory cell models have shown that uro-A can improve the biosynthesis of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) by activating the AhR-Nrf2 dependent pathway, up-regulate the expression of tight junction proteins, prevent inflammation, and improve the barrier function of intestinal epithelium in IBD disease (*54*). Uro-A and its synthetic analog UAS03 enhance the barrier function of intestinal epithelial cells by up-regulating Nrf2 dependent epithelial cell connexin, and protect IBD by reducing inflammatory response (*55*).

4.4. Urolithins inhibit CRC and IBD

Urolithins are mainly produced in the small intestine and colon. Therefore, urolithins are expected to play a role in the intestine and intestinal wall (*56*). *In vitro* studies have proved that uro-A has the inhibitory effects on CRC and IBD. The anticancer effect of uro-A may come from autophagy induction, because autophagy will be triggered after eating polyphenols, thus inhibiting the growth and metastasis of CRC cells (*57*). The mixture of uro-A and uro-B acts on colorectal adenocarcinoma Caco-2 cells both in the long and short term, and the drug reaching the intestinal cavity helped to reduce oxidative stress, and prevented the damage caused by reactive oxygen species (*58*). Uro-A has a significant inhibitory effect on the growth of colon cancer cell line HCT116 with IC_{50} of 19 μM (72 h) and has a synergistic effect with oxaliplatin, which can induce the stability of p53 and the expression of p53 target gene, resulting in p53/p21 dependent aging like growth arrest (*59*). At an achievable concentration in the human colon and rectum, uro-A can enhance the sensitivity of 5-fluorouracil to the anticancer effect of human colon cancer cells, block the cells at G2/M and cause the activation of caspases 8 and 9 (*60*).

IBD is a chronic disease that causing inflammation in the small or large intestines, and is thought to increase the risk of CRC. Uro-A has been reported to prevent the intestinal inflammation by attenuating the inflammatory signaling and upregulating of the tumor suppressor genes (*61*), to increase the permeability of tight junctions (*62*), and to prevent the detrimental effect of inflammation on the cells' viability (*63*). Those finding have given evidence of urolithins, especially uro-A, in the protection of intestinal diseases such as CRC and IBD.

5. The safety of urolithins

Urolithins are the metabolites of tannic polyphenols *in vivo*, which exist in blood, urine and feces in a free form or phase II conjugation, and have extensive biological activities *in vivo* and *in vitro*. Therefore, the experiments based on direct oral administration can verify their safety. The genetic and toxicological toxicity of oral uro-A in rats were studied. The results suggested that high-dose oral synthetic uro-A did not show any toxicity to the target organs at the histopathological level, indicating the clinical safety of uro-A (*64*). Andreux *et al.* recruited 60 elderly people and randomly divided them into four groups: placebo group, uro-A 250 mg, 500 mg and 1,000 mg daily for 28 days. The effects of uro-A on the body were evaluated by the levels of health biomarkers of cells and mitochondria in blood and muscle tissue. The results indicated that uro-A can help slow down the aging process by improving the function of cell mitochondria. It was also found that intake of uro-A had no risk to human health (*65*). And now, the safety evaluation of urolithin *in vivo* is limited to uro-A. So, many problems need to be studied, such as the safety and biological activity of other uros to human body, whether they can enter the blood-brain barrier, their existing form and concentration, the specific function in human body and the relationship with human health.

6. Conclusions

Intestinal microbiota regulates the material and energy metabolism of the host, "You are what you eat" (*66*). Different eating habits have a great impact on the types of intestinal microorganisms in human. At the same time, the types of intestinal microorganisms also determine the metabo-types. At present, the production process of urolithins *in vivo*, the mechanism of intestinal diseases and the interaction with intestinal flora are still in the exploratory stage. It is of great significance to analyze the pharmacological effect and mechanism of urolithins *in vivo* through metabonomics, culturomics and microbiomics, to explore the development of relevant microbial preparations and drugs, and promote its application in the prevention and treatment of intestinal diseases.

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References

1. Yang W, Yu T, Huang X, *et al.* Intestinal microbiota-derived short-chain fatty acids regulation of immune cell IL-22 production and gut immunity. Nat Commun. 2020;

11:4457.
2. Yao Y, Cai X, Fei W, Ye Y, Zhao M, Zheng C. The role of short-chain fatty acids in immunity, inflammation and metabolism. Crit Rev Food Sci Nutr. 2022; 62:1-12.
3. Postler TS, Ghosh S. Understanding the holobiont: how microbial metabolites affect human health and shape the immune system. Cell Metab. 2017; 26:110-130.
4. Guevara-Cruz M, Godinez-Salas ET, Sanchez-Tapia M, *et al.* Genistein stimulates insulin sensitivity through gut microbiota reshaping and skeletal muscle AMPK activation in obese subjects. BMJ Open Diabetes Res Care. 2020; 8:e000948.
5. Amrein M, Li XS, Walter J, *et al.* Gut microbiota-dependent metabolite trimethylamine N-oxide (TMAO) and cardiovascular risk in patients with suspected functionally relevant coronary artery disease (fCAD). Clin Res Cardiol. 2022; 111:692-704.
6. Barrea L, Annunziata G, Muscogiuri G, Di Somma C, Laudisio D, Maisto M, de Alteriis G, Tenore GC, Colao A, Savastano S. Trimethylamine-N-oxide (TMAO) as novel potential biomarker of early predictors of metabolic syndrome. Nutrients. 2018; 10:1971.
7. Dalmasso G, Cougnoux A, Delmas J, Darfeuille-Michaud A, Bonnet R. The bacterial genotoxin colibactin promotes colon tumor growth by modifying the tumor microenvironment. Gut Microbes. 2014; 5:675-680.
8. Kallio T, Kallio J, Jaakkola M, Maki M, Kilpelainen P, Virtanen V. Urolithins display both antioxidant and pro-oxidant activities depending on assay system and conditions. J Agric Food Chem. 2013; 61:10720-10729.
9. Piwowarski JP, Granica S, Kiss AK. Influence of gut microbiota-derived ellagitannins' metabolites urolithins on pro-inflammatory activities of human neutrophils. Planta Med. 2014; 80:887-895.
10. Xia B, Shi XC, Xie BC, Zhu MQ, Chen Y, Chu XY, Cai GH, Liu M, Yang SZ, Mitchell GA, Pang WJ, Wu JW. Urolithin A exerts antiobesity effects through enhancing adipose tissue thermogenesis in mice. PLoS Biol. 2020; 18:e3000688.
11. Zhao W, Wang L, Haller V, Ritsch A. A novel candidate for prevention and treatment of atherosclerosis: Urolithin B decreases lipid plaque deposition in apoE(-/-) mice and increases early stages of reverse cholesterol transport in ox-LDL treated macrophages cells. Mol Nutr Food Res. 2019; 63:e1800887.
12. Kujawska M, Jodynis-Liebert J. Potential of the ellagic acid-derived gut microbiota metabolite - Urolithin A in gastrointestinal protection. World J Gastroenterol. 2020; 26:3170-3181.
13. Pfundstein B, El Desouky SK, Hull WE, Haubner R, Erben G, Owen RW. Polyphenolic compounds in the fruits of Egyptian medicinal plants (*Terminalia bellerica*, *Terminalia chebula* and *Terminalia horrida*): characterization, quantitation and determination of antioxidant capacities. Phytochemistry. 2010; 71:1132-1148.
14. Pei YX, Wang SS, Wang WW, Zhou YH, Zhao HH, Jia L. Isolation and structure-activity relationship of the antioxidant chemical constituents from the clowers of *Rosa Chinensis* Jacq. Int J Food Prop. 2014; 17:38-44.
15. Bai N, He K, Roller M, Zheng B, Chen X, Shao Z, Peng T, Zheng Q. Active compounds from *Lagerstroemia speciosa*, insulin-like glucose uptake-stimulatory/inhibitory and adipocyte differentiation-inhibitory activities in 3T3-L1 cells. J Agric Food Chem. 2008; 56:11668-11674.
16. Nawwar MAM, Hussein SAM, Merfort I. NMR spectral-analysis of polyphenols from *Punica-Granatum*. Phytochemistry. 1994; 36:793-798.
17. Huang X, Xu M, Shirahata T, Li W, Koike K, Kojima-Yuasa A, Yuasa I, Kobayashi Y. Anti-steatosis compounds from leaves of *Mallotus furetianus*. Nat Prod Res. 2018; 32:1459-1462.
18. Totiger TM, Srinivasan S, Jala VR, *et al.* Urolithin A, a novel natural compound to target PI3K/AKT/mTOR pathway in pancreatic cancer. Mol Cancer Ther. 2019; 18:301-311.
19. Garcia-Villalba R, Beltran D, Espin JC, Selma MV, Tomas-Barberan FA. Time course production of urolithins from ellagic acid by human gut microbiota. J Agric Food Chem. 2013; 61:8797-8806.
20. Maini Rekdal V, Nol Bernadino P, Luescher MU, Kiamehr S, Le C, Bisanz JE, Turnbaugh PJ, Bess EN, Balskus EP. A widely distributed metalloenzyme class enables gut microbial metabolism of host- and diet-derived catechols. eLife. 2020; 9:e50845.
21. Lederer E. Chemistry and biochemistry of some mammalian secretions and excretions. J Chem Soc. 1949; 2115-2125.
22. Gonzalez-Sarrias A, Gimenez-Bastida JA, Nunez-Sanchez MA, Larrosa M, Garcia-Conesa MT, Tomas-Barberan FA, Espin JC. Phase-II metabolism limits the antiproliferative activity of urolithins in human colon cancer cells. Eur J Nutr. 2014; 53:853-864.
23. Kujawska M, Jourdes M, Witucki L, Karazniewicz-Lada M, Szulc M, Gorska A, Mikolajczak PL, Teissedre PL, Jodynis-Liebert J. Pomegranate juice ameliorates dopamine release and behavioral deficits in a rat model of Parkinson's disease. Brain Sci. 2021; 11:1127.
24. Tomas-Barberan FA, Selma MV, Espin JC. Interactions of gut microbiota with dietary polyphenols and consequences to human health. Curr Opin Clin Nutr Metab Care. 2016; 19:471-476.
25. Garcia-Villalba R, Selma MV, Espin JC, Tomas-Barberan FA. Identification of novel urolithin metabolites in human feces and urine after the intake of a pomegranate extract. J Agric Food Chem. 2019; 67:11099-11107.
26. Garcia-Villalba R, Espin JC, Tomas-Barberan FA. Chromatographic and spectroscopic characterization of urolithins for their determination in biological samples after the intake of foods containing ellagitannins and ellagic acid. J Chromatogr A. 2016; 1428:162-175.
27. Tomas-Barberan FA, Garcia-Villalba R, Gonzalez-Sarrias A, Selma MV, Espin JC. Ellagic acid metabolism by human gut microbiota: consistent observation of three urolithin phenotypes in intervention trials, independent of food source, age, and health status. J Agric Food Chem. 2014; 62:6535-6538.
28. Espin JC, Larrosa M, Garcia-Conesa MT, Tomas-Barberan F. Biological significance of urolithins, the gut microbial ellagic acid-derived metabolites: the evidence so far. Evid Based Complement Alternat Med. 2013; 2013:270418.
29. Cortes-Martin A, Garcia-Villalba R, Gonzalez-Sarrias A, Romo-Vaquero M, Loria-Kohen V, Ramirez-de-Molina A, Tomas-Barberan FA, Selma MV, Espin JC. The gut microbiota urolithin metabotypes revisited: the human metabolism of ellagic acid is mainly determined by aging. Food Funct. 2018; 9:4100-4106.
30. Romo-Vaquero M, Cortes-Martin A, Loria-Kohen V, Ramirez-de-Molina A, Garcia-Mantrana I, Collado

MC, Espin JC, Selma MV. Deciphering the human gut microbiome of urolithin metabotypes: association with enterotypes and potential cardiometabolic health implications. Mol Nutr Food Res. 2019; 63:e1800958.
31. Cerda B, Tomas-Barberan FA, Espin JC. Metabolism of antioxidant and chemopreventive ellagitannins from strawberries, raspberries, walnuts, and oak-aged wine in humans: identification of biomarkers and individual variability. J Agric Food Chem. 2005; 53:227-235.
32. Mertens-Talcott SU, Jilma-Stohlawetz P, Rios J, Hingorani L, Derendorf H. Absorption, metabolism, and antioxidant effects of pomegranate (*Punica granatum* L.) polyphenols after ingestion of a standardized extract in healthy human volunteers. J Agric Food Chem. 2006; 54:8956-8961.
33. Cerda B, Periago P, Espin JC, Tomas-Barberan FA. Identification of urolithin A as a metabolite produced by human colon microflora from ellagic acid and related compounds. J Agri Food Chem. 2005; 53:5571-5576.
34. Selma MV, Beltran D, Luna MC, Romo-Vaquero M, Garcia-Villalba R, Mira A, Espin JC, Tomas-Barberan FA. Isolation of human intestinal bacteria capable of producing the bioactive metabolite isourolithin A from ellagic acid. Front Microbiol. 2017; 8:1521.
35. Martinez-Blanch JF, Ramon D, Beltran D, Romo-Vaquero M, Garcia-Villalba R, Espin JC, Tomas-Barberan FA, Codoner FM, Selma MV. Complete genome sequence of the new urolithin-producing bacterium *Gordonibacter urolithinfaciens* DSM 27213(T). Genome Announc. 2017; 5:e01120-01117.
36. Selma MV, Tomas-Barberan FA, Beltran D, Garcia-Villalba R, Espin JC. *Gordonibacter urolithinfaciens* sp. nov., a urolithin-producing bacterium isolated from the human gut. Int J Syst Evol Microbiol. 2014; 64:2346-2352.
37. Garcia-Villalba R, Beltran D, Frutos MD, Selma MV, Espin JC, Tomas-Barberan FA. Metabolism of different dietary phenolic compounds by the urolithin-producing human-gut bacteria *Gordonibacter urolithinfaciens* and *Ellagibacter isourolithinifaciens*. Food Funct. 2020; 11:7012-7022.
38. Gaya P, Peiroten A, Medina M, Alvarez I, Landete JM. *Bifidobacterium pseudocatenulatum* INIA P815: The first bacterium able to produce urolithins A and B from ellagic acid. J Funct Foods. 2018; 45:95-99.
39. Amrane S, Raoult D, Lagier JC. Metagenomics, culturomics, and the human gut microbiota. Expert Rev Anti Infect Ther. 2018; 16:373-375.
40. Garcia-Villalba R, Gimenez-Bastida JA, Cortes-Martin A, Avila-Galvez MA, Tomas-Barberan FA, Selma MV, Espin JC, Gonzalez-Sarrias A. Urolithins: a comprehensive update on their metabolism, bioactivity, and associated gut microbiota. Mol Nutr Food Res. 2022; e2101019.
41. Toney AM, Fox D, Chaidez V, Ramer-Tait AE, Chung S. Immunomodulatory role of urolithin A on metabolic diseases. Biomedicines. 2021; 9:192.
42. Zhang M, Cui S, Mao B, Zhang Q, Zhao J, Zhang H, Tang X, Chen W. Ellagic acid and intestinal microflora metabolite urolithin A: A review on its sources, metabolic distribution, health benefits, and biotransformation. Crit Rev Food Sci Nutr. 2022.
43. Chen L, Wang D, Garmaeva S, Kurilshikov A, Vich Vila A, Gacesa R, Sinha T, Lifelines Cohort S, Segal E, Weersma RK, Wijmenga C, Zhernakova A, Fu J. The long-term genetic stability and individual specificity of the human gut microbiome. Cell. 2021; 184:2302-2315 e2312.
44. Gimenez-Bastida JA, Truchado P, Larrosa M, Espin JC, Tomas-Barberan FA, Allende A, Garcia-Conesa MT. Urolithins, ellagitannin metabolites produced by colon microbiota, inhibit Quorum sensing in *Yersinia enterocolitica*: Phenotypic response and associated molecular changes. Food Chem. 2012; 132:1465-1474.
45. Lu C, Shen Y, Li T. Application of 3,4,8,9,10-pentahydroxydibenzo [b, d] pyran-6-one in the preparation of antibiotics. In: China Intellectual Property Office, 2019; pp.1.
46. Diop EHA, Queiroz EF, Marcourt L, Kicka S, Rudaz S, Diop T, Soldati T, Wolfender JL. Antimycobacterial activity in a single-cell infection assay of ellagitannins from *Combretum aculeatum* and their bioavailable metabolites. J Ethnopharmacol. 2019; 238:111832.
47. Cheng G, Hao H, Xie S, Wang X, Dai M, Huang L, Yuan Z. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Front in Microbiol. 2014; 5:217.
48. Larrosa M, Gonzalez-Sarrias A, Yanez-Gascon MJ, Selma MV, Azorin-Ortuno M, Toti S, Tomas-Barberan F, Dolara P, Espin JC. Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. J Nutr Biochem. 2010; 21:717-725.
49. Garcia-Villalba R, Vissenaekens H, Pitart J, Romo-Vaquero M, Espin JC, Grootaert C, Selma MV, Raes K, Smagghe G, Possemiers S, Van Camp J, Tomas-Barberan FA. Gastrointestinal simulation model TWIN-SHIME shows differences between human urolithin-metabotypes in gut microbiota composition, pomegranate polyphenol metabolism, and transport along the intestinal tract. J Agr Food Chem. 2017; 65:5480-5493.
50. Abdulrahman AO, Alzubaidi MY, Nadeem MS, Khan JA, Rather IA, Khan MI. Effects of urolithins on obesity-associated gut dysbiosis in rats fed on a high-fat diet. Int J Food Sci Nutr. 2021; 72:923-934.
51. Zhao R, Long X, Yang J, Du L, Zhang X, Li J, Hou C. Pomegranate peel polyphenols reduce chronic low-grade inflammatory responses by modulating gut microbiota and decreasing colonic tissue damage in rats fed a high-fat diet. Food Funct. 2019; 10:8273-8285.
52. Li Z, Henning SM, Lee RP, Lu QY, Summanen PH, Thames G, Corbett K, Downes J, Tseng CH, Finegold SM, Heber D. Pomegranate extract induces ellagitannin metabolite formation and changes stool microbiota in healthy volunteers. Food Funct. 2015; 6:2487-2495.
53. Xian YB, Fan R, Shao J, Toney AM, Chung S, Ramer-Tait AE. Polyphenolic fractions isolated from red raspberry whole fruit, pulp, and seed differentially alter the gut microbiota of mice with diet-induced obesity. J Funct Foods. 2021; 76:104288.
54. Hering NA, Luettig J, Jebautzke B, Schulzke JD, Rosenthal R. The punicalagin metabolites ellagic acid and urolithin A exert different strengthening and anti-Inflammatory effects on tight junction-mediated intestinal barrier function in vitro. Front Pharmacol. 2021; 12:610164.
55. Singh R, Chandrashekharappa S, Bodduluri SR, *et al.* Enhancement of the gut barrier integrity by a microbial metabolite through the Nrf2 pathway. Nat Commun. 2019; 10:89.
56. Kawabata K, Yoshioka Y, Terao J. Role of intestinal microbiota in the bioavailability and physiological functions of dietary polyphenols. Molecules. 2019; 24:370.

57. Zhao W, Shi F, Guo Z, Zhao J, Song X, Yang H. Metabolite of ellagitannins, urolithin A induces autophagy and inhibits metastasis in human sw620 colorectal cancer cells. Mol Carcinog. 2018; 57:193-200.
58. Kojadinovic M, Arsic A, Petovic-Oggiano G, Gavrovic-Jankulovic M, Glibetic M, Popovic M. Effect of urolithins on oxidative stress of colorectal adenocarcinomacells-Caco-2. Int J Food Sci Nutr. 2017; 68:952-959.
59. Norden E, Heiss EH. Urolithin A gains in antiproliferative capacity by reducing the glycolytic potential *via* the p53/TIGAR axis in colon cancer cells. Carcinogenesis. 2019; 40:93-101.
60. Gonzalez-Sarrias A, Tome-Carneiro J, Bellesia A, Tomas-Barberan FA, Espin JC. The ellagic acid-derived gut microbiota metabolite, urolithin A, potentiates the anticancer effects of 5-fluorouracil chemotherapy on human colon cancer cells. Food Funct. 2015; 6:1460-1469.
61. Larrosa M, Gonzalez-Sarrias A, Yanez-Gascon MJ, Selma MV, Azorin-Ortuno M, Toti S, Tomas-Barberan F, Dolara P, Espin JC. Anti-inflammatory properties of a pomegranate extract and its metabolite urolithin-A in a colitis rat model and the effect of colon inflammation on phenolic metabolism. J Nutr Biochem. 2010; 21:717-725.
62. Singh R, Chandrashekharappa S, Bodduluri SR, *et al.* Enhancement of the gut barrier integrity by a microbial metabolite through the Nrf2 pathway. Nat Commu. 2019; 10:89.
63. Gimenez-Bastida JA, Larrosa M, Gonzalez-Sarrias A, Tomas-Barberan F, Espin JC, Garcia-Conesa MT. Intestinal ellagitannin metabolites ameliorate cytokine-induced inflammation and associated molecular markers in human colon fibroblasts. J Agric Food Chem. 2012; 60:8866-8876.
64. Heilman J, Andreux P, Tran N, Rinsch C, Blanco-Bose W. Safety assessment of urolithin A, a metabolite produced by the human gut microbiota upon dietary intake of plant derived ellagitannins and ellagic acid. Food Chem Toxicol. 2017; 108:289-297.
65. Andreux PA, Blanco-Bose W, Ryu D, Burdet F, Ibberson M, Aebischer P, Auwerx J, Singh A, Rinsch C. The mitophagy activator urolithin A is safe and induces a molecular signature of improved mitochondrial and cellular health in humans. Nat Metab. 2019; 1:595-603.
66. Moszak M, Szulinska M, Bogdanski P. You Are what you eat-the relationship between diet, microbiota, and metabolic disorders-a review. Nutrients. 2020; 12:1096.

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Mini-Review

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Potential applications of Chinese herbal medicines with hemostatic properties

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SUMMARY Various herbal medicines with hemostatic properties have been applied for centuries to accelerate hemostasis and control bleeding. However, the mechanisms of action and active constituents remain unknown. This report provides an overview of current clinical hemostatic agents and their disadvantages, then focuses on the clinical value of Chinese herbal medicines with unique hemostatic features that modern medicines lack. A comprehensive review of hemostatic agents derived from Chinese herbal medicines and their potential medical applications is also presented.

Keywords blood coagulation, hemorrhage, Pollen Typhae, mechanism

1. Introduction

Seepage from ruptured blood vessels results in the loss of valuable blood from the circulatory system. When blood flows out of damaged vessels, local blood vessels contract to suppress bleeding and platelets simultaneously aggregate to form thrombus that blocks damaged sites. Fibrin generated through the activation of a series of blood coagulation factors, strengthens platelet aggregation to form stable thrombus at wounds to stop bleeding (hemostasis) (Figure 1) (*1,2*). Bleeding is not only caused by injury, but also by abnormalities among clotting factors or platelets (*3*). An excessive loss of blood by bleeding can lead to death. Thus, hemostasis is critical to maintain healthy biological activities, prevent blood loss, and function as a physiological self-defense mechanism that impedes bacterial and viral invasion (*4*). This review provides an overview of current clinical hemostatic agents and their disadvantages, then summarizes the features of Chinese herbal hemostatic agents and their potential applications to contemporary and future medicine.

2. Hemostatic system and hemostasis

Hemostasis is achieved when procoagulants and anticoagulants are balanced (*2,3*). The human hemostatic system comprises platelets, coagulation and fibrinolytic factors, vessels, and endothelial cells that line the insides of vessels (*4*). Local blood vessels constrict to contain bleeding from damaged vessels and activated platelets rapidly aggregate to form soft plugs at sites of damage. This process is primary hemostasis, which starts immediately after platelets adhere to the subendothelial matrix (*5*). After primary hemostasis, coagulation factors on the surface of the phospholipid bilayer membranes of activated platelets convert fibrinogen through a proteolytic coagulation cascade into insoluble cross-linked fibrin, which forms a mesh that is incorporated into and around a soft aggregate plug. The mesh strengthens and stabilizes blood clots during the process of secondary hemostasis (*5*). Fibrinolysis also plays a significant role in hemostasis by dissolving blood clots formed during wound healing after hemostasis (*6*).

3. Characteristics of clinical hemostatic agents and issues

Table 1 shows the classification of clinical hemostatic agents. Carbazochrome and adrenochrome are hemostatic capillary stabilizers that treat hemorrhage by improving capillary fragility (*7-10*). These agents might be applicable to stopping extravascular blood leakage. Carbazochrome is clinically administered orally and by intravenous injection. Although details of the mechanism(s) remain obscure, this agent exerts hemostatic effects regardless of the impact on the coagulation and fibrinolytic systems and platelet activity. Administering these agents orally can control purpura, as well as bleeds from the skin and mucous membranes (*9,10*). However, the effects are not potent, and this agent is not applicable to bleeds due to tissue wounds.

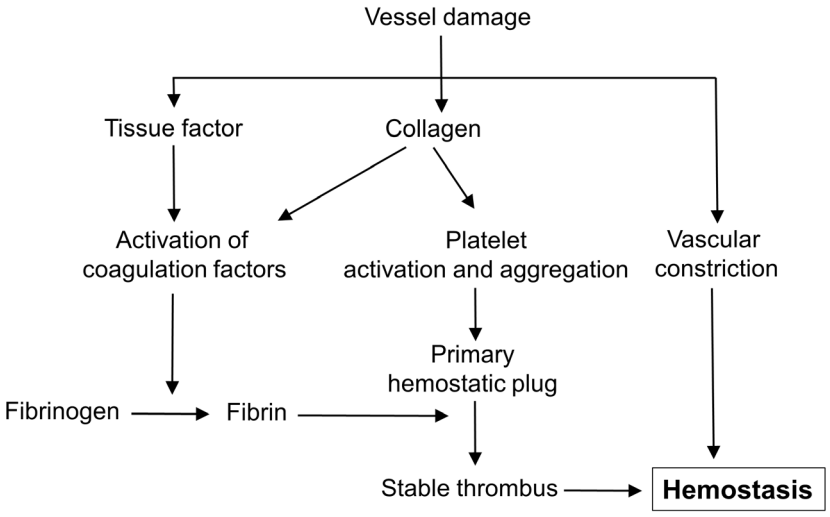

Figure 1. Overview of hemostasis and its components.

Table 1. Classification of hemostatic agents

Mode of action	Hemostatic agent(s)
Capillary stabilizers	Carbazochrome and adrenochrome
Coagulation accelerator	Hemocoagulase
Anti-fibrinolytic	Tranexamic acid
Topical	Thrombin, collagen, oxidized regenerated cellulose, liquid fibrin glue, adrenaline and water-insoluble sponge prepared from gelatin
Vasoconstrictor	Epinephrine (adrenaline)

The enzyme hemocoagulase (reptilase) is a blood coagulation accelerator purified from snake venom that has been recognized for ~18 centuries (*11*). Its activity is associated with batroxobin, a defibrinogenating hemostatic agent derived from *Bothrops atrox moojeni* (pit viper) venom (*12*). Hemocoagulase works by causing the cleavage of fibrinogen into fibrin monomers which eventually leads to the formation of fibrin polymers (*13*). The characteristics and nature of batroxobin differ from those of thrombin. Batroxobin remains systemically active as well as at local sites of application in the absence of important clotting factors. Snake venom has evolved to contain several enzymes, including proteases, several of which specifically act on the blood coagulation system (*14,15*). Although much effort has been directed towards using these enzymes to regulate the blood coagulation system, hemocoagulase seems to remain the sole clinically applied venom-derived agent. Hemocoagulase is used to control pulmonary hemorrhage, hemorrhage in alcoholic cirrhotic liver disease, oral, genital, dental bleeds, and bleeds from wounds and is administered intramuscularly or intravenously to control renal hemorrhage (*16-18*). Biopharmaceutial hemocoagulase is used many bleeding situations, but it cannot be orally administered due to high risk of anaphylaxis-like symptoms.

Tranexamic and ε-aminocaproic acids are anti-fibrinolytic agents that inhibit plasmin, a protease that dissolves fibrin clots and has antithrombotic activity. These agents to the lysine-binding site of plasminogen, thus inhibiting its binding to fibrin. They also inhibit plasminogen activator action on fibrin, thus preventing fibrin degradation and exerting hemostatic effects (*19*). Tranexamic acid is a prevalent hemostatic agent that is clinically administered as an internal medicine and by injection (*20,21*). It is applied when bleeding tendencies might be associated with systemic hyperfibrinolysis (due to leukemia, aplastic anemia, purpura for example, and abnormal peri- and post-operative bleeding), and abnormal bleeding possibly associated with focal hyperfibrinolysis (epistaxis pulmonary, genital, and renal bleeds, as well as abnormal peri- and post-operative bleeds) (*22*). Tranexamic acid can be delivered orally, but its therapeutic value is limited for bleeding tendencies associated with systemic hyperfibrinolysis and abnormal bleeds that might be associated with hyperfibrinolysis,.

Topical hemostatic agents applied to the skin can stop bleeding from small blood vessels and capillaries. Thrombin, collagen microfibers, oxidized regenerated cellulose, liquid fibrin glue, adrenaline and water-insoluble sponges prepared from gelatin are all functional topical agents (*23-26*). Thrombin purified from human or bovine blood dissolved in physiological saline or powdered, can be sprayed onto bleeding

Table 2. Hemostatic herbal medicine and active components (Kosuge *et al*.)

Crude drug	Origin	Active compounds	*Ref.*
地楡 (Chiyu)	*Sanguisorba officinalis* L.; roots	3,3',4-Tri-O-methylellagic acid	*42*
田七 (Denshichi)	*Panax pseudo-ginseng Wall.* var. Notoginseng (BURKILL) HOO and TSENG; radix	Dencitine (β-N-oxalo-L-α-b-diaminopropionic acid)	*41*
蒲黄 (Hoou)	*Typha lactifolia* L.; pollen	Isorhamnetin 3-rutinoside-7-rhamnoside, unknown,	*40*
槐花 (Kaika)	*Sophora japonica* L.; ground buds	Quercetin	*37*
蓮房 (Rembo)	*Nelumbo nucifera* GAERT.; ground toruses	Quercetin	*39*
小連翔 (Shorengyo)	*Hypericum erectum* Thunb.; ground herb	Wedelolactone, Demethylwedelolactone	*43*
側柏葉 (Sokuhakuyo)	*Biota orientalis* (L.) ENDL.; leaves and branches	Quercitrin	*44*
大薊 (Taikei)	*Cirsium japonicum* DC.; ground herb	Pectolinarin	*38*

sites (*23*). Microfibrillar collagen is a hemostatic agent that maintains the three-dimensional structure of bovine dermal collagen, exerts powerful platelet aggregation activity, and adheres tightly to wounds (*27*). Oxidized cellulose is a common surgical hemostat that seems to work *via* caustic action by decreasing the pH and generating an artificial clot (*28*). Fibrin glue is a mixture of fibrinogen, factor XIII, thrombin, and calcium that mimics the last stages of the clotting cascade and forms fibrin clots (*29*). These topical agents achieve hemostasis by generating adhesive cross-linked fibrin in wounds. Epinephrine (adrenaline), a hormone secreted by the medulla of the adrenal glands, works as a topical hemostatic agent by exerting vasoconstrictor effects that stop bleeding (*30,31*).

4. Chinese herbal medicines

Various herbs have a long history of ethno-medical application to control bleeds and are popular worldwide, so they might be valuable sources of new hemostatic drugs (*32*). Here, we focused on the hemostatic potential of Chinese herbal medicines. Several Chinese herbs with hemostatic action have been applied for many years. Kosuge *et al*. rigorously evaluated the active components in 12 Chinese hemostatic herbal medicines during the 1980s and analyzed their activities using mouse tail bleeding assays *in vivo* (*33,34*). Tail bleeding reflects platelet activity (*35*). Kosuge *et al*. identified hemostatic activities in Denshichi, *Panax pseudo-ginseng* Wall. var. *notoginseng* (Burkill) Hoo & Tseng; Chiyu, *Sanguisorba officinalis* L; Kanrenso, *Eclipta prostrata* L; Seikon, *Rubia cordifolia* L; Sokuhakuyo, *Biota orientalis* (L.) ENDL; Renbo and Gusetsu, *Nelumbo nucifera* Gaertn (*36*). Table 2 shows the hemostatic components in these herbal medicines and their active compounds (*37-44*) (Table 2). Although Kosuge *et al*. isolated and determined the structures of these compounds, they did not elucidate their hemostatic mechanisms and sites of action.

Among these Chinese herbal medicines, Denshichi is renowned for treating hemorrhagic diseases and it might help to stop uncontrollable bleeds. For example, the livers of patients with hepatitis produce less blood coagulation factors (*45*), and patients medicated with antithrombotic agents have decreased hemostatic activity (*46*); hence, bleeding can be difficult to stop after tooth extraction. Denshichi hemostasis has been applied in dental practice in Japan (*47,48*). Denshichi notably also has many other pharmacological activities in addition to hemostatic activity (*49-51*). Sun *et al*. subsequently reported that terpene glycosides in Chiyu exert hemostatic activity by inhibiting α2-plasmin inhibitor, an inhibitor of fibrinolysis (*52*), but other herbal medicines have not yet been reported.

5. Hemostatic activity of Pollen Typhae

Several cattail species in the family Typhaceae, including *Typha angustifolia* L. (narrow leaf cattail, lesser bulrush, or jambu), *T. latifolia* L. (common cattail), and *T. orientalis* Preel (broadleaf cumbungi or raupo) are perennial herbaceous plants that inhabit North and South America, Europe, Asia, and Africa (*53*). Dried pollens of a series of cattails (Pollen Typhae) comprise an established Chinese herbal medicine used to treat internal and external hemorrhagic conditions (*32*). Hematuria, blood discharge from the anus, metrorrhagia and erythrocyturia have been treated with oral Pollen Typhae for centuries. Pollen Typhae has also served as a hemostyptic agent to treat excoriations or cuts on the skin surface (*54*). Pollen Typhae is believed to work against bleeding and has been administered orally and topically for centuries. Although not fully understood, more is known about the mechanism of action and the active substance in Pollen Typhae than any other herbal medicine. The hemostatic activity of Pollen Typhae extract (PTE) has been assessed in mice *in vivo* and in human blood *in vitro* (*55*). The effects of PTE on the extrinsic and intrinsic coagulation pathways have been assessed by measuring prothrombin (PT), activated partial thrombin (APTT) and plasma recalcification times (*55,56*). Pollen Typhae extract significantly and dose-dependently decreases the amount of time required to form clots in PT and APTT tests and in recalcified plasma (*55*). These findings suggested that PTE promotes the coagulation system in human plasma. Indeed, negatively charged polysaccharide in PTE activates factor XII, which is a proenzyme in the intrinsic coagulation pathway that

accelerates coagulation (*2*). Activation of the intrinsic coagulation pathway by acidic polysaccharide in PTE partly contributes to the hemostatic activity of topical Pollen Typhae. The time-dependent activation of factor XII to factor XIIa by acidic polysaccharide from PTE has shown that Pollen Typhae has potential as a rapid hemostyptic (*56*).

The oral effects of PTE have also been assessed in mouse tail-bleeding models. Oral PTE significantly decreases the duration of tail bleeding compared with control mice (*55*).Pollen Typhae extract with and without an acidic polysaccharide that might contribute to oral hemostatic activity was administered to mice. Hemostatic activity persisted in PTE without acidic polysaccharide (*55*), indicating that compounds other than acidic polysaccharides are responsible for the hemostatic properties of orally administered Pollen Typhae. Isorhamnetin, a flavonoid extracted from Pollen Typhae, shortens the duration of tail bleeds in mice when injected intraperitoneally (*i.p.*) (*46*). Therefore, the compound in oral Pollen Typhae responsible for the reduced tail bleeding duration is likely to be isorhamnetin or a derivative (*55*). Topical PTE also has hemostatic effects in the mouse tail bleeding model. Blood loss measured in the tips of mouse tails immersed in PTE revealed significantly reduced blood loss (*55*).

Although the detailed mechanisms and components involved in the hemostatic action require validation, the hemostatic action of topical and oral Pollen Typhae have been confirmed *in vivo* and *in vitro*. The hemostatic activity of Pollen Typhae applied to wounds is due to activation of the intrinsic coagulation pathway, whereas the oral effects are due to platelet activation and/or vasoconstriction.

Biocompatible carbon dots (CDs) are quasi-spherical charcoal nanoparticles with high stability and low toxicity, the efficacy and safety of which are under evaluation. Fusing traditional Chinese medicine (TCM)-based CDs have attracted considerable interest to treat common diseases (*57*) and they have hemostatic properties (*58,59*). Hemorrhagic states have been treated with charcoal-processed products of Pollen Typhae for many years in China. The hemostatic bioactivity of CDs derived from Pollen Typhae Carbonisata have been identified and their pharmacodynamics have been investigated (*60*). This provides new insights into potential biomedical applications of Pollen Typhae to hemostasis for future drug discovery.

6. Discoveries of novel hemostatic agents from Chinese herbal medicines

Many plants and plant-derived agents have hemostatic activity and perhaps even more novel hemostatic substances in Chinese medical herbs await discovery. We screened extracts derived from 114 plant species in a library of common herbal crude extracts and identified potential hemostatic agents by measuring blood coagulation activities (*61*). Seventeen herbal extracts induced extrinsic blood coagulation. Among them, Goboushi and Gaiyou activated coagulation factor XII, which is the key enzyme in the intrinsic blood coagulation pathway and promoted blood coagulation. Because we found the activities of these plants using the procoagulant effect *in vitro* as an index, an actual hemostatic effect *in vivo* was not apparent. Nonetheless, these crude extracts remain novel hemostatic candidates from known medicinal plants.

Ebrahimi *et al*. comprehensively reviewed the ethno-pharmaceutical applications of medicinal plants or their isolates that stimulate the hemostatic process (62). Although that report included non-Chinese herbal medicine, the authors found *via* a literature search that several plants could be considered as sources of new herbal hemostatic medicines.

7. Conclusions

Many hemostatic agents are clinically applied depending on the situation, but they do not always meet medical needs. Some herbal medicines with hemostatic properties have characteristics that complement modern medicines. Pollen Typhae is of interest because it can be applied internally and externally. The mechanisms of the effects of orally-administered Pollen Typhae obviously differ from those of clinical oral hemostatic agents such as capillary stabilizers and anti-fibrinolytic agents, but its hemostatic effects are clearly comparable.

Although the mechanisms of action and active constituents of plant-derived hemostatic compounds await further investigation, many plants contain natural compounds that have been applied for centuries to control bleeding. New hemostatic sources in Chinese herbal medicines that might be discovered in the future should become clinically valuable under various conditions after their mechanisms of action and active principles are elucidated.

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References

1. Rasche H. Haemostasis and thrombosis: an overview. Eur Heart J Suppl. 2001; 3:Q3-Q7.
2. Stassen JM, Arnout J, Deckmyn H. The hemostatic system. Curr Med Chem. 2004; 11:2245-2260.
3. Triplett DA. Coagulation and bleeding disorders: review and update. Clin Chem. 2000; 46:1260-1269.
4. Antoniak S. The coagulation system in host defense. Res Pract Thromb Haemost. 2018; 2:549-557.
5. Gale AJ. Continuing education course #2: current

understanding of hemostasis. Toxicol Pathol. 2011; 39:273-280.
6. Longstaff C, Kolev K. Basic mechanisms and regulation of fibrinolysis. J Thromb Haemost. 2015; 13 (Suppl 1):S98-105.
7. Matsumoto Y, Hayashi T, Hayakawa Y, Shinbo M, Niiya K, Sakuragawa N. Carbazochrome sodium sulphonate (AC-17) decreases the accumulation of tissue-type plasminogen activator in culture medium of human umbilical vein endothelial cells. Blood Coagul Fibrinolysis. 1995; 6:233-238.
8. Hayakawa M, Gando S. Systemic and local hemostatic agents. Jpn J Thromb Hemost. 2009; 20:278-280.
9. Riddle AC Jr. Adrenosem salicylate: a systemic hemostatic. Oral Surg Oral Med Oral Pathol. 1955; 8:617-620.
10. Perkins RE. A clinical investigation of adrenochrome monosemicarbazone sodium salicylate. Oral Surg Oral Med Oral Pathol. 1957; 10:230-236.
11. Serrano SM. The long road of research on snake venom serine proteinases. Toxicon. 2013; 62:19-26.
12. Madhu CP, Naik D, Vishak Nair TU. Effect of topical hemocoagulase therapy in wound healing. Int J Surg Sci. 2019; 3:151-156.
13. Shenoy AK, Ramesh KV, Chowta MN, Adhikari PM, Rathnakar UP. Effects of botropase on clotting factors in healthy human volunteers. Perspect Clin Res. 2014; 5:71-4.
14. Estevao-Costa MI, Sanz-Soler R, Johanningmeier B, Eble JA. Snake venom components in medicine: From the symbolic rod of Asclepius to tangible medical research and application. Int J Biochem Cell Biol. 2018; 104:94-113.
15. Matsui T, Fujimura Y, Titani, K. Snake venom proteases affecting hemostasis and thrombosis. Biochim Biophys Acta. 2000; 1477:146-156.
16. Lodha A, Kamaluddeen M, Akierman A, Amin H. Role of hemocoagulase in pulmonary hemorrhage in preterm infants: A systematic review. Indian J Pediatr. 2011; 78:838-44.
17. Ragni MV, Lewis JH, Spero JA, Hasiba U. Bleeding and coagulation abnormalities in alcoholic cirrhotic liver disease. Alcohol Clin Exp Res. 1982; 6:267-274.
18. Gupta G, Muthusekhar MR, Kumar SP. Efficacy of hemocoagulase as a topical hemostatic agent after dental extractions: A systematic review. Cureus. 2018; 10:e2398.
19. Nilsson IM. Clinical pharmacology of aminocaproic and tranexamic acids. J Clin Pathol Suppl (R Coll Pathol). 1980; 14:41-7.
20. Tengborn L, Blombäck M, Berntorp E. Tranexamic acid - an old drug still going strong and making a revival. Thromb Res. 2015; 135:231-242.
21. Ng W, Jerath A, Wasowicz M. Tranexamic acid: a clinical review. Anaesthesiol Intensive Ther. 2015; 47:339-350.
22. McCormack PL. Tranexamic acid: a review of its use in the treatment of hyperfibrinolysis. Drug. 2012; 72:585-617.
23. Wasilko SM, Quinlan NJ, Shafritz AB. Topical hemostatic agents and their role in upper extremity surgery. J Hand Surg Am. 2015; 40:602-604.
24. Groenewold MD, Gribnau AJ, Ubbink DT. Topical haemostatic agents for skin wounds: a systematic review. BMC Surg. 2011; 11:15.
25. Seyednejad H, Imani M, Jamieson T, Seifalian AM. Topical haemostatic agents. Br J Surg. 2008; 95:1197-225.
26. Palm MD, Altman JS. Topical hemostatic agents: A review. Dermatol Surg. 2008; 34:431-445.
27. Zucker WH, Mason RG. Ultrastructural aspects of interactions of platelets with microcrystalline collagen. Am J Pathol. 1976; 82:129-142.
28. Schonauer C, Tessitore E, Barbagallo G, Albanese V, Moraci A. The use of local agents: bone wax, gelatin, collagen, oxidized cellulose. Eur Spine J. 2004; 13 (Suppl 1):S89-S96.
29. Thompson DF, Letassy NA, Thompson GD. Fibrin glue: a review of its preparation, efficacy, and adverse effects as a topical hemostat. Drug Intell Clin Pharm. 22:946-952.
30. Orlandi RR, Warrier S, Sato S, Han JK. Concentrated topical epinephrine is safe in endoscopic sinus surgery. Am J Rhinol Allergy. 2010; 24:140-142.
31. Nesbitt NB, Noller MW, Watson NL, Soneru CP, McCoul ED, Riley CA. Outcomes and complications with topical epinephrine in endoscopic sinus surgery: A systematic review and meta-analysis. Otolaryngol Head Neck Surg. 2020; 163:410-417.
32. Hung HY, Wu TS. Recent progress on the traditional Chinese medicines that regulate the blood. J Food Drug Anal. 2016; 24:221-238.
33. Tajima TO, Ogo T, Miyao K. The hemostatic effects of Menaquinone-4 (K2) and other drugs on hemorrhage caused by hemorrhagenic agents with different mechanism in rats. Folia Pharmacol Jpn. 1971; 46:478-485.
34. Liu Y, Jennings NL, Dart AM, Du XJ. Standardizing a simpler, more sensitive and accurate tail bleeding assay in mice. World J Exp Med. 2012; 2:30-36.
35. Harker LA, Slichter SJ. The bleeding time as a screening test for evaluation of platelet function. N Engl J Med. 1972; 287:155-159.
36. Kosuge T, Yokota M, Yoshida M, Ochiai A. Studies on antihemorrhagic principles in the crude drugs for hemostatics. I. On hemostatic activities of the crude drugs for hemostatics. Yakugaku Zasshi. 1981; 101:501-503.
37. Ishida H, Umino T, Tsuji K, Kosuge T. Studies on antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. VI. On the antihemorrhagic principle in *Sophora japonica* L. Chem Pharm Bull. 1987; 35:857-860.
38. Ishida H, Umino T, Tsuji K, Kosuge T. Studies on antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. VII. On the antihemorrhagic principle in *Cirsium japonicum* DC. Chem Pharm Bull. 1987; 35:861-864.
39. Ishida H, Umino T, Tsuji K, Kosuge, T. Studies on the antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. VIII. On the antihemorrhagic principle in Nelumbins Receptaculum. Chem Pharm Bull. 1988; 36:585-4587.
40. Ishida H, Umino T, Tsuji K, Kosuge T. Studies on the antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. IX. On the antihemorrhagic principles in *Typha lactifolia* L. Chem Pharm Bull. 1988; 36:4414-4420.
41. Kosuge T, Yokota M, Ochiai A. Studies on antihemorrhagic principles in the crude drugs for hemostatics. II. On antihemorrhagic principle in Sanchi Ginseng Radix. Yakugaku Zasshi. 1981; 101:629-632.
42. Kosuge T, Ishida H, Yokota M, Yoshida M. Studies on antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. III. On the antihemorrhagic principle in *Sanguisorba officinalis* L. Chem Pharm Bull. 1984; 32:4478-4481.

43. Kosuge T, Ishida H, Satoh T. Studies on antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. IV. On antihemorrhagic principles in *Hypericum erectum* Thunb. Chem Pharm Bull. 1985; 33:202-205.
44. Kosuge T, Ishida H, Satoh T. Studies on antihemorrhagic substances in herbs classified as hemostatics in Chinese medicine. V. On antihemorrhagic principle in *Biota orientalis* (L.) ENDL. Chem Pharm Bull. 1985; 33:06-209.
45. Allison MG, Shanholtz CB, Sachdeva A. Hematological issues in liver disease. Crit Care Clin. 2016; 32:385-396.
46. Scharf RE. Management of bleeding in patients using antithrombotic agents: Prediction, prevention, protection and problem-oriented intervention. Hamostaseologie. 2009; 29:388-398.
47. Tsunoda M, Fujiwara H, Saitoh M, Nagayama M. As for anticoagulated patients who succeeded in having adequate hemostasis by means of San-chi ginseng. J Jap Dent Soc Oriental Med. 2007; 26:23-27.
48. Tsunoda M, Fujiwara H, Saito M, Nagayama M. Three cases of hemostasis completed by applying *Panax notoginseng* (Burk.) F. H. Chen following invasive treatment. J Jap Dent Soc Oriental Med. 2008; 27:23-27.
49. Ng TB. Pharmacological activity of sanchi ginseng (*Panax notoginseng*). J Pharm Pharmacol. 2006; 58:1007-1019.
50. Wang T, Guo R, Zhou G, Zhou X, Kou Z, Sui F, Li C, Tang L, Wang Z. Traditional uses, botany, phytochemistry, pharmacology and toxicology of *Panax notoginseng* (Burk.) F.H. Chen: A review. J Ethnopharmacol. 2016; 188:234-258.
51. White CM, Fan C, Song J, Tsikouris JP, Chow M. An evaluation of the hemostatic effects of hydrophilic, alcohol, and lipophilic extracts of *notoginseng*. Pharmacotherapy. 2001; 21:773-777.
52. Sun W, Zhang ZL, Liu X, Zhang S, He L, Wang Z, Wang GS. Terpene glycosides from the roots of *Sanguisorba officinalis* L. and their hemostatic activities. Molecules. 2012; 17:7629-7636.
53. Smith SG. The cattails (Typha): interspecific ecological differences and problems of identification. Lake Reservoir Manag. 1986; 2:357-362.
54. Mitich LM. Common Cattail, *Typha latifolia* L. Weed Technol. 2000; 14:446-450.
55. Ohkura N, Tamura K, Tanaka A, Matsuda J, Atsumi G. Experimental study on the hemostatic activity of Pollen Typhae: a traditional folk medicine used by external and oral application. Blood Coagul Fibrinolysis. 2011; 22:631-636.
56. Ohkura N, Tauchi C, Nakayama A, Atsumi G. Pollen Typhae is a rapid hemostyptic. Blood Coagul Fibrinolysis. 2012; 23:254-255.
57. Li D, Xu KY, Zhao WP, Liu MF, Feng R, Li DQ, Bai J, Du WL. Chinese medicinal herb-derived carbon dots for common diseases: Efficacies and potential mechanisms. Front Pharmacol. 2022; 13:815479.
58. Liu X, Wang Y, Yan X, Zhang M, Zhang Y, Cheng J, Lu F, Qu H, Wang Q, Zhao Y. Novel Phellodendri Cortex (Huang Bo)-derived carbon dots and their hemostatic effect. Nanomedicine. 2018; 13:391-405.
59. Luo J, Zhang M, Cheng J, Wu S, Xiong W, Kong H, Zhao Y, Qu H. Hemostatic effect of novel carbon dots derived from *Cirsium setosum* Carbonisata. RSC Adv. 2018; 8:37707-37714.
60. Yan X, Zhao Y, Luo J, Xiong W, Liu X, Cheng J, Wang Y, Zhang M, Qu H. Hemostatic bioactivity of novel Pollen Typhae Carbonisata-derived carbon quantum dots. J Nanobiotechnology. 2017;15:60.
61. Ohkura N, Yokouchi H, Mimura M, Nakamura R, Atsumi G. Screening for hemostatic activities of popular Chinese medicinal herbs *in vitro*. J Intercult Ethnopharmacol. 2015; 4:19-23.
62. Ebrahimi F, Torbati M, Mahmoudi J, Valizadeh H. Medicinal plants as potential hemostatic agents. J Pharm Pharm Sci. 2020; 23:10-23.

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Original Article

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Evaluation of Japanese university students' perception of smoking, interest in quitting, and smoking behavior: An examination and public health challenges during the COVID-19 pandemic

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SUMMARY This study examined college students' perceptions of the association between smoking and novel coronavirus disease 2019 (COVID-19), changes in smoking behavior, and interest in quitting categorized by smoking device, to identify public health challenges. A questionnaire survey was conducted among 8,547 students in a Japanese university in March and April 2021. In response to "Awareness of the increased risk of COVID-19 infection due to smoking and the tendency to develop severe disease", current smokers (70.2%) were more aware of the risk than non-smokers (49.8%) ($p < 0.001$), with no significant difference according to smoking device ($p = 0.213$). "Interest in quitting smoking" ($p = 0.323$), and "Changes in smoking behavior during the COVID-19 pandemic" ($p = 0.146$) did not differ by smoking device. However, approximately 50% of the respondents answered that they were not interested in quitting smoking, while two-thirds reported that the number of cigarettes they smoked did not change during the pandemic. During the COVID-19 pandemic, college students were found to be less interested in quitting and not likely to change their smoking behavior, despite the knowledge of the increased risk of COVID-19 transmission and severity of disease from smoking, regardless of smoking device.

Keywords COVID-19 pandemic, college students, perceptions of smoking, interest in quitting, smoking behavior

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes novel coronavirus disease 2019 (COVID-19), was first reported in Wuhan, China, in December 2019 (*1*) and is responsible for a global pandemic. A previous study reported that college students in the United States showed increased stress and anxiety due to COVID-19, including fear and worry about their health, disruption of sleep patterns, and concerns about academic performance (*2*). Japanese university students may have similar concerns, as they may not be able to lead the student life they envisioned due to online lectures, restrictions on school entry, restrictions on clubs, and other activities.

The WHO estimates that there are currently about 1.1 billion smokers worldwide, and this is expected to increase to 1.3 billion worldwide by 2025 (*3*). The United Nations has established the Sustainable Development Goals (SDGs), particularly Goal 3, which is to "ensure healthy lives and promote well-being for all people of all ages" including "the implementation of the WHO Framework Convention on Tobacco Control be strengthened in all countries, as appropriate" (target 3.a). Smoking has been reported as a possible risk for COVID-19 infection (*4*). Furthermore, a study reported that patients with a history of smoking have a higher rate of rapid deterioration in health status during hospitalization than non-smokers (*5*).

Previous studies have reported that there was no noticeable change in the smoking status of college students during the pandemic (*6*). However, there are

no studies that have examined changes in smoking behavior by smoking devices, such as cigarettes or new types of cigarettes (heated cigarettes/electronic cigarettes). The use of new types of cigarettes, including electronic and heated cigarettes, is increasing rapidly in every country in the world (*7-9*). Youth smokers reportedly hold misconceptions such as how easy it is to quit using e-cigarettes and perceived benefits such as having more friends and looking cool (*10*). Therefore, it is important to clarify the characteristics of different smoking tools, including new types of cigarettes, in terms of the perception of harm, concern, and behavioral changes in smokers during the COVID-19 pandemic.

Therefore, we conducted a survey of smoking among university students in Japan during the COVID-19 pandemic. Its purpose was to compare and evaluate the perceptions of the association between smoking and COVID-19, interest in quitting, and changes in smoking behavior by smoking devices.

2. Methods

2.1. Subjects

From March to April 2021, a self-administered, anonymous, questionnaire was distributed to all 8,547 students from University A in Japan. Eligible students included undergraduate and graduate students. Responses were received from 8,117 students (95%). The valid response rate was 100%. The age of the students was 19.5 ± 2.87 years (mean ± SD). The survey was conducted between March and April 2021, which was between the third and fourth expansion periods of COVID-19 infection in Japan. At this time, other countries were taking measures to introduce lockdowns (city blockades), but there were no city blockades in Japan. The Japanese government had already lifted its declaration of a state of emergency, requesting the restriction of activities.

2.2. Survey items

For all subjects, responses were obtained for age, sex, and smoking history. To determine why current smokers started smoking, participants were able to check all that applied from the following: "Influence from friends, classmates, and older adults", "Curiosity", "Stress relief", "Influence from people at part-time jobs", and "My family smoked". Participants could select "Yes, I know" or "No, I did not know" about their awareness of the increased risk of COVID-19 infection and the increased severity of the disease due to smoking. Interest in quitting smoking was indicated as follows: "I would like to quit smoking now", "I am interested and plan to quit within a month", "I am interested but want to start after a month", "I am interested but do not plan to quit within 6 months", and "I am not interested in quitting smoking at all". Respondents were asked to select one of the following items regarding changes in smoking behavior during the COVID-19 pandemic: "I would like to quit smoking", "I have reduced the number of times I smoke", "The number of times I smoke has not changed", or "The number of times I smoke has increased".

2.3. Statistical analysis

To analyze the association between smoking history and "perception of increased risk of COVID-19 infection and susceptibility to severe disease due to smoking", smoking history was divided into three groups: never smokers, former smokers, and current smokers, and Pearson's χ^2 test was performed. In addition, smoking devices were divided into three groups: cigarette smokers, new types of cigarettes smokers, and cigarette smokers who smoked both cigarettes and new types of cigarettes (dual users). In addition, to analyze the relationship between smoking devices and "awareness of increased risk of COVID-19 infection and susceptibility to severe disease due to smoking", "interest in quitting among current smokers", and "changes in smoking behavior during the COVID-19 pandemic", we performed Pearson's χ^2 test. SPSS statistics 26 (IBM Corp Armonk. NY. USA) was used for analysis, and $p < 0.05$ was set as statistically significant.

2.4. Ethics

The purpose, methods, and management of personal information of the study were explained in writing to the subjects, and their consent was obtained by answering the questionnaire. This study was approved by the Chubu University Ethics Review Board (Approval No. 20200095).

3. Results

3.1. Attributes of respondents

The attributes of the respondents are listed in Table 1. The number of current smokers was 356 (4.4%). When the current smokers were divided by smoking devices, 128 (36.0%) were cigarette smokers, 68 (19.1%) were new types of cigarettes smokers, and 160 (44.9%) were dual users.

3.2. Current smokers' reasons for smoking

Table 2 shows the reasons for smoking among current smokers. The results indicate that "influence from friends, classmates, and older adults" was the primary reason for smoking (68.3%), followed by "curiosity" (29.2%).

Table 1. Basic attributes of respondents (*n* = 8,117)

Gender	Non-smokers (*n* = 7,202)	Former smokers (*n* = 559)	Current smokers (*n* = 356)		
			Cigarette smokers (*n* = 128)	New type types of cigarettes smokers (*n* = 68)	Dual users (*n* = 160)
Male (*n* = 5,551)	4,789 (66.5%)	456 (81.6%)	116 (90.6%)	56 (82.4%)	134 (83.8%)
Female (*n* = 2,500)	2,357 (32.7%)	95 (17.0%)	11 (8.6%)	11 (16.2%)	26 (16.3%)
Non-response (*n* = 66)	56 (0.8%)	8 (1.4%)	1 (0.8%)	1 (0.4%)	0 (0.0%)

Table 2. Current smokers' reasons for smoking (multiple answers) (*n* = 356)

Items	*n*	%
Influenced by friends, classmates, and older adults	243	68.3
Curiosity	104	29.2
Stress relief	76	21.3
Influenced by people at my part-time job	74	20.8
A family member was smoking	31	8.7

Table 3. Perception of increased risk of COVID-19 infection and susceptibility to severe disease due to smoking (*n* = 8,117)

Items	Non-smokers (*n* = 7,202)	Former smokers (*n* = 559)	Current smokers (*n* = 356)	Total	*p*-value
I knew it.	3,587 (49.8%)	339 (60.6%)	250 (70.2%)	4,176 (51.4%)	< 0.001
I didn't know.	3,615 (50.2%)	220 (39.4%)	106 (29.8%)	3,941 (48.6%)	

3.3. Awareness that smoking increases the risk of COVID-19 infection and the likelihood of severe disease

The increased risk of COVID-19 infection due to smoking and the perception of susceptibility to severe disease are shown in Table 3. Among non-smokers, former smokers, and current smokers, the results demonstrate that the number of respondents who "knew" that smoking increased the risk of COVID-19 infection and increased the severity of the disease was significantly higher among current smokers compared to non-smokers 250 (70.2%) and 3,587 (49.8%) respectively; ($p < 0.001$).

In addition, we divided current smokers according to smoking types and compared their perceptions of increased risk of COVID-19 infection and susceptibility to severe disease due to smoking, as shown in Table 4. There were no significant differences when comparing cigarettes, new types of cigarettes, and dual users ($p = 0.213$). However, the percentage of "knew" was more than half for all smoking equipment groups, especially for the new types of cigarettes, with nearly 80% of students knowing that smoking caused an increased risk of COVID-19 infection and the likelihood of severe disease.

Furthermore, other factors (year, gender) and awareness that smoking increases the risk of COVID-19 infection and the likelihood of severe disease were explored by regression analysis but no significance was found (data not shown).

3.4. Interest in quitting among current smokers

Table 4 shows the results of the responses to the question about interest in quitting smoking among current smokers. There was no association with smoking paraphernalia in terms of interest in quitting ($p = 0.323$). "I want to quit smoking now" was answered by a small number of students for all smoking devices. However, responses of "I am interested but will not quit smoking within six months" were answered by traditional cigarette smokers, 35 students (27.3%), new types of cigarettes smokers, 22 (32.4%), and dual users, 38 (23.8%). The number of students who answered, "I am not interested in quitting smoking at all" was 61 (47.7%) of cigarette smokers, 30 (44.1%) of new types of cigarettes smokers, and 91 (56.9%) among dual users.

3.5. Changes in smoking behavior during the COVID-19 pandemic

The changes in smoking behavior during the COVID-19 pandemic, shown in Table 4, were not associated with smoking device ($p = 0.146$). A small number of students answered, "I will try to quit smoking," for all smoking devices. However, more than half of the students answered that the number of cigarettes smoked did not change during the COVID-19 pandemic: 87 (68.0%) among cigarette smokers, 38 (55.9%) among new types of cigarettes smokers, and 116 (72.5%) among dual

Table 4. Perceptions of increased risk of COVID-19 infection and susceptibility to severe disease due to smoking, interest in quitting smoking among current smokers, changes in smoking behavior during the COVID-19 pandemic by smoking device (n = 356)

Items	Cigarette smokers (n = 128)	New types of cigarettes smokers (n = 68)	Dual users (n = 160)	Total	p-value
Perceptions of increased risk of COVID-19 infection and susceptibility to severe disease due to smoking					0.213
I knew it.	87 (68.0)	54 (79.4)	109 (68.1)	250 (70.2)	
I didn't know.	41 (32.0)	14 (20.6)	51 (31.9)	106 (29.8)	
Interest in quitting smoking among current smokers					0.323
I currently want to work on quitting smoking.	15 (11.7)	7 (10.3)	12 (7.5)	34 (9.6)	
I'm interested and planning to quit smoking within a month.	13 (9.4)	5 (7.4)	8 (5.0)	26 (7.3)	
I'm interested but want to start after 1 month.	4 (3.1)	4 (5.9)	11 (6.9)	19 (5.3)	
I'm interested, but not planning to quit smoking within 6 months.	35 (27.3)	22 (32.4)	38 (23.8)	95 (26.7)	
I have no interest in quitting smoking.	61 (47.7)	30 (44.1)	91 (56.9)	182 (51.1)	
Changes in smoking behavior during the COVID-19 pandemic					0.146
I'm going to quit smoking.	6 (4.7)	4 (4.9)	3 (1.9)	13 (3.7)	
I reduced the number of times I smoke.	29 (22.7)	22 (32.4)	30 (18.8)	81 (22.8)	
The number of cigarettes I smoked (times) remains the same.	87 (68.0)	38 (55.9)	116 (72.5)	241 (67.7)	
The number of cigarettes I smoked (times) increased.	6 (4.7)	4 (4.9)	11 (6.9)	21 (5.9)	

users. There were also a few students who answered, "the number of times I smoke has increased.

4. Discussion

We surveyed students from one university in Japan during the period between the third and fourth expansion of COVID-19 infection in Japan. This study found that although university students who smoke were aware that smoking increases the risk and severity of COVID-19 infection, many of them tended not to change their smoking cessation interest or smoking behavior during the COVID-19 epidemic. This result was true for all smokers. To the best of our knowledge, this is the first study to evaluate COVID-19 awareness, smoking cessation concerns, and changes in smoking behavior among college students during the pandemic.

Many smokers start smoking as adolescents or young adults (*11,12*). Therefore, it is important to prevent the initiation and continuation of smoking among adolescents. The largest number of current smokers in this study started smoking because they were "influenced by friends, classmates and older adults". In previous studies, the most common reason for initiating smoking was reported to be "smoking by friends" (*13*), which is consistent with the results of this study.

Smokers were more likely than non-smokers to know that smoking increases the risk and severity of COVID-19. We believe that this result is due to the fact that non-smokers are less interested in smoking than current smokers and have fewer opportunities to gain knowledge about the harmful effects of smoking.

The results of the comparison of awareness of the increased risk of COVID-19 infection due to smoking and the increased severity of the disease by smoking habit showed that the percentage of students who were aware was nearly 80%, especially among smokers who use new types of cigarettes. Prior research has shown that new types of cigarette smokers have lower awareness of the harmfulness of new types of cigarettes (*10*) and that those who perceived them to be less harmful than cigarettes had higher rates of e-cigarette use (*14*). Interestingly, our results differed from the results of this previous study in terms of the perceived harmfulness of new tobacco products. Previous studies have reported that beliefs that e-cigarette use helps people quit smoking, tastes good, and looks cool is associated with e-cigarette use, and the belief that heated cigarettes taste good and help people quit smoking is associated with heated cigarette use (*15*). In summary, we found the new types of cigarettes smokers are aware that smoking increases the risk of COVID-19 infection and the severity of the disease, but they continue to smoke because they prioritize the benefits such as helping them to quit smoking and the attractiveness of the taste.

For interest in quitting smoking, there was no

relationship with smoking device, but the results showed that the percentage of respondents who answered "I am not interested in quitting smoking at all" was over 50%. Additionally, more than 70% of the smokers in this study reported that they were aware of the increased risk of COVID-19 infection and susceptibility to severe disease, suggesting that many students are not at all interested in quitting smoking, even if they are aware of the negative health effects of smoking.

In terms of changes in smoking behavior during the COVID-19 pandemic, there was no association with smoking devices, and more than half of the students reported that the number of cigarettes smoked (times) remained the same for all smoking devices, with a few students reporting an increase. A previous study on changes in smoking behavior during the COVID-19 pandemic reported that 40% of smokers did not change their smoking behaviors (*16*). This is similar to our results. Furthermore, previous studies have reported that increased smoking is associated with depression, anxiety, and stress symptoms during the COVID-19 pandemic (*17*). Although this study did not explore the factors that prevented the target students from reducing the number of times they smoked during the pandemic, it did suggest the need for smoking cessation education during the pandemic as well as psychological support for students who smoke.

This study had several limitations. First, the subjects of this study were university students, most of who had probably been smoking for a few months to a few years. Different results may occur when targeting students with a longer smoking history. Second, this study did not distinguish between e-cigarettes and heated cigarettes for new types of cigarettes. Heated cigarettes sold in Japan contain nicotine, whereas e-cigarettes do not. Therefore, different characteristics of cigarettes may result in different perceptions of COVID-19, interest in quitting, and changes in smoking behavior. Third, the questionnaire survey was conducted during the period between the third and fourth expansion of COVID-19 infection in Japan. This was the time when the declaration of a state of emergency to prevent the spread of infection and take measures to secure the medical system had been lifted. The results may vary depending on the future spread of the infection and the measures taken, such as vaccines.

However, the strength of this study is that the questionnaire survey was conducted on a large scale with over 8,000 people. In addition, University A is a general university with many faculties, including humanities, social sciences, natural sciences, and medical sciences. Furthermore, most of the students are from all over Japan. While these results are insufficient to apply to the country as a whole, as they are only an assessment within a single Japanese university, we believe that they are important data for the smoking behavior of college students.

In recent years, a wide variety of smoking devices have been marketed. The current COVID-19 pandemic should be viewed as an opportunity to motivate smokers to quit smoking and to further improve smoking cessation education for smokers who use all types of smoking devices. In addition, from the perspective of the SDGs, since smoking cessation is one factor in achieving the health and well-being of all people, we believe that providing guidance on smoking cessation to smokers, including young people such as college students who have just started smoking, will ultimately lead to the achievement of a sustainable society.

5. Conclusions

In this study, we found that college students who smoked during the COVID-19 pandemic were less interested in quitting smoking and not likely to change their smoking behavior, despite awareness that smoking increases the risk of COVID-19 infection and the severity of the disease. This trend applies to smokers of cigarettes, new types of cigarettes, and dual users. To address these emerging public health challenges identified by this study, the current COVID-19 pandemic should be viewed as an opportunity to motivate smokers to quit and to further improve smoking cessation education for smokers who use all types of smoking devices.

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References

1. Zhu N, Zhang D, Wang W*, et al.* A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727-733.
2. Son C, Hegde S, Smith A, Wang X, Sasangohar F. Effects of COVID-19 on college students' mental health in the United States: Interview survey study. J Med Internet Res. 2020; 22:e21279.
3. Bilano V, Gilmour S, Moffiet T, d'Espaignet ET, Stevens GA, Commar A, Tuyl F, Hudson I, Shibuya K. Global trends and projections for tobacco use, 1990-2025: an analysis of smoking indicators from the WHO Comprehensive Information Systems for Tobacco Control. Lancet. 2015; 385:966-976.
4. Brake SJ, Barnsley K, Lu W, McAlinden KD, Eapen

MS, Sohal SS. Smoking upregulates angiotensin-converting enzyme-2 receptor: A potential adhesion site for novel coronavirus SARS-CoV-2 (Covid-19). J Clin Med. 2020; 9:841.
5. Liu W, Tao ZW, Wang L, Yuan ML, Liu K, Zhou L, Wei S, Deng Y, Liu J, Liu HG, Yang M, Hu Y. Analysis of factors associated with disease outcomes in hospitalized patients with 2019 novel coronavirus disease. Chin Med J (Engl). 2020; 133:1032-1038.
6. Gallè F, Veshi A, Sabella EA, Çitozi M, Da Molin G, Ferracuti S, Liguori G, Orsi GB, Napoli C. Awareness and behaviors regarding COVID-19 among Albanian undergraduates. Behav Sci (Basel). 2021; 11:45.
7. Agaku IT, King BA, Husten CG, Bunnell R, Ambrose BK, Hu SS, Holder-Hayes E, Day HR, (CDC) CfDCaP. Tobacco product use among adults – United States, 2012-2013. MMWR Morb Mortal Wkly Rep. 2014; 63:542-547.
8. Goniewicz ML, Gawron M, Nadolska J, Balwicki L, Sobczak A. Rise in electronic cigarette use among adolescents in Poland. J Adolesc Health. 2014; 55:713-715.
9. Lee CM. The impact of heated tobacco products on smoking cessation, tobacco use, and tobacco sales in South Korea. Korean J Fam Med. 2020; 41:273-281.
10. Bernat D, Gasquet N, Wilson KO, Porter L, Choi K. Electronic cigarette harm and benefit perceptions and use among youth. Am J Prev Med. 2018; 55:361-367.
11. Almutairi KM. Smoking among Saudi students: a review of risk factors and early intentions of smoking. J Community Health. 2014; 39:901-907.
12. Rudatsikira E, Muula AS, Siziya S. Current cigarette smoking among in-school American youth: results from the 2004 National Youth Tobacco Survey. Int J Equity Health. 2009; 8:10.
13. Oh DL, Heck JE, Dresler C, Allwright S, Haglund M, Del Mazo SS, Kralikova E, Stucker I, Tamang E, Gritz ER, Hashibe M. Determinants of smoking initiation among women in five European countries: a cross-sectional survey. BMC Public Health. 2010; 10:74.
14. Adkison SE, O'Connor RJ, Bansal-Travers M, Hyland A, Borland R, Yong HH, Cummings KM, McNeill A, Thrasher JF, Hammond D, Fong GT. Electronic nicotine delivery systems: international tobacco control four-country survey. Am J Prev Med. 2013; 44:207-215.
15. Morgan JC, Cappella JN. Harm Perceptions and Beliefs about Potential Modified Risk Tobacco Products. Int J Environ Res Public Health. 2021; 18:576.
16. Sidor A, Rzymski P. Dietary Choices and Habits during COVID-19 Lockdown: Experience from Poland. Nutrients. 2020; 12:1657.
17. Stanton R, To QG, Khalesi S, Williams SL, Alley SJ, Thwaite TL, Fenning AS, Vandelanotte C. Depression, anxiety and stress during COVID-19: Associations with changes in physical activity, sleep, tobacco and alcohol use in Australian adults. Int J Environ Res Public Health. 2020; 17:4065.

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Original Article

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Efficacy and safety of sotrovimab for vaccinated or unvaccinated patients with mild-to-moderate COVID-19 in the omicron era

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SUMMARY Although sotrovimab, one of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) neutralizing antibodies has been shown to be effective in patients with mild-to-moderate coronavirus disease 2019 (COVID-19) with risk factors, their efficacy in mRNA COVID-19 vaccinated patients in omicron era is unknown. To evaluate the effectiveness of sotrovimab clinical data from both COVID-19 vaccinated and unvaccinated patients who were hospitalized and receiving sotrovimab at the Japanese Red Cross Medical Center were compared. The efficacy and adverse events were evaluated. Of the total 60 patients enrolled in this study, 45 had received the mRNA COVID-19 vaccine and 15 were unvaccinated. The clinical progression with low nasal cannula or face mask was not significantly different between groups (occurring in one patient in each group; $p = 0.44$), with no further progression in both groups. The duration of hospitalization was eight days for both groups ($p = 0.90$). Two patients in each group experienced adverse events (7%, $p = 0.26$). The results suggested that the efficacy and safety of sotrovimab against mild-to-moderate COVID-19 with risk factors in the omicron era might not be different regardless of the vaccination status. The results of the present study are encouraging; however, further randomized clinical studies are needed.

Keywords COVID-19, severe acute respiratory syndrome coronavirus 2, adverse event, efficacy, neutralizing antibodies

1. Introduction

The number of people infected with coronavirus disease 2019 (COVID-19) is increasing worldwide (*1*). The number of COVID-19 patients is increasing in Japan as well, and infection control and medical care are essential. From July 2021, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-neutralizing antibody therapeutics and oral drugs were approved for use in patients with COVID-19 with risk factors (*2*). SARS-CoV-2-neutralizing antibodies and oral drugs are therapies used for mild-to-moderate cases, and SARS-CoV-2-neutralizing antibodies are said to be particularly effective against mild-to-moderate COVID-19 with risk factors (*3*). Two SARS-CoV-2-neutralizing antibodies, REGN-COV2 (Regeneron Pharmaceuticals, Inc., USA) and sotrovimab (VIR-7831; Vir Biotechnology and GlaxoSmithKline, United Kingdom), are available in Japan (*4-6*). However, the omicron variant became prevalent in Japan in January 2022, and it is said that the effect of REGN-COV2 on the omicron strain is diminished (*2*). Therefore, sotrovimab became the antibody drug of choice. However, during clinical trials of sotrovimab, only patients unvaccinated against COVID-19 were included as target patients, and the efficacy and safety of sotrovimab in vaccinated patients remains unknown (*5*). Therefore, we investigated the efficacy and safety of sotrovimab in the omicron era for patients with COVID-19 with risk factors, and compared the results between patients who were vaccinated or unvaccinated against COVID-19.

2. Materials and Methods

2.1. Eligibility criteria

Patients diagnosed with COVID-19 and admitted to the Japanese Red Cross Medical Center were screened for the analysis. In accordance with the National Institutes of Health classification criteria, patients with COVID-19 were classified into four categories as follows: 1) mild illness group, which included patients with different signs and symptoms of COVID-19 excluding shortness of breath, dyspnea, or abnormal chest imaging finding;

2) moderate illness group, which included patients with lower respiratory diseases diagnosed based on clinical assessment or imaging examination and a blood oxygen saturation level (SpO_2) ≥ 94% on room air at sea level; 3) severe illness group, which included patients with respiratory rate > 30 breaths per minute, SpO_2 < 94% on room air at sea level, arterial partial pressure of oxygen to fraction of inspired oxygen ratio < 300 Torr, or lung infiltrates > 50%; and 4) critical illness group, which included patients with respiratory failure, septic shock, and/or multi-organ dysfunction (*7*).

From January 2022 to February 2022 of the omicron era, patients who demonstrated mild or moderate illness with COVID-19 risk factors on admission and who received sotrovimab were consecutively enrolled in this study. Patients with a compatible symptom onset no more than seven days before the administration and who had at least one of the following risk factors: age ≥ 55 years; body mass index (BMI) ≥ 30 kg/m^2; or comorbidity with diabetes, chronic kidney disease (estimated glomerular filtration rate, < 60 mL per minute per 1.73 m^2 of body-surface area) including hemodialysis, congestive heart failure (New York Heart Association class II, III, or IV), cancer, chronic obstructive pulmonary disease, moderate-to-severe asthma, hypertension, hyperlipidemia, pregnancy, or long term use of steroids or immunosuppressants were included in the study.

2.2. Procedures

Consecutive patients received a single 500 mg, 30 minutes infusion of sotrovimab on the day of, or the day after, admission. This study did not mandate any treatment for COVID-19 other than sotrovimab; as a result, the patients received another treatment at the discretion of their physicians according to the local standard of care if the patient's condition worsened.

2.3. COVID-19 vaccines

Two kinds of mRNA vaccines (*8*); mRNA-1273 (developed by Moderna Ltd., USA) and BNT162b (developed by Pfizer and BioNTech Ltd., USA) were the first vaccines approved for emergency use in Japan. In this study, patients who had received at least two doses of either of these vaccines were defined as the vaccinated group, and those who had not received either of these vaccines were defined as the unvaccinated group.

2.4. Statistical analyses

All data are presented as medians with interquartile ranges (IQRs) or absolute numbers with percentages. The Fischer exact test was used for categorical data, and the Mann-Whitney *U* test was used for numeric data to evaluate the difference between the vaccinated and unvaccinated groups. *P*-values < 0.05 were considered significant. Data were analyzed using EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan; *http://www.jichi.ac.jp/saitama-sct/SaitamaHP.files/statmed.html*), a graphical user interface for R software (version 2.13.0; The R Project for Statistical Computing; *http://www.r-project.org*) and a modified version of R Commander (*9*). Adverse events were reported in accordance with the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0.

2.5. Ethics approval and consent to participate

This study was approved by the Ethics Committee for Clinical Studies of the Japanese Red Cross Medical Center (No. 1394; April 28, 2022). Written informed consent for the use of sotrovimab was obtained from all patients. Additional informed consent for this study was waived owing to the nature of the study design, which involved retrospective chart review to obtain clinical information. In accordance with the Japanese ethical guidelines for clinical research, the need for informed consent was waived.

3. Results

In total, 60 patients with mild or moderate COVID-19 who received sotrovimab were enrolled in this study. Of the 60 patients, 43 had received two doses of mRNA COVID-19 vaccine, two had received three doses of mRNA COVID-19 vaccine, and 15 had never received any mRNA COVID-19 vaccine. Thus, 45 patients were enrolled in the vaccinated group and 15 in the unvaccinated group. Details on whether the mRNA COVID-19 vaccine ingested was mRNA-1273 or BNT162b were not available. The clinical characteristics of the patients on admission are presented in Table 1. Briefly, the median patient age was 67 years (IQR: 53-79 years), and 36 patients (60%) were male. Regarding concurrent risk factors, 17 (28%) patients had one, 18 (30%) patients had two, and 26 (42%) patients had three or more. At admission, 46 patients (77%) were diagnosed with mild symptoms and 14 (23%) with moderate symptoms. There was no significant difference in baseline admission data between the vaccinated and unvaccinated groups.

The patient outcomes are presented in Table 2. One patient in each group experienced severe or critical progression (p = 0.44). Both groups had one patient requiring a low nasal cannula or face mask, but they had no further progression. The median duration of hospitalization was 8 days (interquartile range [IQR]: 6-10 days) for both groups, with no significant difference (p = 0.90).

The adverse events are presented in Table 3 and were reported in accordance with CTCAE version 5.0. Adverse events occurred in a total of four patients (7%),

Table 1. Clinical characteristics of the patients on admission

Characteristic	Overall (n = 60)	Vaccinated (n = 45)	Unvaccinated (n = 15)	p value
Age, years (IQR)	67 (53-79)	70 (54-83)	64 (46-70)	0.1
Male sex-No. (%)	36 (60)	27 (60)	9 (60)	1.0
BMI, kg/m^2 (IQR)	22.8 (19.8-26.2)	22.8 (19.6-26.1)	23.5 (20.3-25.7)	0.98
Any risk factor	60 (100)	45 (100)	15 (100)	
Age ≥ 55 years (%)	43 (72)	32 (71)	11 (73)	1.0
Obesity: BMI ≥ 30 (%)	3 (5)	2 (4)	1 (6)	1.0
Diabetes (%)	15 (25)	12 (27)	3 (20)	0.70
Chronic kidney disease (%)	9 (15)	5 (11)	4 (27)	0.21
Congestive heart failure (%)	6 (10)	5 (11)	1 (6)	1.0
Cancer (%)	8 (13)	6 (13)	2 (13)	1.0
Chronic obstructive pulmonary disease (%)	2 (3)	2 (4)	0 (0)	1.0
Moderate-to-severe asthma (%)	4 (7)	4 (9)	0 (0)	0.56
Hypertension (%)	23 (38)	16 (36)	7 (47)	0.54
Hyperlipidemia (%)	13 (22)	8 (18)	5 (33)	0.28
Pregnancy (%)	3 (5)	1 (2)	2 (13)	0.15
Long term use of steroids or immunosuppressants (%)	3 (5)	3 (7)	0 (0)	0.57
No. of concurrent risk factors (%)				0.51
1	17 (28)	13 (29)	4 (27)	
2	18 (30)	15 (33)	3 (20)	
≥3	25 (42)	17 (38)	8 (53)	
Severity on admission (%)				0.73
Mild	46 (77)	35 (78)	11 (73)	
Moderate	14 (23)	10 (22)	4 (27)	

IQR, interquartile range; BMI, Body Mass Index.

Table 2. Patient outcomes

Outcome	Overall (n = 60)	Vaccinated (n = 45)	Unvaccinated (n = 15)	p value
Primary outcome				
Severe or critical progression, No. (%)	2 (3)	1 (2)	1 (7)	0.44
Low flow nasal cannula or face mask	2 (3)	1 (2)	1 (7)	
High flow nasal cannula or noninvasive mechanical ventilation	0 (0)	0 (0)	0 (0)	
Intensive mechanical ventilation	0 (0)	0 (0)	0 (0)	
Admission to ICU for any cause	0 (0)	0 (0)	0 (0)	
Death from any cause	0 (0)	0 (0)	0 (0)	
Secondary outcome				
Duration of hospitalization, days (IQR)	8 (6-10)	8 (6-10)	8 (6-10)	0.90

ICU, intensive care unit; IQR, interquartile range.

Table 3. Adverse events

Event	Overall (n = 60)	Vaccinated (n = 45)	Unvaccinated (n = 15)	p value
All adverse events, No. (%)	4 (7)	2 (4)	2 (14)	0.26
Infusion-related reactions, No. (%)	1 (2)	1 (2)	0 (0)	
Post-dose fever, No. (%)	2 (3)	1 (2)	1 (7)	
Liver dysfunction, No. (%)	1 (2)	0 (0)	1 (7)	

wherein infusion-related reactions occurred in 2% of patients, post-dose fever in 3%, and liver dysfunction in 2%. There were no significant differences between the two groups (p = 0.26).

4. Discussion

To the best of our knowledge, this is the first report to investigate the efficacy and safety of sotrovimab for patients with mild-to-moderate COVID-19 with risk factors in the omicron era according to their vaccination status.

COVID-19 is an infection caused by SARS-CoV-2 (*1*). In patients with severe disease, excessive inflammation and cytokine storm-like conditions are considered serious; therefore, anti-inflammation and antiviral drug combination therapies are currently being used for patients with severe COVID-19 (*10*). However, early intervention is necessary for patients with risk factors to improve the efficacy of COVID-19 therapy (*3*).

The clinical trial of sotrovimab for mild-to-

moderate COVID-19 included 583 patients (291 in the sotrovimab group and 292 in the placebo group) (*5*). That study reported that three patients (1%) in the sotrovimab group and 21 patients (7%) in the placebo group had disease progression leading to hospitalization or death, with sotrovimab significantly reducing disease progression compared to that in the placebo group. The placebo group also reported five intensive care unit admissions, including one death by day 29. Safety was evaluated in 868 patients (430 in the sotrovimab group and 438 in the placebo group); 17% and 19% of the individuals in the sotrovimab and placebo groups, respectively, reported adverse events, with serious adverse events occurring rather less frequently in the sotrovimab group than in the placebo group. Based on these results, the investigators reported that sotrovimab reduced the risk of disease progression in patients with mild-to-moderate Covid-19 with risk factors and had no safety problems.

In the present study, sotrovimab was found to be highly effective, with only a 3% (2 out of 60 patients) clinical progression rate, and no patients required invasive mechanical ventilation or admission to the intensive care unit (ICU). In addition, these effects were not significantly different between the vaccinated and unvaccinated groups, suggesting that sotrovimab could be effective in vaccinated COVID-19 patients with risk factors. Although two sotrovimab-treated patients had progression requiring low flow nasal oxygenation, both patients had improvement with remdesivir and steroid therapies.

The incidence of adverse events was 7%. All adverse events were grade 2 in CTCAE, and no serious adverse events occurred with or without mRNA COVID-19 vaccination. This result suggests that sotrovimab can be safely used with or without mRNA COVID-19 vaccination.

The present study had several limitations. First, the study was conducted at a single center, and only a few patients were included. Second, it was unknown whether patients in the vaccination group received mRNA-1273 or BNT162b vaccine and the duration since vaccination. Third, the COVID-19 variant of all enrolled patients has not been determined, although it is thought to have largely replaced the omicron variant in Japan since January 2022. Further investigations are needed to clarify this aspect.

In conclusion, this study suggested that the efficacy and safety of sotrovimab against mild-to-moderate COVID-19 with risk factors in the omicron era might not be different regardless of the vaccination status. The results of this study are promising because the clinical impact of the neutralizing antibody might be also significant in mild-to-moderate COVID-19 patients with risk factors vaccinated with mRNA COVID-19, although further randomized clinical trials must be conducted to confirm these findings.

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References

1. Zhu N, Zhang D, Wang W, *et al*. A novel coronavirus from patients with pneumonia in China, 2019. N Engl J Med. 2020; 382:727-733.
2. Takashita E, Kinoshita N, Yamayoshi S, *et al*. Efficacy of antibodies and antiviral drugs against Covid-19 Omicron variant. N Engl J Med. 2022; 386:995-998.
3. Gavriatopoulou M, Ntanasis-Stathopoulos I, Korompoki E, Fotiou D, Migkou M, Tzanninis IG, Psaltopoulou T, Kastritis E, Terpos E, Dimopoulos MA. Emerging treatment strategies for COVID-19 infection. Clin Exp Med. 2021; 21:167-179.
4. Weinreich DM, Sivapalasingam S, Norton T, *et al*. REGEN-COV antibody combination and outcomes in outpatients with Covid-19. N Engl J Med. 2021; 385:e81.
5. Gupta A, Gonzalez-Rojas Y, Juarez E, *et al*. Early treatment for Covid-19 with SARS-CoV-2 neutralizing antibody sotrovimab. N Engl J Med. 2021; 385:1941-1950.
6. Amano N, Iwata K, Iwata S. Clinical effectiveness of REGN-COV2 in patients with COVID-19 in Japan: A retrospective cohort study with a Bayesian inference. Infect Chemother. 2021; 53:767-775.
7. Awano N, Inomata M, Kuse N, Tone M, Takada K, Muto Y, Fujimoto K, Akagi Y, Mawatari M, Ueda A, Izumo T. Serum KL-6 level is a useful biomarker for evaluating the severity of coronavirus disease 2019. Respir Investig. 2020; 58:440-447.
8. Park JW, Lagniton PNP, Liu Y, Xu RH. mRNA vaccines for COVID-19: what, why and how. Int J Biol Sci. 2021; 17:1446-1460.
9. Kanda Y. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. Bone Marrow Transplant. 2013; 48:452-458.
10. Izumo T, Kuse N, Awano N, Tone M, Sakamoto K, Takada K, Muto Y, Fujimoto K, Saiki A, Ito Y, Matsumoto H, Inomata M. Clinical impact of combination therapy with baricitinib, remdesivir, and dexamethasone in patients with severe COVID-19. Respir Investig. 2021; 59:799-803.

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Original Article

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Safety verification of a new peripheral intravenous catheter placed in the upper arm vein for administration of drugs with high irritant potential

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SUMMARY Despite the widespread use of peripheral intravenous catheters, unscheduled catheter failure before completion of treatment occurs frequently. If a large vein is selected, catheter failures may be prevented despite administering a highly irritant drug. In this study, we attempted to use a catheter that can be placed in a large upper arm vein. The new catheter was 88 mm long but had no guidewire to reduce contamination risk. This study aimed to evaluate the safety of the first-in-human trial for the new catheter with the administration of highly irritant drugs. This study was conducted at a university hospital in Tokyo, Japan. Eight Japanese adults were hospitalized adults with planned administration of non-cancer drugs with high irritant potential using a peripheral catheter. A trained nurse catheterized with the new catheter in the upper arm using ultrasonography. The catheterization site was monitored by staff and a research nurse once every 24 hours for up to 7 days. No adverse events or catheter failure occurred and the catheter placement success rate was 100%. In two patients, a temporary occlusion alarm of the infusion pump occurred, possibly due to the flexion of the catheter base. The new peripheral intravenous catheter did not interrupt medical treatments as is common after placement, but safety administered the irritant drugs. However, because this catheter may be easily affected by the contraction of the muscle at the fixation position, the position and method of catheter fixation in the upper arm need to be carefully considered.

Keywords adverse event, device malfunction, first-in-human trial, irritant infusates, peripheral intravenous catheter failure

1. Introduction

Peripheral intravenous catheters (PIVCs) are widely used. Almost 70% of hospitalized patients worldwide require a PIVC to secure the venous route for infusion therapy (*1*). In Japan, only a short peripheral catheter (SPC) has been approved as a medical device, while midline catheters and long peripheral intravenous catheters (LPCs) are not available. Physicians and general nurses can choose only a short catheter to secure the peripheral venous route for infusion in Japan.

Despite the widespread use of PIVCs, unscheduled catheter failure (CF) before completion of treatment occurs in up to 69% of patients, even in cases of successful catheter placement (*2-4*). In adult inpatients at a university hospital in Japan, 18% of placed catheters were removed because of CF (*5*). The major reasons for discontinuation of using indwelling catheters are the appearance of symptoms and signs, such as redness, swelling, pain, and induration along the vein, and catheter occlusion and insufficient infusion volume. These signs and symptoms are often observed as early symptoms of complications, such as catheter-related bloodstream infection or thrombophlebitis (*6*), potentially leading to bacteremia, sepsis, and even death (*7*). Preventing CF would not only prevent complications but also reduce the burden due to repeated punctures by needles on the patients.

Contributing factors to complications related to CF can be classified as biological, mechanical, and chemical factors (*8*). In our previous study, we identified the following factors associated with reduced risk of CF: (*i*) selection of a vessel with a large diameter (at least 3.3 times the external diameter of the catheter) (*9*), (*ii*) successful insertion on the first attempt (*10*), (*iii*) securing the catheter tip in a non-stimulatory position on the vessel wall (*11,12*), and (*iv*) use of a catheter made of soft materials such as polyurethane rather than polytetrafluoroethylene (*13*). To meet these requirements, a long catheter that can be safely and easily placed in the upper arm vein, that can reach in the vicinity of the axillary vein, and which has a large blood flow, is required.

Although midline catheters and LPCs are PIVCs meet these requirements (*14,15*), they have several drawbacks mainly associated with the insertion procedure. Existing long catheters (length is 80 mm or over) require a guidewire for insertion which also requires aseptic non-touch technique for additional manipulation of the guidewire. This technique requires more time and effort for catheter placement, and the risk of infection is increased owing to the complicated guidewire procedures (*16*). Furthermore, the inserter needs to use both hands and cannot hold the US probe for catheter insertion.

To improve the ease of catheterization in this study, we used a new indwelling PIVC catheter (new PIVC) that can be placed in an upper arm vein. It does not necessitate a guidewire even though the catheter length is 88 mm. This study is the first-in-human trial to verify its safety in Japan. This study aimed to evaluate the safety of the new PIVC in patients undergoing administration of drugs with high irritancy other than anticancer drugs by assessing the occurrence of adverse events. The adverse events evaluated in this study included catheter-related bloodstream infection and device malfunction at the time of puncture, during the indwelling period, at the time of removal, and up to approximately 24 hours after catheter removal.

2. Methods

2.1. Study setting and samples

This exploratory research was conducted in the hematology and oncology department at The University of Tokyo Hospital in Tokyo, Japan, between August and December 2020. All participants read a description of the study and signed a consent form. The study protocol was approved by the Research Ethics Committee of the Graduate School of Medicine and Institutional Review Board, The University of Tokyo (2019016SP). The study protocol was registered and published in the Japan Registry of Clinical Trials: jRCT (protocol number: jRCTs032200076).

2.2. Inclusion criteria

Participants were male and female patients over 20 years old who had planned administration of hyperstimulant drugs, such as those with an osmotic pressure ratio 3 higher, or irritant or vesicant drugs, excluding anticancer drugs, for longer than 24 hours using a PIVC.

2.3. Exclusion criteria

We excluded patients who could not maintain the position with the shoulder joint abducted and the elbow joint externally rotated, those who had skin disorders at the puncture site, those with peripheral neuropathy, those with a history of vasovagal reflex due to puncture, those with a history of thrombosis, those with stage ≥ G3a chronic kidney disease, and those with an abnormal blood coagulation ability or a bleeding tendency (prothrombin-international normalized ratio ≥ 1.5; activated partial thromboplastin time of ≥ 36.1 seconds, and taking anticoagulant or antiplatelet drugs). Patients scheduled to undergo invasive procedures were also excluded (*e.g.*, endoscopy and bronchoscopy). Furthermore, other patients judged by the physician in charge to be inappropriate for participation in this study were excluded.

2.4. Sample size

The incidence of adverse events with the new PIVC was unknown, including nerve injury, arterial puncture, vasovagal reflex, skin damage due to echo jelly, hematoma formation due to deep vascular injury, catheter-related bloodstream infection, and venous thrombosis. Adverse events might occur not only with the new PIVC but also with the conventional SPC. Additionally, the frequency of a device malfunction was unpredictable. We set 10 cases as the sample size because this survey was an exploratory study to determine the frequency of adverse events for subsequent validation. The exact one-sided 95% confidence interval of the probability of occurrence was calculated to be 0%-25.9% when the number of cases is 0 of 10 for each event. Therefore, at least one event with a true probability of occurrence > 25.9% was identified with a probability of ≥ 95% in this study.

2.5. Outcomes and patients' characteristics

The primary outcomes were the incidence of adverse events: nerve injury, arterial puncture, vasovagal reflex, skin damage due to echo jelly, hematoma formation due to deep vascular injury, catheter-related bloodstream infection, and venous thrombosis, from the time of puncture to 24 hours after catheter removal and the frequency of a device malfunction

by the time of catheter removal or up to a maximum of day 7. Nerve injury, arterial puncture, hematoma, and venous thrombosis were recorded by an interview, macroscopic observation, and ultrasonography with two-dimensional linear-array transducers (6-13 MHz, FC1-X VA; Fujifilm, Tokyo, Japan) to observe blood vessels and subcutaneous tissues. Bacterial culture tests were applied for removed catheters and the skin surface around the catheter insertion point. The visual analogue scale (100 points: maximum pain) was used to evaluate pain. Photographs of the removed catheter were taken immediately after its removal, and then the angle of the base of the catheter was measured using image-J software (NIH) because this could be related to device failure.

The secondary outcomes were the catheter placement success rate, the incidence of CF, and subjective assessment of catheter placement on the upper arm. CF was defined as premature catheter removal before completion of planed fluid therapy, excluding self-removal and accidental removal. CF and catheter removal were determined by physicians and nurses based on interviews and macroscopic observations. Ultrasonography was additionally used to confirm the subtypes of CF (*e.g.*, catheter dislodgement, thrombus formation in the vein, and subcutaneous edema formation) by a trained researcher. Subjective assessment of catheter placement was confirmed by original questionnaires.

The patients' characteristics (diagnosis, medical history, oral medication, age, sex, and body mass index), blood test data (total protein, albumin, hematocrit, platelet count, white blood cell with differential count, C-reactive protein, prothrombin time, prothrombin-international normalized ratio, activated partial thromboplastin time, and fibrinogen), length of hospital stay, rate and dosage of the infusate, infusion pump use, and performance status were collected by chart review.

2.6. Features of the new PIVC

The length of the catheter was 88 mm to enable approaching the peripheral vein in the upper arm and inserting the catheter tip into the vicinity of the axillary vein (Figure 1). The catheter size was 22 gauge (outer diameter: 0.9 mm) and made of polyurethane. There was no guidewire, and catheter insertion was performed by the inserter pushing in the "controlling element" with their finger in the punctured hand, allowing a one-handed catheter insertion operation. This catheter has a backflow prevention valve which can help to protect against blood exposure. Ultrasonography was necessary in order to guide catheter puncture and insertion.

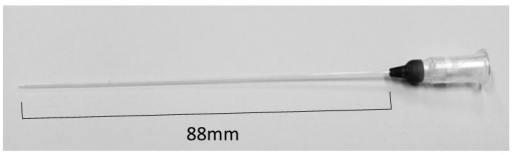

Figure 1. New peripheral intravenous catheter. The length of the catheter is 88 mm. The catheter size is 22 gauge (outer diameter: 0.9 mm) and it is made of polyurethane.

2.7. Research procedure

The patients were admitted to the department, and consent for participation in the study was obtained. Informed consent was obtained in accordance with the Declaration of Helsinki and the Clinical Research Act (Japan). Eligibility was confirmed simultaneously, and then a trained nurse catheterized the new PIVC in the upper arm using ultrasonography under the direction of a physician. Standard infection precautions were applied, with 1% chlorhexidine alcohol as skin antisepsis, and the insertion site was covered with dressing film. The catheterization site was monitored by staff and a research nurse at least once every 24 hours. The catheter was removed after being placed for up to 7 days.

2.8. Statistical analysis

The incidence of adverse events or device malfunction (%) was calculated as the number of catheters with adverse events or device malfunction/the number of placed catheters. The success rate of indwelling catheters (%) was calculated as the number of successful indwelling catheters/the number of punctures. The incidence of CF (%) was calculated as the number of catheters in which CF occurred/the number of placed catheters. Descriptive statistics were used for the patients' characteristics and the points of the visual analogue scale. Qualitative analysis was used for answers by an open-ended questionnaire. Data were analyzed with the Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, ver. 22.0; IBM Corp., Armonk, NY, USA).

3. Results

3.1. Participants

The number of patients who agreed to participate in this study was nine. All of them were enrolled in the study and underwent insertion of the new PIVC and infusion. After catheter removal, one participant was excluded from the analysis because the activated partial thromboplastin time value on the day before placement was prolonged beyond the inclusion criteria. One participant withdrew consent after the initiation of infusion, and removed catheter before completion the study, in which subsequent participation to the study was refused but use of data already collected so far was allowed. Therefore, the number of subjects for analysis was eight.

3.2. Baseline characteristics

Of the eight participants, four were men. The median age was 51.5 years. The mean of the body mass index was 21.6 kg/m^2. The mean of the length of the hospital stay was 32.3 days (Table 1). The performance status score on admission was zero in four patients, 1 in two patients, and 2 in three patients. Table 2 shows the blood test data before catheter placement.

Seven patients were administered BFLUID® injection: amino acid and glucose injection with electrolytes and vitamin B1 (Otsuka Holdings Co., Ltd., Tokyo, Japan), one patient was administered tazobactam/piperacillin hydrate, and three patients were administered vancomycin hydrochloride (Table 3). All patients used an infusion pump for the administration of these irritant drugs.

Table 1. Participants characteristics

Age (years): median	51.5 (range: 29-76)
Sex	male: 4; female: 4
BMI (kg/m^2): mean (SD)	21.6 (3.3)
Length of hospital stay: mean (SD)	32.3 (18.1) days
Diagnoses (*n*)	acute myeloid leukemia (2)
	mycosis fungoides (2)
	acute lymphocblastic leukemia (1)
	lymphoplasmacytic lymphoma (1)
	acute undifferentiated leukemia (1)
	mantle cell lymphoma (1)

BMI, Body Mass Index.

3.3. Outcome measures

No predefined adverse events were observed during puncture, at the indwelling period, at the time of removal, and up to approximately 24 hours after catheter removal. The culture test results of all removed catheters and the skin surface around the catheter insertion site were negative.

A temporary occlusion alarm of the infusion pump, possibly due to flexion of the catheter base, occurred in two of eight (25%) patients. Actually, under the magnification of two catheters, a linear line was observed at the base of the catheter. In one patient, the catheter was used until the completion of the treatment, and the other patient hoped to remove the catheter the next day. Table 4 shows the characteristics of the patients, the vessel where the catheter was placed, and details of the catheter according to catheter malfunction. The body mass index was relatively low, and the depth of the vessel was relatively shallow in patients with catheter malfunctions than in those without catheter malfunction. The angle of the base of the removed catheter was smaller in patients with catheter malfunction than in those without catheter malfunction.

According to the visual analog scale, the mean

Table 2. Blood test values

Blood test items	Patients' values[a]	Reference values[b]
Total protein (g/dL)	5.50 (4.40-6.40)	6.6-8.1
Albumin (g/dL)	3.00 (2.30-3.60)	4.1-5.1
Hematocrit (%)	23.40 (19.20-28.10)	Men: 40.7-50.1, Women: 35.1-44.4
Blood platelet count (×10^4/μL)	7.35 (1.60-35.60)	15.8-34.8
White blood cell count (×10^3/μL)	4.45 (0.20-47.80)	3.3-8.6
C-reactive protein (mg/dL)	1.82 (0.20-5.88)	< 0.3
Prothrombin (%)	92.85 (80.0-100.0)	86.0-124.1
PT-INR	1.05 (0.93-1.13)	–
APTT (seconds)	27.50 (25.30-30.10)	24.0-34.0
Fibrinogen (mg/dL)	421.00 (253.0-700.0)	168-355

Values are median (range). [a]Blood test values on the closest day before catheter placement. [b]Reference values of blood testing by The University of Tokyo Hospital. PT-INR: prothrombin-international normalized ratio; APTT: activated partial thromboplastin time.

Table 3. Total volume of administered irritant drugs

Patient ID	PIVC dwelling time (days)	Irritant drug administration (total)
201	7	BFLUID[a] 6631 mL
301	4	BFLUID 2265 mL
801	7	BFLUID 6880 mL, VCM 14 g
401	7	BFLUID 3668 mL, KCL 60 mEq
901	7	BFLUID 5850 mL, KCL 60 mEq
501	7	BFLUID 7000 mL, VCM 21 g, KCL 70 mEq
601	7	BFLUID 3500 mL, TAZ/PIPC 49.5 g
701	1 (withdrawal)	VCM 4 g

[a]BFLUID (amino acid and glucose injection with electrolytes and vitamin B1) was infused intravenously. PIVC: peripheral intravenous catheter; VCM: vancomycin; KCL: potassium chloride; TAZ/PIPC: tazobactam/piperacillin hydrate.

Table 4. Characteristics of the patients, catheter, and vessel

Items	Total (n = 8)	Without catheter malfunction (n = 6)	With catheter malfunction (n = 2)
Body mass index	21.6 (3.4)	22.3 (3.7)	19.8 (1.2)
Distance between the elbow joint and the puncture point	91.3 (24.2)	88.3 (11.7)	100.0 (56.6)
Vein diameter at the puncture point*	4.4 (1.0)	4.0 (0.6)	5.9 (0.3)
Vein diameter at the catheter tip	5.7 (1.5)	5.3 (1.3)	6.9 (2.0)
Vein depth	7.3 (2.8)	8.4 (2.1)	4.2 (2.1)
Angle of the base of the removed catheter	17.4 (4.4)	18.2 (5.0)	15.1 (0.6)

Data are mean (SD). *Vein diameter = [major axis + minor axis]/2.

pain scores were < 40 at all the time points, including at insertion and removal. Despite the invasive nature of the needle puncture, there was no incidence of an inability to puncture owing to pain or premature removal during indwelling.

A secondary endpoint, the catheter placement success rate, was 100% in all eight patients. The incidence of CF was 0% at all time points. Regarding the subjective assessment of catheter placement, there were no complaints of hindered activities of daily living due to indwelling and fixation of the catheter in the upper arm.

4. Discussion

To reduce the risk of CF occurrence, we used a new PIVC in the upper arm vein without a guidewire despite it being 88 mm long. In this first-in-human trial to verify its safety, no adverse events or CF occurred. The catheter placement success rate was 100%; it allowed no device malfunction occurred at the catheter placement.

In our previous study, the incidence of CF using SPCs was 29% in the control group compared with 11% in the intervention group, where a care bundle was implemented that used ultrasonography to place the catheter in a larger vessel and secure it in the appropriate position in the vessel during SPC placement (*12*). To further reduce CF, a PIVC needs to be placed near the axillary vein in the upper arm, which has a larger diameter and higher blood flow rate than those in the forearm veins (*17*). PIVCs placed near the axillary vein in the upper arm could avoid CF caused by chemical factors due to administering irritant drugs. In fact, a previous study reported an incidence rate of complications of 2.7% with using a catheter that was placed in the upper arm for vancomycin administration (*18*). However, most existing upper arm PIVCs need a guidewire, which is associated with an increased risk of contamination because it can reach in the vicinity of the axillary vein (*16*). A catheter inserter has to perform many steps for inserting the catheter; the skill is complex, the process is time-consuming, and the medical cost involved in maximal sterile barrier precaution is higher than that with the standard precaution. Catheters that can be inserted into the upper arm without a guidewire are expected to reduce the burden on the medical staff and diminish the risk of CF. In this study, we verified the safety of the new PIVC.

A systematic review of studies using LPCs (6-15 cm in length) showed that the insertion success rate ranged from 86% to 100%, and the CF rate in adult patients ranged between 4.3% and 51.5% (*16*). In the current study, no adverse events occurred during the catheterization in all eight patients, the success rate of the first puncture attempt was 100%, and there was no device malfunction in operation during the puncture. There were no interruptions in the catheter placement due to complaints of pain. There were also no adverse events or CF after placement, and no suspected infectious organisms were detected. Our findings suggest that the new PIVC is as safe as a 80 mm LPC with a guidewire.

The patients in this study generally had low blood platelet count because of their underlying hematological diseases or exposure to cytotoxic chemotherapy, making them susceptible developing subcutaneous hemorrhage upon puncture. In our analysis, no patients developed subcutaneous hemorrhage, possibly because the mean diameter of the vessel at the puncture point was 4.4 mm, and the diameter of the catheter tip position was 5.7 mm, which was approximately five times larger than the catheter outer diameter. It leads to an increased puncture success rate and avoids the mechanical stress to the vessel wall. Additionally, the catheter was sufficiently long to prevent dislodgment, even though the mean depth of the vessel was 7.3 mm.

The cause of a temporary occlusion alarm of the infusion pump in our study is unclear. Between the catheter hub and the skin puncture, the base of the catheter could have been temporarily bent, resulting in occlusion, supported by an emergence of a linear line at the base of the catheter observed under magnification. Patients whose alarms went off had a lower average body mass index and shallower blood vessels than those whose alarms did not go off. The subcutaneous fat layer seemed to be relatively thin in these subjects. It was reported that the subcutaneous adipofascial tissue was

made up of two adipofascial layers; the superficial layer forms a solid structure. It is thought to protect against external forces and the deep layer forms a mobile layer and is thought to lubricate the musculoskeletal movement (*19*). So that means, contraction of the muscle leads to movement of the epidermis along with the subcutaneous tissue, furthermore, thinner subcutaneous fat layer may allow the skin surface to move easily. Therefore, the positional relationship between the catheter hub fixed to the skin surface and the vessel puncture site may have expanded and contracted under the influence of contraction of the underlying muscles. We need to consider the position and method of catheter fixation in the upper arm, considering that the catheter is easily affected by muscle contraction at the fixation position. Taking into consideration these countermeasures may help prevent catheter malfunction.

In summary, our novel PIVC was safe and beneficial for the administration of irritant drugs. Furthermore, this catheter is user-friendly, because it does not need a guidewire. Further studies, including a randomized controlled trials are warranted to further evaluate the effectiveness of the new PIVC.

5. Conclusions

A new catheter without a guidewire did not interrupt medical treatments after placement. The catheter placement success rate was 100%, thus resulting in no device malfunction at the catheter placement. Furthermore, no adverse events, including catheter-related bloodstream infection and CF, occurred. According to the results of the first-in-human trial to verify the safety of the new PIVC, this new catheter without a guidewire is safe for catheterization and administering drugs with a high potential for irritancy. In the case of a temporary occlusion alarm of the infusion pump, the causal relationship is unclear. We consider that the new PIVC is easily affected by the muscle contraction at the fixation position. Therefore, the position and the method of catheter fixation in the upper arm need to be considered.

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References

1. Zingg W, Pittet D. Peripheral venous catheters: an under-evaluated problem. Int J Antimicrob Agents. 2009; 34:S38-S42.
2. Bolton D. Improving peripheral cannulation practice at an NHS Trust. Br J Nurs. 2010; 19:1346-1350.
3. Blanco-Mavillard I, Pedro-Gómez JE, Rodríguez-Calero MÁ, Bennasar-Veny M, Parra-García G, Fernández-Fernández I, Bujalance-Hoyos J, Moya-Suárez AB, Cobo-Sánchez JL, Ferrer-Cruz F, Castro-Sánchez E. Multimodal intervention for preventing peripheral intravenous catheter failure in adults (PREBACP): a multicentre, cluster-randomised, controlled trial. Lancet Haematol. 2021; 8:e637-e647.
4. Marsh N, Webster J, Larsen E, Cooke M, Mihala G, Rickard CM. Observational study of peripheral intravenous catheter outcomes in adult hospitalized patients: a multivariable analysis of peripheral intravenous catheter failure. J Hosp Med. 2018; 13:83-89.
5. Murayama R, Uchida M, Oe M, Takahashi T, Oya M, Komiyama C, Sanada H. Removal of peripheral intravenous catheters due to catheter failures among adult patients. J Infus Nurs. 2017; 40:224-231.
6. Gorski LA, Hadaway L, Hagle ME, Broadhurst D, Clare S, Kleidon T, Meyer BM, Nickel B, Rowley S, Sharpe E, Alexanderet M. Infusion therapy standards of practice, 8th edition. J Infus Nurs. 2021; 44(1S Suppl 1):S1-S214.
7. Mattox EA. Complications of peripheral venous access devices: prevention, detection, and recovery strategies. Crit Care Nurse. 2017; 37:e1-e14.
8. Helm RE, Klausner JD, Klemperer JD, Flint LM, Huanget E. Accepted but unacceptable: peripheral IV catheter failure. J Infus Nurs. 2015; 38:189-203.
9. Tanabe H, Takahashi T, Murayama R, Yabunaka K, Oe M, Matsui Y, Arai R, Uchida M, Komiyama C, Sanada H. Using ultrasonography for vessel diameter assessment to prevent infiltration. J Infus Nurs. 2016; 39:105-111.
10. Takahashi T, Murayama R, Oe M, *et al.* Is thrombus with subcutaneous edema detected by ultrasonography related to short peripheral catheter failure? A prospective observational study. J Infus Nurs. 2017; 40:313-322.
11. Murayama R, Takahashi T, Tanabe H, Nakagami G, Tanabe H, Yabunaka K, Arai R, Komiyama C, Uchida M, Sanada H. The relationship between the tip position of an indwelling venous catheter and the subcutaneous edema. Biosci Trends. 2015; 9:414-419.
12. Takahashi T, Murayama R, Abe-Doi M, Miyahara-Kaneko M, Kanno C, Miwa Nakamura M, Mizuno M, Komiyama C, Sanada H. Preventing peripheral intravenous catheter failure by reducing mechanical irritation. Sci Rep. 2020; 10:1550.
13. Tanabe H, Murayama R, Yabunaka K, Oe M, Takahashi T, Komiyama C, Sanada H. Low-angled peripheral intravenous catheter tip placement decreases phlebitis. J Vasc Access. 2016; 17:542-547.
14. Fabiani A, Eletto V, Dreas L, Beltrame D, Sanson G. Midline or long peripheral catheters in difficult venous access conditions? A comparative study in patients with acute cardiovascular diseases. Am J Infect Control. 2020;

48:1158-1165.
15. Scoppettuolo G, Pittiruti M, Pitoni S, Dolcetti L, Emoli A, Mitidieri A, Migliorini I, Giuseppina Annetta MG. Ultrasound-guided "short" midline catheters for difficult venous access in the emergency department: a retrospective analysis. Int J Emerg Med. 2016; 9:3.
16. Qin KR, Ensor N, Barnes R, Englin A, Nataraja R, Pacilliet M. Long peripheral catheters for intravenous access in adults and children: a systematic review of the literature. J Vasc Access. 2021; 22:767-777.
17. Takahashi T, Shintani Y, Murayama R, Noguchi H, Abe-Doi M, Koudounas S, Nakagami G, Mori T, Sanada H. Ultrasonographic measurement of blood flow of peripheral vein in the upper limb of healthy participants: a pilot study. J Jpn WOCM. 2021; 25:576-584.
18. Caparas JV, Hung HS. Vancomycin administration through a novel midline catheter: summary of a 5-year, 1086-patient experience in an urban community hospital. J Assoc Vasc Access. 2017; 22:38-41.
19. Nakajima, H, Imanishi, N, Minabe, T, Kishi, K, Aiso, S. Anatomical study of subcutaneous adipofascial tissue: A concept of the protective adipofascial system (PAFS) and lubricant adipofascial system (LAFS). Scand J Plast Reconstr Surg Hand Surg. 2004; 38:261-266.

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Brief Report

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Proposal of an *in vitro* thrombus-growth model for evaluating anticoagulants

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SUMMARY The general anticoagulant evaluation requires high expense equipment, reagents, and space. Therefore, not all laboratories can perform research related to anticoagulant. In this study, we propose a novel simple method "*in vitro* thrombus-growth model" that can evaluate anticoagulant ability by measuring weight. The *in vitro* thrombus-growth model is prepared by creating a "growth-clot" with citrate plasma, calcium chloride, and thrombin, and then pouring new citrate plasma onto it. The prepared growth-clots were increased in volume in citrated human plasma, including surpluses calcium chloride, which was released slowly, leading to clot coagulation around the plasma. As a result of evaluating the anticoagulant ability of direct thrombin inhibitor using this *in vitro* thrombus-growth model, it was confirmed that clot growth was suppressed in a concentration-dependent manner. Therefore, this thrombus-growth model is useful as a primary anticoagulant test that can to discover compounds with anticoagulant activity perform in any laboratory.

Keywords Thrombus-growth model, anticoagulants, thrombosis, fibrin clot, *in vitro* model

1. Introduction

The first and second leading causes of death in the world are ischemic heart disease and stroke, respectively, with a marked increase in cardiovascular disease, especially in Asian countries (*1,2*). These diseases have a high prevalence in the elderly, and life expectancy is increasing globally, so it is possible that their prevalence will continue to increase (*3*). Proper use of antithrombotic drugs to prevent thrombosis is important for extending healthy life expectancy. Recently, the development of various anticoagulants, such as direct oral anticoagulants (DOAC), has significantly improved the prevention of thrombus-related diseases and prognosis of patients (*4*). However, it may not be enough to prevent blood clot-related diseases; for example, elderly people often have impaired renal function, DOACs are difficult to use because they are excreted by the kidneys, and some DOACs do not have antagonists and cannot be treated to deal with bleeding complications and so on. As such, it is important to improve the case of these anticoagulants and develop new ones.

To accelerate the development of anticoagulants, an experimental system that can easily evaluate the thrombus formation inhibitory effect is needed. Indeed, the general anticoagulant evaluation requires high expense equipment, reagents, and space. Therefore, not all laboratories can perform research related to anticoagulants. Here, we propose a new experimental system that can evaluate anticoagulant ability without requiring special equipment. Factor IV (Ca^{2+}) is involved in blood coagulation cascade. The anticoagulant sodium citrate, which is used for blood collection, exerts an anticoagulant effect by chelating calcium ions. Thus, citrate plasma regains coagulation activity simply by supplementing citrate plasma with calcium ions. Therefore, the proposed model consisted of a fibrin clot containing a high concentration of calcium ions, which release calcium ions in the plasma and coagulate the surrounding plasma, thereby causing the growth of the fibrin clot. In this thrombus-growth model, fibrin clot weight increases in a calcium chloride concentration-dependent manner. The fibrin clot growth was then evaluated by directly adding the direct thrombin inhibitor argatroban to see if the clot stopped growing.

This thrombus-growth model evaluates thrombus growth by weight; thus, it is not suitable for directly elucidating the mechanism of anticoagulants, but it can

clearly evaluate whether thrombus formation can be inhibited.

2. Materials and Methods

2.1. Growth-clot preparation

Growth-clots were prepared with human citrated plasma, CRYOcheck™ Pooled Normal Plasma (CCN-40) (Precision BioLogic, NS, Canada) 193 µL with 2 M calcium dichloride ($CaCl_2$) (FUJIFILM Wako Chemicals, Osaka, Japan) solution 5 µL (final conc. 50 mM), and 0.1 U/µL thrombin (FUJIFILM Wako Chemicals) 2 µL (final conc. 1 U/mL) in a latex rubber tube (Finger Cots Unroll Type S) (AS ONE, Osaka, Japan) and incubated at room temperature for 45 min in a moistened box.

2.2. Effects of calcium chloride concentration on thrombus-growth

The concentration of the calcium chloride solution for preparing the growth-clots was adjusted, and growth-clots with final concentrations of calcium chloride of 30 mM, 50 mM, and 100 mM were prepared. A total of 3 mL of human citrate plasma was poured into each growth-clot and incubated at 37°C in a water bath for 30 min. Human citrate plasma was removed with a micropipette and growth-clots were weighed by microbalance.

2.3. Effects of solvent contamination on thrombus-growth model

A total of 5 or 10 v/v% saline was added to human citrate plasma and poured into growth-clots and incubated at 37°C in a water bath for 30 min. Further, 0.5 or 2.5 v/v% dimethyl sulfoxide (DMSO) was added to human citrate plasma and poured into growth-clots and incubated at 37°C in a water bath for 30 min.

2.4. Suppression of growth-clots' growth by argatroban

Argatroban was dissolved in DMSO (20 mg/mL) and added to human citrate plasma at a rate of 2 v/v%. Finally, the plasma was poured into growth-clot and incubated at 37°C in a water bath for 30 min.

2.5. Statistical analysis

Growth-clot weights were examined using Student's *t*-test. The level of statistical significance was $p < 0.05$.

3. Results and Discussion

To confirm the growth-clot characteristics of the thrombus-growth model, the effects on growth-clot weight were investigated by changing the calcium chloride concentration. Growth-clot weight in the 30 mM group was low, making it unsuitable for experiments compared with controls. Growth-clot weight and variability were both large in the 100 mM group. In contrast, in the 50 mM group, the growth-clot weight was relatively large and the variation was small (Figure 1). Thus, the calcium chloride concentration in the thrombus-growth model was set to 50 mM.

When evaluating an anticoagulant, it is conceivable to prepare an aqueous solution of the anticoagulant and add it to the thrombus-growth model. Therefore, the effect on growth-clot weight when plasma was diluted with an aqueous solvent was evaluated. The addition of 5 v/v% distilled water or saline to the thrombus-growth model did not affect the weight of the growth-clot. However, the addition of 10 v/v% saline significantly reduced growth-clot weight (Figure 2). Therefore, it seems that addition of anticoagulant aqueous solution to the plasma should be kept within 5 v/v%.

Many medicines are fat-soluble, and it is difficult to dissolve these medicines in water or plasma; thus, the use of organic solvents is recommended. Therefore, we evaluated the effect of DMSO on the thrombus-growth model, which is widely used as a solvent in biological

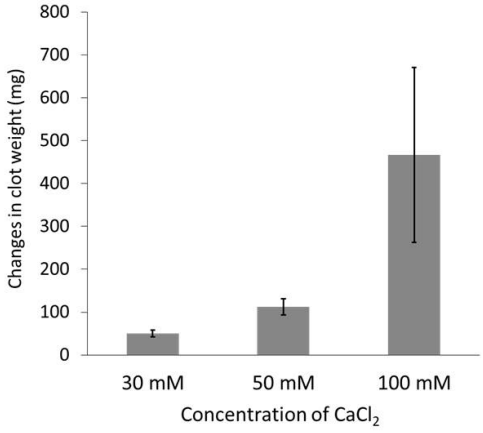

Figure 1. Thrombus-growth weight dependent on $CaCl_2$ concentration. Appropriate concentrations of $CaCl_2$ in thrombus growth were evaluated. mean ± standard deviation (n = 5 for each group).

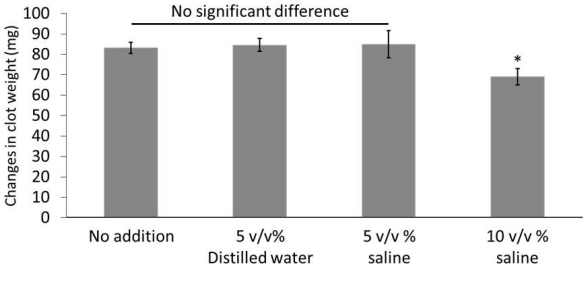

Figure 2. Effect of plasma dilution on thrombus-growth model. An aqueous solvent was added to the thrombus-growth model, and the effect on the thrombus growth amount was evaluated. *$p < 0.05$ *vs*. no addition (Student's *t*-test), mean ± standard deviation (n = 4 for each group).

experiments. The addition of DMSO up to 2.5 v/v% did not affect growth-clot weight, but DMSO at 2.5 v/v% tended to decrease the weight slightly (Figure 3). Therefore, it seems that addition of anticoagulant/DMSO solution to the plasma should be kept below of 2.5 v/v%.

Finally, we evaluated whether clot growth was suppressed when the direct thrombin inhibitor argatroban was added in the thrombus-growth model. Clot growth was suppressed by argatroban in a concentration-dependent manner, and clot growth stopped at 400 μg/mL (Figure 4).

In this study, a thrombus-growth model showed that growth-clots were dependent on calcium chloride concentration and that the mechanism of clot growth was thrombin-dependent as in a living body.

The growth-clots include surpluses of factor IV (Ca^{2+}) of blood coagulation factors. Factor IV was slowly released and coagulated the surrounding plasma into new clots in human citrated plasma. In contrast, growth-clots prepared with 10-fold concentrations (10 U/mL) of thrombin did not change the weight of thrombus growth (data not shown). The fibrin network pore size is affected by thrombin concentration and ionic strength (*5*), but the pore size is about 4-5 μm (*6*) and it is unlikely that thrombin cannot be release. We consider the effects of factor XIII to understand why thrombin cannot be involved in thrombus growth. Factor XIII catalyzes the cross-linking of fibrin and stabilizes the fibrin clot. However, Factor XIII has relatively low substrate specificity, and the substrate of Factor XIII exceeds 140 (*7-9*). Therefore, we guessed that thrombin forms a complex with the fibrin monomer in fibrin clot and thus cannot release. We would like to examine the possibility of cross-linking between Factor XIII and thrombin in the future. We would like to examine the possibility of cross-linking between Factor XIII and thrombin in the future.

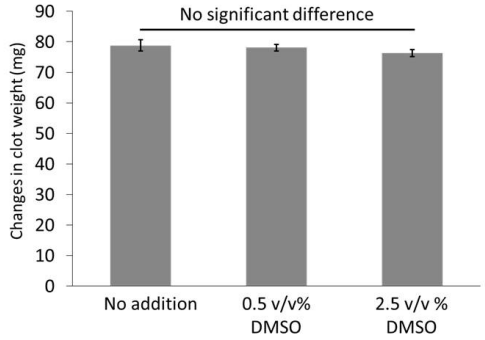

Figure 3. Effect of dimethyl sulfoxide on thrombus-growth model. Dimethyl sulfoxide as fat-solvent was added to the thrombus-growth model, and the effect on the thrombus growth amount was evaluated. mean ± standard deviation (n = 4 for each group).

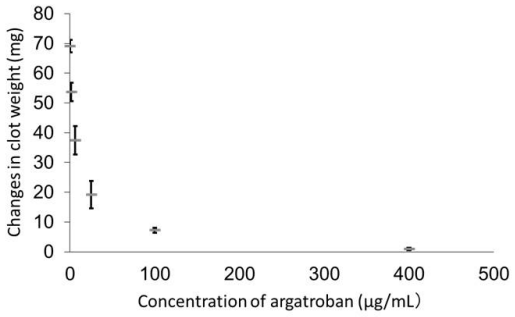

Figure 4. Thrombus growth suppression of thrombus-growth model by argatroban. A thrombus-growth model was tested if it can be suppression of clots growth by direct thrombin inhibitor. mean ± standard deviation (n = 5 for each group).

In this study, a latex rubber tube was used as a container for preparing a thrombus-growth model. This is because we used ultrasonic waves in a previous study (*10*) and decided to use latex rubber because the material does not easily reflect ultrasonic waves. Therefore, the thrombus-growth model does not necessarily have to use latex rubber, and there is no problem with using another container, such as synthetic resin. We also confirmed that growth-clots grow in 5 mL polypropylene and polystyrene test tubes. In addition, although this report proposes a thrombus-growth model using human citrate plasma, similar experiments can be performed using relatively inexpensive bovine citrate plasma (P4639-10ML, Sigma-Aldrich, Tokyo, Japan).

The limitation of this thrombus-growth model is that the weight of growth-clots varies slightly depending on the plasma lot. Therefore, when evaluating the ability of anticoagulants, a comparison target is always necessary. Further, to correct the error between experiments, it is necessary to calculate the thrombus-growth suppression ratio (R) equation as below:

$$R = ((\Delta \text{ control} - \Delta \text{ sample})/\Delta \text{ control}) \times 100\%,$$

where Δ control (Δ sample) is change in clot weight (mg).

There are several tips for growing a growth-clot with good reproducibility. When preparing a growth clot, the surface shape of the growth-clots should be hemispherical with a bulge. Clots do not grow much if they are flat or dented. Thus, careful manipulation is required when pipetting thrombin and plasma. However, slow pipetting increases the viscosity of plasma due to the coagulation reaction, thereby increasing the probability of trapping air bubbles in the growth-clots. Moreover, the reagents used to prepare growth-clots should be kept cold to slow down the blood clotting reaction.

The principle of the instrument that measures the parameters of blood coagulation mainly captures changes in plasma based on the coagulation reaction, such as transparency and viscosity. On the other hand, the thrombus-growth model is a novel evaluation method that can evaluate the formation of fibrin clot by clot weight (mg) without the need for expensive equipment or special reagents. Therefore, the thrombus-growth model

we propose is a utility experimental method that can be performed in any laboratory with common laboratory tool such as microbalance and micropipette.

As a limitation of the evaluation of anticoagulants in this thrombus-growth model, antiplatelet drugs (aspirin, clopidogrel, *etc*.) cannot be evaluated because this model does not contain platelets. It is also not suitable for evaluating vitamin K antagonists such as warfarin. This model is suitable for assessing substances that directly inhibit blood coagulation factors or directly activate anticoagulation factors.

In addition, prothrombin time, activated partial thromboplastin time, and the like are measured in the evaluation of general anticoagulants. Although it is not suitable to analyze these detailed parameters and mechanisms in the thrombus-growth model, it is possible to clearly evaluate the inhibition of thrombus formation. Conversely, even if the mechanism is unclear, it is possible to evaluate the presence or absence of inhibition of thrombus formation.

In conclusion, this thrombus-growth model serves as a primary anticoagulant test that can clearly assess anticoagulant ability even with drugs of both fat-soluble or water-soluble. We hope that this model will contribute to the development of novel antithrombotic drugs.

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References

1. The WHO CVD Risk Chart Working Group. World Health Organization cardiovascular disease risk charts: revised models to estimate risk in 21 global regions. Lancet Glob Health. 2019; 7:e1332-e1345.
2. Zhao D. Epidemiological features of cardiovascular disease in Asia. JACC Asia. 2021; 1:1-13.
3. Go AS, Hylek EM, Phillips KA, Chang Y, Henault LE, Selby JV, Singer DE. Prevalence of diagnosed atrial fibrillation in adults: national implications for rhythm management and stroke prevention: the AnTicoagulation and Risk Factors in Atrial Fibrillation (ATRIA) Study. JAMA. 2001; 285:2370-2375.
4. Bauersachs RM, Herold J. Oral anticoagulation in the elderly and frail. Hamostaseologie. 2020; 40:74-83.
5. Blomback B, Carlsson K, Hessel B, Liljeborg A, Procyk R, Aslund N. Native fibrin gel networks observed by 3D microscopy, permeation and turbidity. Biochim Biophys Acta. 1989; 997:96-110.
6. Carr ME, Jr., Hardin CL. Fibrin has larger pores when formed in the presence of erythrocytes. Am J Physiol. 1987; 253:H1069-1073.
7. Sakata Y, Aoki N. Cross-linking of alpha 2-plasmin inhibitor to fibrin by fibrin-stabilizing factor. J Clin Invest. 1980; 65:290-297.
8. Mosher DF. Cross-linking of cold-insoluble globulin by fibrin-stabilizing factor. J Biol Chem. 1975; 250:6614-6621.
9. Shi DY, Wang SJ. Advances of coagulation factor XIII. Chin Med J (Engl). 2017; 130:219-223.
10. Sawaguchi Y, Wang Z, Itou S, Kikuchi R, Yamamoto H, Tachibana K, Nakajima T, Nakata N. Basic research for development of ultrasonic prevention of vascular occlusion. Neurosonology. 2017; 30:4-7.

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Communication

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Oral high-dose acetylcysteine: Effective against the Omicron variant of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)?

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SUMMARY The Omicron variant of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has a high rate of transmission and it exhibits immune escape characteristics. N-acetyl-L-cysteine (NAC) is a precursor of reduced glutathione (GSH), which can enter cells to play an antioxidant role, so it is better than glutathione. Patients tolerate NAC well, and adverse reactions are rare and mild, so this type of drug with multiple actions is considered to be a mucolytic agent as well as a drug for the prevention/treatment of various diseases, including COVID-19. Previous studies indicated that the clinical effectiveness of NAC is dose-dependent. Low-dose NAC (0.2 g tid for adults) is a mucolytic expectorant, high-dose NAC (0.6 g bid or tid) has expectorant action as well as antioxidant action, and extreme-dose NAC (300 mg/kg.d) is used for detoxification in cases of an acetaminophen overdose. Presumably, orally administered high-dose NAC (0.6 g tid for adults and 10 mg/kg tid for children) could be used as an adjuvant to treat an Omicron infection. It should reduce the time to negative conversion and prevent severe COVID-19, reducing the duration of hospitalization and increasing the bed turnover rate.

Keywords Omicron, N-acetyl-L-cysteine (NAC), high-dose, oral, effectiveness

In November 24, 2021, a highly variable variant of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) named "Omicron (B.1.1.529)" by the World Health Organization (WHO) was discovered in South Africa. Omicron has a high rate of transmission and it exhibits immune escape characteristics. Omicron has a higher rate of infection than the Delta variant, but fortunately Omicron's symptoms are mild, and the rate of hospitalization, the rate of severe disease, and its mortality rate are relatively low (*1*). However, serious cases of Omicron have been reported. Italy reported the first case of severe acute respiratory syndrome caused by Omicron in November 2021 (*2*). As of March 30, 2022, Shenzhen's Third People's Hospital, the only hospital designated to treat COVID-19 in Shenzhen, China, has admitted about 2,600 patients with COVID-19, including about 1,800 with an Omicron infection. Elderly patients and patients with comorbidities, including diabetes, cardiovascular disease, and compromised immunity, are known to be at risk of becoming severely or critically ill. In addition, 20% of patients infected with Omicron were children, who accounted for a very low proportion of patients infected with the Delta variant. Fever and gastrointestinal symptoms in children were more severe than those in adults, and the time to negative conversion was longer. Therefore, the following questions need to be considered: 1. How can patients test negative for the nucleic acids of SARS-CoV-2 as soon as possible and meet the discharge criteria; 2. How can mild cases be prevented from developing into severe or critical cases? (This especially applies to the elderly and children).

COVID-19 can lead to complications such as pneumonia, acute respiratory distress syndrome, cardiovascular disease, and multiple organ failure (possibly due to a cytokine storm), a systemic inflammatory response, and an immune system attack. In addition, excessive oxidative stress is one factor that contributes to the pathophysiology of COVID-19 (*3*). N-acetyl-L-cysteine (NAC) is a precursor of reduced glutathione (GSH), which can enter cells to play an antioxidant role, so it is better than glutathione. Patients tolerate NAC well, and adverse reactions are rare and mild, so this type of drug with multiple actions is considered to be a mucolytic agent as well as a drug for the prevention/treatment of various diseases, including the consumption of glutathione supplements (glutathione

is an antioxidant) in order to combat oxidative stress. Therefore, NAC is a treatment or adjuvant therapy for the following diseases: infectious diseases such as influenza and acquired immunodeficiency syndrome, digestive system diseases such as Crohn's disease and ulcerative colitis, nervous system diseases such as schizophrenia, bipolar disorder, obsessive compulsive disorder, Parkinson's disease, multiple sclerosis, peripheral neuropathy, and stroke (*4*).

Previous studies have reported that NAC inhibits the replication of influenza viruses such as H5N1, it reduces the release of inflammatory factors, it reduces oxidative stress and oxidative damage caused by inflammatory mediators, and it reduces damage to the human body (*5,6*). Studies have also confirmed that NAC can be used as an adjuvant therapy for idiopathic pulmonary fibrosis and pulmonary fibrosis after COVID-19 (*7,8*). A clinical trial indicated that NAC supplements can quickly alleviate a GSH deficiency, antioxidant stress, and oxidative damage in patients with COVID-19 (*9*). Therefore, NAC has recently been proposed as a potential adjuvant therapy for COVID-19 (*10,11*). The mechanisms of NAC therapy for COVID-19 include: 1. inhibiting the envelope (E) protein and thorn (s) protein of the virus and reducing its binding to angiotensin II receptors (AT2R); 2. inhibiting angiotensin-converting enzyme (ACE); 3. inducing the synthesis of endogenous GSH so as to enhance antioxidation and reduce intracellular protein glycosylation; and 4. inhibiting the production of proinflammatory mediators and cytokines (*12*). In addition, high-dose intravenous NAC is reported to play a key role in the treatment of severe cases of COVID-19 (*3,13,14*).

Further research has indicated that the clinical effectiveness of NAC is dose-dependent. Low-dose NAC (0.2 g tid for adults) is a mucolytic expectorant, high-dose NAC (0.6 g bid or tid) has expectorant action as well as antioxidant action, and extreme-dose NAC (300 mg/kg.d) is used for detoxification in cases of an acetaminophen overdose. For patients suffering from an acetaminophen overdose, the initial intravenous dose of NAC is 300 mg/kg over 21 hours, and then 100 mg/kg is infused for more than 16 hours until the level of acetaminophen is less than 20 μg/mL and aspartate aminotransferase (AST) and alanine transaminase (ALT) levels tend to decrease (*15*). For patients with idiopathic pulmonary fibrosis, the NAC dose is 1,800 mg/d (600 mg, tid) orally for more than 1 year, and no adverse reactions were noted (*7*). Twelve patients with chronic obstructive pulmonary disease (COPD) were randomly treated with NAC 1,800 mg per day for 3 months; the treatment was effective without causing adverse reactions (*16*). That said, a study has suggested that whether low-dose NAC (less than or equal to 600 mg per day) is effective in treating COPD is uncertain (*17*). Clinical experiments have indicated that low concentrations of NAC (< 1 μM) in the blood cannot regulate the imbalance of bronchial oxidation, that high concentrations of NAC (2,300 μM) can inhibit the airway inflammatory response caused by nocturnal lipopolysaccharide (LPS) stimulation, and that extreme concentrations of NAC (21 μM) can reduce the release of IL-6 induced by LPS, indicating that high concentrations of NAC can alleviate oxidative damage (*18*). Lai *et al*. confirmed that oral high-dose NAC (1,200 mg, bid) can rapidly increase the level of glutathione in lymphocytes of patients with systemic lupus erythematosus with chronic inflammation, while low-dose NAC (600 mg, bid) cannot achieve this effect (*19*). There is little literature on the dosage for children. The dosage of NAC for treating β-thalassemia in children is 10 mg/kg.d (maximum dose of 600 mg) orally (*20*). Intravenous NAC was used to treat an acetaminophen overdose in children at a dose of 300 mg/kg.d, and the dose per kilogram of body weight of children is the same as that of adults (*21,22*).

NAC can be taken orally, intravenously, or atomized. Numerous studies have reported that oral NAC has the same effectiveness as an injection (*23-26*). NAC is well-tolerated. Compared to an injection, oral administration causes fewer adverse reactions. In most clinical trials, adverse reactions to NAC did not differ significantly from those to a placebo. The most common adverse reaction is mild gastrointestinal symptoms; adverse reactions are uncommon when the dose is less than 2.5 g/day (*27*).

Based on the findings above, orally administered high-dose NAC (0.6 g tid for adults and 10 mg/kg tid for children) could be used as an adjuvant instead of a low-dose expectorant (0.2 g tid for adults and 10 mg/kg qd for children) to treat an Omicron infection. It should reduce the time to negative conversion and prevent severe COVID-19, reducing the duration of hospitalization and increasing the bed turnover rate. The grounds for that contention can be summarized as follows: 1. NAC has been proven to have antiviral action in patients with influenza and adjuvant action in a variety of diseases; 2. Compared to an injection, oral administration is more convenient, safer, and causes fewer adverse reactions; 3. Low doses are only used as an expectorant and NAC's effectiveness as an adjuvant therapy for COVID-19 is uncertain, but only high doses have antioxidant, anti-inflammatory, and antiviral actions. Moreover, high doses of NAC also have expectorant action; and 4. Even if a high dose of NAC, such as 300 mg/kg.d, is injected, adverse reactions are rare and the drug is well-tolerated.

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References

1. Vitiello A, Ferrara F, Auti AM, Di Domenico M,

Boccellino M. Advances in the Omicron variant development. J Intern Med. 2022. doi: 10.1111/joim.13478
2. Micheli V, Bracchitta F, Rizzo A, Mancon A, Mileto D, Lombardi A, Stefanelli P, Gismondo MR. First identification of the new SARS-CoV-2 Omicron variant (B.1.1.529) in Italy. Clin Infect Dis. 2022. doi: 10.1093/cid/ciab1044
3. De Flora S, Balansky R, La Maestra S. Rationale for the use of N-acetylcysteine in both prevention and adjuvant therapy of COVID-19. FASEB J. 2020; 34:13185-13193.
4. Schwalfenberg GK. N-acetylcysteine: A review of clinical usefulness (an old drug with new tricks). J Nutr Metab. 2021; 2021:9949453.
5. Mata M, Morcillo E, Gimeno C, Cortijo J. N-acetyl-L-cysteine (NAC) inhibit mucin synthesis and pro-inflammatory mediators in alveolar type II epithelial cells infected with influenza virus A and B and with respiratory syncytial virus (RSV). Biochem Pharmacol. 2011; 82:548-555.
6. Geiler J, Michaelis M, Naczk P, Leutz A, Langer K, Doerr HW, Cinatl J Jr. N-acetyl-L-cysteine (NAC) inhibits virus replication and expression of pro-inflammatory molecules in A549 cells infected with highly pathogenic H5N1 influenza A virus. Biochem Pharmacol. 2010; 79:413-420.
7. Demedts M, Behr J, Buhl R, *et al*. High-dose acetylcysteine in idiopathic pulmonary fibrosis. N Engl J Med. 2005; 353:2229-2242.
8. Menicagli R, Limodio M, Limodio M, Casotti MT, Menicagli L. Pulmonary Covid fibrosis a new pharmaceutic approach. Int J Prev Med. 2021; 12:35.
9. Kumar P, Osahon O, Vides DB, Hanania N, Minard CG, Sekhar RV. Severe glutathione deficiency, oxidative stress and oxidant damage in adults hospitalized with COVID-19: Implications for GlyNAC (Glycine and N-acetylcysteine) supplementation. Antioxidants (Basel). 2021; 11:50.
10. Jorge-Aarón RM, Rosa-Ester MP. N-acetylcysteine as a potential treatment for COVID-19. Future Microbiol. 2020; 15:959-962.
11. Poe FL, Corn J. N-acetylcysteine: A potential therapeutic agent for SARS-CoV-2. Med Hypotheses. 2020; 143:109862.
12. Wong KK, Lee SWH, Kua KP. N-acetylcysteine as adjuvant therapy for COVID-19 - A perspective on the current state of the evidence. J Inflamm Res. 2021; 14:2993-3013.
13. Elhidsi M, Fachrucha F, Irawan RY. N-acetylcysteine for COVID-19: A potential adjuvant therapy. J Health Sci. 2021; 11:1-6.
14. Mohanty RR, Padhy BM, Das S, Meher BR. Therapeutic potential of N-acetyl cysteine (NAC) in preventing cytokine storm in COVID-19: Review of current evidence. Eur Rev Med Pharmacol Sci. 2021; 25:2802-2807.
15. Downs JW, Cumpston KL, Kershner EK, Troendle MM, Rose SR, Wills BK. Clinical outcome of massive acetaminophen overdose treated with standard-dose N-acetylcysteine. Clin Toxicol (Phila). 2021; 59:932-936.
16. De Backer J, Vos W, Van Holsbeke C, Vinchurkar S, Claes R, Parizel PM, De Backer W. Effect of high-dose N-acetylcysteine on airway geometry, inflammation, and oxidative stress in COPD patients. Int J Chron Obstruct Pulmon Dis. 2013; 8:569-579.
17. Shen Y, Cai W, Lei S, Zhang Z. Effect of high/low dose N-acetylcysteine on chronic obstructive pulmonary disease: A systematic review and meta-analysis. COPD. 2014; 11:351-358.
18. Cazzola M, Calzetta L, Facciolo F, Rogliani P, Matera MG. Pharmacological investigation on the anti-oxidant and anti-inflammatory activity of N-acetylcysteine in an *ex vivo* model of COPD exacerbation. Respir Res. 2017; 18:26.
19. Lai Z, Hanczko R, Bonilla E, *et al*. N-acetylcysteine reduces disease activity by blocking mammalian target of rapamycin in T cells from systemic lupus erythematosus patients: A randomized, double-blind, placebo-controlled trial. Arthritis Rheumatology. 2012; 64:2937-2946.
20. Ozdemir ZC, Koc A, Aycicek A, Kocyigit A. N-acetylcysteine supplementation reduces oxidative stress and DNA damage in children with β-thalassemia. Hemoglobin. 2014; 38:359-364.
21. Doyon S, Klein-Schwartz W. Hepatotoxicity despite early administration of intravenous N-acetylcysteine for acute acetaminophen overdose. Acad Emerg Med. 2009; 16:34-39.
22. Pauley KA, Sandritter TL, Lowry JA, Algren DA. Evaluation of an alternative intravenous N-acetylcysteine regimen in pediatric patients. J Pediatr Pharmacol Ther. 2015; 20:178-185.
23. Kanter MZ. Comparison of oral and *i.v.* acetylcysteine in the treatment of acetaminophen poisoning. Am J Health Syst Pharm. 2006; 63:1821-1827.
24. Tsai CL, Chang WT, Weng TI, Fang CC, Walson PD. A patient-tailored N-acetylcysteine protocol for acute acetaminophen intoxication. Clin Ther. 2005; 27:336-341.
25. Green JL, Heard KJ, Reynolds KM, Albert D. Oral and intravenous acetylcysteine for treatment of acetaminophen toxicity: A systematic review and meta-analysis. West J Emerg Med. 2013; 14:218-226.
26. Shi Z, Puyo CA. N-acetylcysteine to combat COVID-19: An evidence review. Ther Clin Risk Manag. 2020; 16:1047-1055.
27. Amini A, Masoumi-Moghaddam S, Morris DL. Utility of bromelain and N-acetylcysteine in treatment of peritoneal dissemination of gastrointestinal mucin-producing malignancies. Anticancer Res. 2016; 36:3224-3225.

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Letter to the Editor

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Safety of linezolid, rifampicin, and clindamycin combination therapy in patients with prosthetic joint infection

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SUMMARY We investigated adverse events in patients with prosthetic joint infections receiving combination therapy with linezolid, rifampicin, and clindamycin for ≥ 7 days. Twenty-two patients were evaluated. The combination therapy was administered for 15.5 (7–29) days at dosages of 1200, 450, and 450–1200 mg/day for linezolid, rifampicin, and clindamycin, respectively. Adverse events (gastrointestinal, eye, and skin disorders; liver damage; myelosuppression; hyponatremia, and others) were recorded. The incidence rates of leukopenia, neutropenia, anemia, thrombocytopenia, and hyponatremia were 36.4%, 31.8%, 40.9%, 18.2%, and 18.2%, respectively. Common Terminology Criteria for Adverse Events version 5.0 Grade 3 neutropenia, anemia, and hyponatremia were observed. The incidence rate of myelosuppression was higher following combination therapy compared with that previously reported following single-drug administration. All patients were discharged after the infection was under control. It is important to monitor these adverse events during combination therapy with the aforementioned agents; these conditions may be relieved by discontinuing linezolid.

Keywords Myelosuppression, toxicity, hyponatremia, anemia

To the Editor,

For intractable infectious diseases, such as prosthetic joint infections (PJIs), combination therapy with antibacterial agents, such as rifampicin combined with linezolid or clindamycin belonging to rifamycin, oxazolidinone, and lincomycin classes, respectively, is recommended to ensure the effect of the agents at the lesion site (*1*). The combined use of linezolid and rifampicin has been reported to be more likely to cure biofilm-forming PJI compared with linezolid alone while preserving the implant of the orthopedic device (*2*). Clindamycin prevents the emergence of rifampin resistance; the combination displayed synergetic or additive bactericidal activity, and favorable cure rates (*3*). Serious adverse events, such as myelosuppression from linezolid, liver damage from rifampicin, and pseudomembranous enteritis from clindamycin, have been reported, even with single-agent administration (*4-7*). Moreover, rifampicin affects linezolid and clindamycin blood levels; therefore, it is necessary to observe drug interactions (*8,9*). Safety information on single-agent or two-agent combination therapy has been reported; however, that on the combination of three agents is scarce. Therefore, we retrospectively investigated adverse events in patients with PJI who received combination therapy with linezolid, rifampicin, and clindamycin.

This study was conducted according to the Code of Ethics of the World Medical Association (Declaration of Helsinki) and following the approval of the ethics committee of Nissan-Kohseikai Institute of Medicine (approval number: 2021-006). The ethics committee did not request the acquisition of informed consent but posted an opt-out of the study. The subjects were patients with PJI who received a combination of linezolid, rifampicin, and clindamycin for ≥ 7 days at our hospital from May 2017 to February 2021. Data regarding sex, age, aspartate aminotransferase and alanine aminotransferase levels, and estimated glomerular filtration rate before combination therapy were extracted. The duration of combination therapy, dose administered, and clinical outcome were recorded. In addition, the severity, incidence, and number of days until the onset of newly developed adverse events after the start of combination therapy were documented. Severity was assessed using the Common Terminology Criteria for Adverse Events version 5.0. Numerical values are indicated by the number of cases or median (percentage or range).

Twenty-two cases with a median age of 72.6 (40.2-

Table 1. Severity, incidence, and days to onset of adverse events in combination therapy with linezolid, rifampicin, clindamycin

Adverse events	CTCAE severity	*n* (%)	Days to onset[a]
Gastrointestinal disorders			
Stomach pain	Grade 1	3 (13.6)	1 (0-10)
Anorexia	Grade 1	2 (9.1)	13 (13-13)
Dysgeusia	Grade 1	1 (4.5)	13
Nausea	Grade 1	2 (9.1)	1 (1-1)
	Grade 2	1 (4.5)	13
Vomiting	Grade 2	1 (4.5)	6
Diarrhea	Grade 1	2 (9.1)	7 (6-8)
	Grade 2	1 (4.5)	16
Constipation	Grade 2	1 (4.5)	25
Oropharyngeal pain	Grade 1	1 (4.5)	8
Esophageal pain	Grade 1	1 (4.5)	13
Eye disorders			
Blurred vision	Grade 1	2 (9.1)	5 (4-6)
Skin disorders			
Maculo-papular rash	Grade 1	1 (4.5)	13
Laboratory test			
White blood cell count decreased	Grade 1	4 (18.2)	11 (11-13)
	Grade 2	4 (18.2)	6 (4-16)
Neutrophil count decreased	Grade 1	4 (18.2)	15 (11-25)
	Grade 2	1 (4.5)	13
	Grade 3	2 (9.1)	5 (4-6)
Anemia	Grade 2	5 (22.7)	6 (5-21)
	Grade 3	4 (18.2)	16.5 (6-22)
Platelet count decreased	Grade 1	4 (18.2)	13 (2-15)
Aspartate aminotransferase increased	Grade 1	2 (9.1)	9 (5-13)
Alanine aminotransferase increased	Grade 1	2 (9.1)	9 (5-13)
Blood bilirubin increased	Grade 1	3 (13.6)	3 (1-14)
Metabolic and nutritional disorders			
Hyponatremia	Grade 1	2 (9.1)	9 (6-12)
	Grade 2	1 (4.5)	16
	Grade 3	1 (4.5)	13
Black tongue[b]	-	1 (4.5)	7

[a]Number of days from the start of combined use of linezolid, rifampicin, and clindamycin to the onset adverse events. [b]No CTCAE. CTCAE: Common Terminology Criteria for Adverse Events.

93.7) years were evaluated, seven of which (31.8%) involved male patients. Before the combination therapy, aspartate aminotransferase was 16.5 (8.0-51.0) U/L, alanine aminotransferase was 12.0 (3.0-47.0) U/L, and estimated glomerular filtration was 76.1 (45.0-130.6) mL/min. The number of days of combination therapy was 15.5 (7-29) days. The dose was 1,200 mg/day for linezolid and 450 mg/day for rifampicin, and clindamycin varied from 450 to 1,200 mg/day. Adverse events, such as gastrointestinal, eye, and skin disorders; liver damage; myelosuppression; and hyponatremia, among others, were observed and are listed in Table 1. Grade 3 neutropenia, anemia, and hyponatremia were observed. Three patients who developed diarrhea did not have pseudomembranous enteritis. Of the five patients who developed Grade 3 adverse events, two who developed neutropenia and anemia were administered linezolid after the administration of the other two drugs was initiated, whereas the three-agent combination therapy was initiated in the other three patients. In all five Grade 3 adverse event cases, only linezolid was discontinued, and all adverse events subsequently improved. All cases were discharged from the hospital 49.5 (5-282) days after the start of combination therapy.

Although the definitions of adverse events and treatments vary among the cases, the incidence of myelosuppression was higher when the three drugs were used in combination, compared with previous reports on single-drug administration (*4-7*). In previous studies and our study, leukopenia incidence was 0.1-10% and 36.4%, neutropenia was 1%-10% and 31.8%, anemia was 7.1% and 40.9%, and thrombocytopenia was 1-10% and 18.2%, respectively (*4-7*). The incidence of hyponatremia was 18% in a report on linezolid alone (*10*), which was equivalent to 18.2% when the three drugs were used in combination. The high incidence of myelosuppression may have been due to the combination of the three drugs; however, the exact cause could not be established. All cases with Grade 3 adverse events improved after linezolid discontinuation, suggesting that linezolid was the cause. Contrarily, although linezolid is not a substrate for cytochrome P450, it has been reported that the area under the plasma concentration–time curve is reduced by 32% and the incidence of anemia and thrombocytopenia is reduced when linezolid is used in combination with rifampicin (*8*), which contradicts our results. Linezolid blood concentration monitoring was useful in the recovery and amelioration of toxicity when used in combination with rifampicin. Clindamycin, another antibacterial agent used in the combination therapy in this study, may have increased linezolid blood concentration and worsened myelosuppression.

However, there are no reports of an interaction between linezolid and clindamycin.

Conclusively, monitoring of myelosuppression, especially anemia and hyponatremia, is essential during combination therapy with linezolid, rifampicin, and clindamycin; these conditions may be relieved by discontinuing linezolid.

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References

1. Ariza J, Cobo J, Baraia-Etxaburu J, *et al*. Executive summary of management of prosthetic joint infections. Clinical practice guidelines by the Spanish Society of Infectious Diseases and Clinical Microbiology (SEIMC). Enferm Infecc Microbiol Clin. 2017; 35:189-195.
2. Sendi P, Zimmerli W. Antimicrobial treatment concepts for orthopaedic device-related infection. Clin Microbiol Infect. 2012; 18:1176-1184.
3. Leijtens B, Elbers JBW, Sturm PD, Kullberg BJ, Schreurs BW. Clindamycin-rifampin combination therapy for staphylococcal periprosthetic joint infections: A retrospective observational study. BMC Infect Dis. 2017; 17:321.
4. Food and Drug Administration. ZYVOX®: Highlights of prescribing information. *https://www.accessdata.fda.gov/drugsatfda_docs/label/2021/021131s040lbl.pdf* (accessed December 15, 2021).
5. Electronic Medicines Compendium (EMC), clindamycin 600 mg/50 ml solution for infusion - Summary of product characteristics (SmPC) - (emc). (2021c). *https://www.medicines.org.uk/emc/product/11007* (accessed December 15, 2021).
6. Electronic Medicines Compendium (EMC), Dalacin C Capsules 150 mg - Summary of product characteristics (SmPC) - (emc). (2021b). *https://www.medicines.org.uk/emc/product/1210* (accessed December, 15 2021).
7. Electronic Medicines Compendium (EMC), Rifadin 150 mg Capsules - Summary of product characteristics (SmPC) - (emc). (2021a). *https://www.medicines.org.uk/emc/product/6382/smpc* (accessed December 15, 2021).
8. Gandelman K, Zhu T, Fahmi OA, Glue P, Lian K, Obach RS, Damle B. Unexpected effect of rifampin on the pharmacokinetics of linezolid: *In silico* and *in vitro* approaches to explain its mechanism. J Clin Pharmacol. 2011; 51:229-236.
9. Join-Lambert O, Ribadeau-Dumas F, Jullien V, Kitzis MD, Jais JP, Coignard-Biehler H, Guet-Revillet H, Consigny PH, Delage M, Nassif X, Lortholary O, Nassif A. Dramatic reduction of clindamycin plasma concentration in hidradenitis suppurativa patients treated with the rifampin-clindamycin combination. Eur J Dermatol. 2014; 24:94-95.
10. Tanaka R, Suzuki Y, Takumi Y, Iwao M, Sato Y, Hashinaga K, Hiramatsu K, Kadota JI, Itoh H. A retrospective analysis of risk factors for linezolid-associated hyponatremia in Japanese patients. Biol Pharm Bull. 2016; 39:1968-1973.

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Letter to the Editor

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Neuroleptic malignant syndrome in a case of extra-pontine myelinolysis: On the horns of dilemma

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SUMMARY Osmotic demyelination syndrome (ODS) and neuroleptic malignant syndrome (NMS) lead to severe neurological sequalae. Though currently thought to be different syndromes, literature suggests a relation between the two. We present the case of a 45-year-old male who was found to have chronic severe hyponatremia and underwent rapid correction of sodium and developed parkinsonism features. Magnetic resonance imaging (MRI) confirmed extrapontine myelinolysis (a type of ODS). The patient received haloperidol for agitated behavior and developed new features of rigidity, fever, tachycardia and elevated creatine phosphokinase (CPK) levels and thus neuroleptic malignant syndrome was suspected to overlap with ODS. We report this case highlighting the difficulty in differentiating the between ODS and NMS and their relationship.

Keywords extrapontine myelinolysis, neuroleptic malignant syndrome, hyponatremia, parkinsonism

To the Editor,

A 45-year-old alcoholic, hypertensive male receiving chlorthalidone presented to us with complaints of headache and altered sensorium after an episode of generalized tonic-clonic seizure (GTCS). On examination the notable findings were a Glasgow coma score (GCS) E4V2M5, poorly discernible speech, reactive pupils, with normal reflexes.

Initial investigations revealed hyponatremia (91 mEq/L), for which he received hypertonic saline with frequent electrolyte measurements. Over the next 2 days, sodium levels increased rapidly despite stopping all further correction ($[Na^+]$ = 97 mEq/L at 24 h and 117 mEq/L at 48 h). The correction of sodium and further hospital course is depicted in Figure 1.

He suffered from 3 episodes of GTCS on the 3rd day of admission that were managed with benzodiazepines. Given the rapid rate of sodium correction and overt risk factors, a high suspicion of osmotic demyelination syndrome (ODS) was kept. Magnetic resonance imaging (MRI) of the brain on the fourth day revealed subtle hyperintensities in bilateral putamen, thalamus, and caudate lobe with a normal pons, consistent with extrapontine myelinolysis (EPM).

Post recovery from his seizures, he developed mutism and parkinsonism: slow shuffling gait, bradykinesia, resting tremors involving the oro-facio-lingual muscles, and cogwheel rigidity. With suspicion of organic delirium, he was prescribed low-dose haloperidol 0.5 mg on an as-needed basis. After 4 doses of haloperidol, on the 7th day of admission, he had worsening of sensorium (GCS: E3V1M4), fever, generalized rigidity, hyperreflexia, tachycardia beginning on the 9th day. Creatine phosphokinase (CPK) levels were elevated (by 2.5 times), with mild elevation of liver aminotransferases and leukocytosis. Due to recent haloperidol use, neuroleptic malignant syndrome (NMS) was suspected, and lorazepam with bromocriptine were initiated after withholding haloperidol. There was a gradual decrease in rigidity along with the resolution of fever, altered mentation, tachycardia after 3 days, and biochemical parameters also improved gradually. At discharge, he remained irritable with residual rigidity and bradykinesia for which he was prescribed quetiapine. At 3 months, he displayed residual parkinsonian features but was able to return to his job and perform his activities of daily living.

Rapid correction of hyponatremia can result in ODS (*1*). Patho-physiologically, it is characterized by oligodendrocyte damage and subsequent demyelination (*2*). Risk factors include chronic hyponatremia with very low levels of serum $[Na^+] < 105$ mEq/L, rapid correction, alcoholism, hypokalemia *etc*. (*3*). Pons is commonly involved in ODS - called as central pontine myelinolysis (CPM), while half have extension beyond the pons resulting in EPM, and that may be the sole manifestation in up to 13% cases (*4*). The clinical presentation of EPM includes a myriad of movement disorders such as parkinsonism, dystonia, and catatonia.

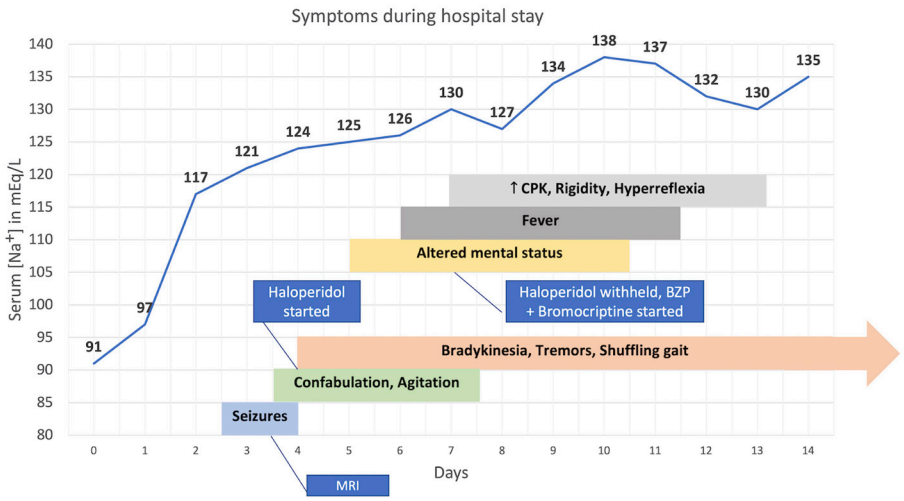

Figure 1. Hospital course of the patient showing the trend of serum sodium [Na^+] levels with time, and the temporal correlation between the symptomatology of EPM and NMS, along with the exposure to haloperidol. BZP, benzodiazepines; CPK, creatine phosphokinase; MRI, magnetic resonance imaging.

Our patient's hospital stay was complicated by fever, worsening mental status, generalized rigidity and persistent tachycardia following haloperidol use. NMS is a distinct clinical syndrome occurring after use of dopaminergic antagonist agents, presenting with a tetrad of altered mental status, fever, rigidity, and autonomic dysfunction. Two dilemmas arose in the present case – first, whether the clinical picture suggested NMS or catatonia; and secondly – should we consider NMS secondary to the haloperidol use or due to EPM.

Differentiating between the NMS and catatonia has remained an enigma, and some authors consider the two illnesses to lie on a spectrum (*5*). NMS diagnosis is considered more likely when fever, rigor, tremors, laboratory evidence of muscle injury, leucocytosis, and diaphoresis are found. In contrast, catatonia is favored when features of negativism, posturing, waxy flexibility, stereotypy, stupor, or agitation are present (*6*). In our patient the symptomatology was more suggestive of NMS.

To address the second difficulty, we reviewed the literature and identified two reports describing ODS presenting as NMS (*7,8*). The first report described a patient on long term quetiapine therapy and developed altered mental status, fever, stiffening, raised CPK values after rapid correction of hyponatremia and an MRI brain found T2 hyperintensities in the caudate nucleus and putamen. The second report describes a hypertensive male with chronic alcoholism and pancreatitis who developed NMS 1 day after receiving neuroleptics for alcohol withdrawal, and an MRI showing prominent findings of CPM with EPM. In both of these reports, patients were exposed to neuroleptics, but NMS seemed unlikely because of specific features like stable doses, use of atypical antipsychotics, compounded by imaging findings consistent with EPM. Although NMS following antipsychotic use is an idiosyncratic reaction, a parenteral route of administration, higher individual doses, and the total dose of drug administered have been linked to increased risk of NMS (*9*).

NMS is a diagnosis of exclusion and can occur even after a single dose of typical antipsychotics. However, an erroneous diagnosis of NMS can result in delayed recognition of other serious medical disorders with similar symptoms and may inhibit future antipsychotic treatment because of unwarranted concerns about recurrent episodes (*7*). We conclude that EPM may clinically mimic NMS, making differentiation between superimposed NMS and pure EPM difficult.

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References

1. Martin RJ. Central pontine and extrapontine myelinolysis: the osmotic demyelination syndromes. J Neurol Neurosurg Psychiatry. 2004; 75 (Suppl 3):iii22-28.
2. Lien YH, Shapiro JI, Chan L. Study of brain electrolytes and organic osmolytes during correction of chronic hyponatremia. Implications for the pathogenesis of central pontine myelinolysis. J Clin Invest. 1991; 88:303-309.
3. Aegisdottir H, Cooray C, Wirdefeldt K, Piehl F, Sveinsson O. Incidence of osmotic demyelination syndrome in Sweden: A nationwide study. Acta Neurol Scand. 2019; 140:342-349.
4. Singh TD, Fugate JE, Rabinstein AA. Central pontine and extrapontine myelinolysis: A systematic review. Eur J Neurol. 2014; 21:1443-1450.
5. Northoff G, Krill W, Wenke J, Gille B, Russ M, Eckert J, Pester U, Bogerts B, Pflug B. Major differences in subjective experience of akinetic states in catatonic and Parkinsonian patients. Cognit Neuropsychiatry. 1998; 3:161-178.

6. Lang FU, Lang S, Becker T, Jäger M. Neuroleptic malignant syndrome or catatonia? Trying to solve the catatonic dilemma. Psychopharmacology (Berl). 2015; 232:1-5.
7. Groff KE, Lam C, Caroff SN. Extrapontine myelinolysis resembling neuroleptic malignant syndrome. J Clin Psychopharmacol. 2005; 25:620-621.
8. González Robledo J, Ballesteros Herráez JC, Chamorro Fernández A, Martín Polo J. Mielinolisis central pontina tras síndrome neuroléptico maligno. Med Intensiva. 2007; 31:342-343.
9. Strawn JR, Keck PE, Caroff SN. Neuroleptic malignant syndrome. Am J Psychiatry. 2007; 164:870-876.

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