ISSN 1881-7831 Online ISSN 1881-784X

DD&T

Drug Discoveries & Therapeutics

Volume 18, Number 4
August, 2024

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DD&T
Drug Discoveries & Therapeutics

ISSN: 1881-7831
Online ISSN: 1881-784X
CODEN: DDTRBX
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.

Drug Discoveries & Therapeutics is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group. It is published bimonthly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA.

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Editorial

DOI: 10.5582/ddt.2024.01055

Tailored to fit: China optimizes policies and regulations regarding drug registration and review to promote innovation in traditional Chinese medicine

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SUMMARY The classification system for drug registration and the review and approval process influence the innovation and development of pharmaceuticals. China's previous classification standards for registration of traditional Chinese medicines (TCMs) overly emphasized the material basis while neglecting the clinical value of TCM. Moreover, the review and approval system did not fully consider the characteristics of new TCM drugs, such as the clinical experience already available for many TCM formulations guided by TCM theories. This resulted in suboptimal quality and quantity in the development of new TCM drugs. Since 2019, China has introduced a series of policies and regulations aimed at reforming the classification system for registration of TCMs and establishing a review system tailored to TCM characteristics. The new classification system for registration of TCMs emphasizes that the development of new TCM drugs should be oriented towards clinical value, focusing on meeting unmet clinical needs. The policies and regulations promote the conversion of prescriptions in ancient classics into new drugs and encourages the conversion of preparations from medical facilities into new TCM drugs. Secondary development of already marketed TCM products is encouraged to enhance the advantages of their clinical use. The new review system places importance on the role of TCM theories and clinical experience in supporting the registration of new TCM drugs. These reform measures have paved a path for registration and review of the characteristics of TCMs and will positively promote the development of new TCMs.

Keywords traditional Chinese medicine, registration, classification, policy, regulations

Traditional Chinese medicine (TCM) is a treasure of the Chinese nation, having made enormous contributions to the health and well-being of the Chinese people over thousands of years. Compared to modern medicine, TCM has a unique theoretical system and plays a distinct role in maintaining and promoting people's health. It has particularly notable advantages in the treatment of specific conditions and specialties such as orthopedics, proctology, pediatrics, dermatology, gynecology, cardiovascular diseases, kidney diseases, and peripheral vascular diseases. During the fight against the COVID-19 pandemic, TCM gained significant attention for its ability to effectively alleviate symptoms, reduce the progression of mild cases to severe cases, increase cure rates, and promote the recovery of individuals in the convalescent phase (*1*). Despite this, the development of innovative TCM drugs faces several challenges and issues. As an example, the classification of TCMs for registration is not sufficiently rational, leading to an overemphasis on the material basis while undervaluing their clinical significance. In addition, the policies and regulations related to the review and approval process are incomplete, causing suboptimal quality and quantity in the development of new TCMs.

In October 2019, the government issued the Opinions on Promoting the Passing Down, Innovation, and Development of Traditional Chinese Medicine (*2*). Addressing various issues and deficiencies in the development of TCM, such as an incomplete TCM care system, inadequate supply of quality TCMs, the shortage of skilled personnel, an imperfect innovation system, and lack of prominent development characteristics, the document proposed comprehensive requirements for reform. In promoting quality drug development, the document emphasized optimizing

the management of TCM evaluation and approval and refining the management of TCM classification and registration. Revised Measures for the Administration of Drug Registration in China were promulgated in January 2020, introducing a new, more rational classifications for TCM registration (*3*). In September 2020, detailed classifications for TCM registration and corresponding application requirements were issued by the National Medical Products Administration (NMPA) (*4*). In February 2023, the NMPA issued the Special Provisions for Registration of Traditional Chinese Medicines, which further refined the requirements for TCM research and development based on the general provisions of drug registration management (*5*). This enhanced the management of the development and registration of new TCMs.

After the reform, the system to manage drug classification categorizes TCM registration into four types: innovative TCMs, improved TCMs, TCMs in ancient classics, and drugs with the same name and formula (*4*). The first three categories fall under the scope of new TCMs. Innovative TCMs include preparations of Chinese herbal medicines, extracts, and preparations obtained from single plants, animals, minerals, *etc*., as well as new herbal materials and their preparations (*4*). Improved TCMs modify the administration route or dosage form of existing marketed TCMs, with advantages and characteristics in terms of clinical use such as enhanced therapeutic functions or new indications (*4*). TCMs in ancient classics refer to formulas recorded in ancient TCM classics that are still currently widely used with proven efficacy, distinctive features, and advantages (*4*). Unlike the previous classification system for registration of TCMs that emphasized the material basis for innovation in TCM, the revised system underscores that the development of new TCM should be guided by clinical value (*5*). It emphasizes assessing clinical benefits and risks, capitalizing on the unique advantages of TCM in disease prevention and treatment, and addressing unmet clinical needs. The strategy for developing novel TCMs focuses on promoting research and development of preparations based on TCM formulas in ancient classics and encouraging the conversion of preparations from medical facilities into new TCMs (*5*). Secondary development of existing marketed TCMs is encouraged to enhance the advantages of their clinical use (*5*).

Another highlight of the reform is the emphasis on the support role of TCM theories and clinical experience in the registration of new TCMs. Unlike chemical drugs, Chinese herbal medicines are often clinically used to treat related diseases before registration. Therefore, the policies and regulations emphasize the supporting role of clinical experience in the safety and effectiveness of TCMs. During the registration and approval process, an integrated system for evaluating evidence that combines TCM theories, clinical experience, and clinical trials is used to comprehensively assess the safety, effectiveness, and quality controllability of TCMs (*5*). A point worth noting is that preparations based on TCMs in ancient classics generally do not require clinical trials (*5*). The approval of these new TCMs mainly relies on the opinions of experts (*5*). This reform reflects respect for the clinical use of TCM formulas from ancient classics in TCM practice and highlights the academic inheritance and clinical characteristics of TCM. For other new TCMs used in clinical practice, if clinical experience can provide supporting evidence in terms of clinical positioning, patient selection, consideration of the duration of treatment and dosage, *etc*., such medicines may skip Phase II clinical trials but generally require Phase III clinical trials to confirm the safety and efficacy (*5*). In contrast, if the development of a new TCM is based on studies involving pharmacological screening rather than TCM theories and clinical experience, necessary Phase I clinical trials should be conducted, followed sequentially by Phase II and Phase III clinical trials (*5*). These reform measures open up a registration and evaluation pathway that considers the characteristics of TCM.

Compared to modern medicine, TCM has its own characteristics regarding indicators of efficacy and methods of assessment. The effectiveness of TCM is not only reflected in the improvement of indicators of disease pathology but also in the improvement of TCM syndromes. Therefore, assessment of the efficacy of TCM can now involve a combination of diseases and syndromes (*5*). TCMs can be classified into three specific categories based on their therapeutic indications (*5*): (*i*) Syndrome-based TCMs: These are formulations guided by TCM theories to specifically treat TCM syndromes. Their therapeutic functions and indications are described using TCM terminology. (*ii*) Combined disease-syndrome-based TCMs: These address both modern medical diseases and TCM syndromes. Their indications are described in terms that combine modern medical diseases and TCM syndromes. (*iii*) Disease-based TCMs: These are medications for specific modern medical diseases formulated in accordance with TCM theories. Their therapeutic functions are described using TCM terminology, while their indications are based on modern medical diseases. In clinical trials, the assessment of efficacy should be based on corresponding indicators of disease pathology or TCM syndromes, fully reflecting the unique characteristics of the efficacy of TCMs. Establishing methods of evaluating safety and efficacy and technical standards that align with the characteristics of TCM is a goal of the reform.

In response to specific situations in clinical use of TCM, recent policy reforms have clarified the criteria for implementing priority review and approval, conditional approval, and special approval. (*i*) Priority review and approval: TCMs that have clear clinical positioning and

significant clinical value and that are used to treat major diseases, rare diseases, clinical emergencies as a result of market shortages, or for pediatric use are eligible for priority review and approval (*5*). (*ii*) Conditional approval: TCMs to treat diseases that are life-threatening and for which no effective treatment is available may be conditionally approved if clinical trial data or quality clinical experience with the TCM indicates its efficacy and predicts its clinical value (*5*). (*iii*) Special approval: TCMs already on the market that require additional indications to address urgent needs during sudden major public health emergencies may undergo special approval to expand their therapeutic indications (*5*). These reforms aim to streamline the regulatory processes for TCMs, ensuring timely access to effective treatments for critical medical conditions and public health emergencies.

In order to fully capitalize on the advantages of the healthcare security system and support the innovation and development of TCM, the National Healthcare Security Administration and the National Administration of Traditional Chinese Medicine jointly issued the Guiding Opinions on Medical Insurance Supporting the Passing down, Innovation, and Development of Traditional Chinese Medicine in December 2021 (*6*). This guidance includes coverage of suitable TCMs by health insurance payments. In order to enhance the full lifecycle management of TCMs and promote continuous improvement in their quality, the NMPA drafted the Regulations on Protection of Traditional Chinese Medicine Varieties (Draft for Public Comments) in December 2022 and solicited public opinions (*7*). These policies and regulations comprehensively and systematically create a TCM management system in order to positively promote the development of new TCMs.

Funding: None.

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

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Received July 15, 2024; Accepted July 18, 2024.

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Released online in J-STAGE as advance publication July 20, 2024.

Review

DOI: 10.5582/ddt.2024.01050

Rethinking the use of direct oral anticoagulants for secondary thromboprophylaxis in patients with thrombotic antiphospholipid syndrome

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SUMMARY Patients with thrombotic antiphospholipid syndrome (APS) are at high risk for recurrent thrombosis, and indefinite anticoagulation is recommended. Patients with APS merit indefinite anticoagulation, and vitamin K antagonists (VKAs) have historically been the standard treatment. Direct oral anticoagulants (DOACs) present an appealing alternative to VKAs. Due to their pharmacokinetic and pharmacodynamic characteristics, DOACs offer advantages over VKAs, namely the lack of need for laboratory monitoring, the usage of a fixed dosage, and the absence of significant interaction with dietary components and drugs. The efficacy and safety of DOACs in patients with APS have been studied in four phase II/III clinical trials (three with rivaroxaban and one with apixaban). These studies showed DOACs' inferiority compared to VKAs in preventing recurrent thrombosis. Recurrence was significantly greater in patients with arterial thrombotic events and a triple positivity for antiphospholipid antibodies. No differences were observed in the incidence of venous thromboembolism between both groups. Major bleeding was similar in patients treated with DOACs or VKAs. Several observational studies have reported similar results. This review aims to analyse the existing evidence on the efficacy and safety of DOACs for secondary prevention in patients with APS.

Keywords Antiphospholipid syndrome, arterial and venous thrombosis, bleeding, direct-acting oral anticoagulants, secondary prevention, vitamin K antagonists

1. Introduction

Antiphospholipid syndrome (APS) is a complex autoimmune disease characterised by arterial, venous, or small vessel thrombosis in any tissue or organ that can present as obstetric morbidity. Meeting the clinical criteria previously mentioned and determining the presence of antiphospholipid antibodies (APLAs) as laboratory criteria is essential for diagnosis. APLAs include lupus anticoagulant (LA) measured by functional clotting assays, anticardiolipin antibody (aCL) and anti-β2 glycoprotein I (aβ2GPI) immunoglobulin (Ig) type G and/or type IgM assayed by solid phase enzyme-linked immunosorbent assay (ELISA) at medium (40-70 GPL units) or high (≥ 80 GPL units) titres. Laboratory testing must be positive on at least two separate occasions 12 weeks apart. New APS classification criteria have recently been published jointly by The American College of Rheumatology (ACR) and The European Alliance of Associations for Rheumatology (EULAR) (*1*). The 2023 criteria are intended to be more specific than the Sydney 2006 criteria (*2*) to better identify patients with APS (99% and 86%, respectively).

APS management is based on long-term anticoagulation due to the high risk of recurrent thrombosis among non-treated patients (*3,4*). Patients with APS should receive indefinite oral anticoagulation with vitamin K antagonists (VKAs). In patients with venous thrombosis the goal is to achieve and maintain an International Normalised Ratio (INR) between 2-3 and in patients with arterial thrombosis to maintain an INR of 2-3 or 3-4, depending on the thrombotic and bleeding risk. In case of recurrence despite an adequate INR in both, arterial and venous thrombosis, the recommendation is to maintain an INR of 3-4 or to add low doses of acetylsalicylic acid (*5*).

Direct oral anticoagulants (DOACs) have shown a similar, in some case even superior, efficacy and safety

profile compared to VKAs in the treatment and secondary prevention of venous thromboembolism (VTE) (*6-8*) and in the prevention of stroke and systemic embolism in patients with non-valvular atrial fibrillation (*6,8-10*). However, controversy has arisen around the use of these drugs in APS. This study aims to review the currently available literature to determine the efficacy and safety of these drugs in secondary thromboprophylaxis in APS to detect groups of patients who may benefit from this antithrombotic therapy.

2. Evidence from clinical trials

Four randomized controlled trials (RCT) have been conducted to evaluate the efficacy and safety of DOACs in secondary prevention of patients with APS (Table 1) (*11-14*). These RCTs include RAPS (rivaroxaban in antiphospholipid syndrome) (*11*), TRAPS (trial on rivaroxaban in antiphospholipid syndrome) (*12*), rivaroxaban versus VKA in APS: a randomized non-inferiority trial (*13*), and ASTRO-APS (apixaban for the secondary prevention of thrombosis among patients with antiphospholipid syndrome) (*14*). RCTs involved 474 patients, comprising 234 assigned to DOACs and 240 to VKAs. Studies were conducted in the United Kingdom (*11*), Italy (*12*), Spain (*13*), and the United States (*14*). Women accounted for 70.8%, and 56% of the participants had triple positive for APLAs. The mean follow-up time was 18.8 months.

RAPS study, the first in this field, was a prospective, open-label, non-inferiority clinical trial in patients with APS and a history of VTE, whose main objective was to demonstrate the non-inferiority intensity anticoagulation achieved with rivaroxaban when compared to warfarin, using endogenous thrombin measurement (*11*). One hundred and sixteen patients treated with warfarin for at least three months after the VTE event were included. Although rivaroxaban did not meet the primary endpoint of the study versus warfarin, no recurrent thrombotic events were observed and both drugs were equally safe in terms of major bleeding.

TRAPS (*Trial on Rivaroxaban in AntiPhospholipid Syndrome at high-risk for thromboembolic recurrence*) study was a prospective, randomized, non-inferiority trial comparing the efficacy and safety of rivaroxaban versus warfarin for the prevention of recurrent thrombotic events, major bleeding and death from vascular cause in patients with triple-positive APLAs (LA, aCL and aβ2GPI) (*12*). Patients were randomized to receive rivaroxaban 20 mg/day or warfarin (target INR 2.5). The trial was prematurely stopped by the adjudication and safety committee for an excess of events in the rivaroxaban group. At the time of trial termination, 120 patients had been randomized: 59 in the rivaroxaban group and 61 in the warfarin group. Seven patients experienced arterial events in the rivaroxaban arm, whereas there were no cases of arterial thrombosis in the warfarin group. In the rivaroxaban arm, ischemic stroke occurred in 4 (7%) patients, and myocardial infarction occurred in 3 (5%) patients. No episode of VTE was recorded in either arm. There were 4 and 2 cases of major bleeding in the rivaroxaban and warfarin groups, respectively (hazard ratio [HR], 2.5; 95% confidence interval [CI], 0.5-13.6; $p = 0.3$). The use of rivaroxaban in patients with APS and triple-positive APLAs were associated with an increased rate of arterial thrombotic events compared with warfarin.

A Spanish study evaluated the efficacy and safety of rivaroxaban administered at doses of 20 mg or 15 mg daily depending on renal function, compared with VKAs in patients with thrombotic APS (*13*). After three years of follow-up, recurrent thrombosis occurred in 11 of 95 patients (11.6%) treated with rivaroxaban and in 6 of 95 (6.3%) treated with VKAs (relative risk [RR], 1.83; 95% CI, 0.71-4.76; $p = 0.21$). Notably, 9 of the 11 recurrences in the DOAC arm were strokes (81.8% *vs.* 0%; $p < 0.001$). Rivaroxaban did not meet the specified criterion for non-inferiority to VKAs. The incidence of major bleeding was 6.3% (6/95) in the rivaroxaban group and 7.4% (7/95) in the VKA group (RR 0.86; 95% CI, 0.30-2.46; $p = 0.77$). In this study, rivaroxaban showed a near doubling of the risk for recurrent thrombosis compared to VKAs for thrombotic APS.

More recently, in the ASTRO-APS study, 48 patients with APS were randomised to receive apixaban 2.5 mg/12 h, apixaban 5 mg/12 h or warfarin (*14*). The primary efficacy objective was the combined endpoint of thrombosis (arterial and venous) and death from vascular cause, and the primary safety objective was major bleeding and clinically relevant minor bleeding. The primary efficacy endpoint occurred in 6 of 23 patients (26%) treated with apixaban (3 in the 2.5 mg group and 3 at the 5 mg dose). All were strokes. None of the patients randomised to warfarin had thrombotic recurrence. The incidence of major bleeding was 4% (1/25) in the warfarin group with no events in apixaban-treated patients. Apixaban showed inferiority compared to warfarin in preventing recurrent thrombosis, especially strokes, among patients with APS.

3. Direct oral anticoagulants in secondary prevention in arterial thrombosis in patients with antiphospholipid syndrome

RCTs have consistently demonstrated a significantly increased risk of subsequent arterial thrombosis in patients treated with DOACs. The overall incidence of new arterial thrombosis in the group treated with DOACs was 10.3% versus 1.3% in those receiving VKAs (odds ratio [OR], 5.43; 95% CI, 1.87-15.75; $p < 0.001$) (*15*). Compared with VKAs, rivaroxaban and apixaban showed a higher rate of stroke (8.6% versus 0%; OR, 10.74; 95% CI, 2.29-50.38; $p < 0.001$). No differences were observed for myocardial infarction (1.3% versus 0%; OR, 2.15;

Table 1. Results of clinical trials investigating the efficacy and safety of direct oral anticoagulants for secondary prevention of thrombosis in patients with thrombotic antiphospholipid syndrome

	RAPS (*11*)	TRAPS (*12*)	Ordi-Ros *et al*. (*13*)	ASTRO-APS (*14*)
Year	2016	2018	2019	2022
Country	United Kingdom	Italy	Spain	United States
Initial thrombosis event	VTE	Arterial and venous	Arterial and venous	Arterial and venous
Median follow-up in months	7	20.4	36	12
DOACs/comparison	Rivaroxaban 20 mg day / Warfarin (INR 2.5)	Rivaroxaban 20 or 15 mg day / Warfarin (INR 2.5)	Rivaroxaban 20 or 15 mg day / VKA (INR 2-3)	Apixaban 5 or 2,5 mg/12 h / Warfarin (INR 2-3)
Sample size	57 / 59	59 / 61	95 / 95	23 / 25
Mean age	47 / 50	46.5 /46.1	47* / 51*	46 / 48.5
Female gender, %	74 / 71	66 /62	64 / 63	83 / 84
APLAs profile, %				
Simple	60 / 48	0 / 0	34 / 32	22 / 28
Double	28 / 32	0 / 0	5 / 8	17 / 8
Triple	12 / 20	100 / 100	61 / 60	30 / 28
Efficacy primary endpoint	Intensity of anticoagulation achieved by thrombin measurement	Recurrent thrombosis	Recurrent thrombosis	Combined endpoint for arterial and venous thrombosis and death from vascular cause
Recurrent thrombosis	None for both groups	12% versus 0%	11.6% versus 6.3%, (p = 0.21)	26% versus 0%
Major bleeding	None for both groups	7% versus 3%, (p = 0.30)	6.3% versus 7.4%, (p = 0.77)	0% versus 4%
Study findings	Rivaroxaban did not meet the primary efficacy endpoint versus warfarin (endogenous thrombin 1086 nmol/L per minute versus 548 nmol/L per minute, respectively; $p < 0.0001$); however, no recurrent thrombotic events were observed.	Rivaroxaban was inferior to warfarin for prevention of recurrent thrombosis.	Rivaroxaban did not demonstrate non-inferiority to VKAs. The recurrent thrombosis rate was twice as high as with VKAs.	Apixaban was inferior to warfarin for prevention of recurrent thrombosis.

*Ordi-Ros *et al*. used median and interquartile range. Abbreviations: APLAs, antiphospholipid antibodies; DOACs, direct oral anticoagulants; VKAs, vitamin K antagonists; VTE, venous thromboembolism.

95% IC, 0.35-13.11; $p = 0.41$) or peripheral embolism (0.4% versus 1.3%; OR, 0.58; 95% CI, 0.12-2.92; $p = 0.51$).

Observational cohorts comparing DOACs to VKAs also show an increased incidence of arterial events in patients treated with DOACs. In the study by Williams *et al*., a nearly 3-fold increased risk of arterial recurrence was observed with rivaroxaban, apixaban and edoxaban compared to warfarin (*16*). This ratio was previously detected with rivaroxaban in a prospective series (*17*).

Meta-analyses have reported a significantly increased risk of new arterial thrombosis with DOACs compared to VKAs. Except for the meta-analysis by Sánchez-Redondo *et al*. (*18*), a higher incidence of new arterial events have been demonstrated in patients treated with DOACs with varying follow-up periods (6 to 72 months). Reviews by Wu *et al*. (OR, 2.27; 95% CI, 1.28-4.00; $p < 0.005$) (*19*), Gullapalli *et al*. (OR, 2.61; 95% CI, 1.44-4.71; $p < 0.001$) (*20*) and Attachaipanich *et al*. (OR, 4.06; 95% CI, 1.33-12.40) (*21*) consistently show a significant increase in arterial recurrence in patients treated with DOACs. Gullapalli *et al*. also analysed the risk of recurrent arterial thrombosis in the patient subgroup additionally receiving antiplatelet therapy. A higher risk was reported in the DOACs arm, although statistical significance was not reached (6.7% in the DOACs arm versus 2.3% in the VKAs arm) (*20*).

4. Direct oral anticoagulants in secondary prevention of venous thromboembolism in patients with antiphospholipid syndrome

Clinical trials found that the incidence of VTE was low in individuals with APS treated with either DOACs or VKAs (1 pulmonary embolism [PE] and 3 cases of deep vein thrombosis [DVT] with DOACs, and 3 events of DVT with VKAs). No differences were observed in the incidence of VTE between both groups (1.7% versus 1.3%; OR, 1.20; 95% CI, 0.31-4.55; $p = 0.79$) (*15*). The risk of PE (0.4% versus 0%; OR, 1.49; 95% IC, 0.23-9.53; $p = 0.68$) and DVT (1.3% versus 1.3%; OR, 1.03; 95% CI, 0.23-4.57; $p = 0.97$) was similar.

In observational studies that have compared DOACs to VKAs, the results regarding the incidence of VTE are heterogeneous. Three cohorts' studies (*16,17,22*) report a similar incidence of VTE, a statement not corroborated by Sato *et al*. (*23*). Notably, in this series two of the three events were cerebral venous sinus thrombosis (CVST).

Several meta-analyses have demonstrated that the incidence of VTE among both treatments is similar. A recent meta-analysis analysing 1,145 patients from 9 studies conclude that the incidence of VTE is similar in individuals with APS treated with either DOACs or warfarin (OR, 1.22; 95% CI, 0.68-2.17; $p = 0.51$) (*19*). Another analysis pooling 12 studies with a total of 1,437 patients showed an incidence of venous thrombosis (DVT/PE/CVST) of 3.8% in patients treated with DOACs and 2.6% in those treated with VKAs (OR, 1.17; 95% CI, 0.66-2.07; $p = 0.60$) (*20*).

5. Influence of immune phenotype on recurrent thrombosis

APLAs profile has prognostic implications. Triple positivity (AL, aCL and aβ2GPI) increases the risk of recurrent thrombosis. In a Chinese study of patients with APS, the recurrence was significatively higher in triple positive patients treated with DOACs versus VKAs (RR, 3.65; 95% CI, 1.49-8.93) (*19*). In the series by Dufrost *et al*. involving 122 patients with APS, there was a clear trend towards new thrombosis in triple-positive patients treated with DOACs versus warfarin (OR, 3.69; 95% CI, 1.12-12.14; $p = 0.05$) (*24*). In the meta-analysis by Gullapalli *et al*. involving a total of 1,437 patients with APS, the recurrence was significantly higher in the triple positive DOACs arm (RR, 4.50; 95% CI, 1.91-10.63; $p = 0.0006$) (*20*). In another recent meta-analysis triple positivity was associated with a 4-fold increased risk for thrombotic recurrence (*25*). A study comparing the occurrence of new thrombosis with rivaroxaban, apixaban and dabigatran versus VKAs showed a significantly higher incidence of recurrence in triple positive patients (56% versus 23%, respectively; OR, 4.3; 95% CI, 2.3-7.7; $p < 0.0001$) (*26*).

The results the regarding the recurrence of thrombosis in patients with single/double positive APLAs are controversial. In the previously mentioned RAPS study, 85% of patients were single/double positive (*11*). The authors found no difference in the development of new thrombotic events between rivaroxaban and warfarin in this subgroup of patients. The study by Legault *et al*. analysed the safety of rivaroxaban (single arm) in 82 patients with APS, none of whom were triple positive. After a median follow-up of 19 months, four recurrences were observed (two strokes and two VTE events) (*27*). The authors conclude that the incidence of recurrent thrombosis is comparable to VKA treatment. In a retrospective cohort of patients with APS treated with DOACs, the incidence of a new thrombosis was 1.7 events/year in triple positive patients compared to 0.7 for single/double-positive patients (RR 2.72; 95% CI, 0.41-18.0) (*28*). More recently, a retrospective study including 50 patients with non-triple positive APS and VTE treated with DOACs observed a low incidence of new thrombosis (0.64 events per 100 patients/year) (*29*). In contrast, in other series no differences were observed between single/double and triple positive groups, although without reaching statistical significance (*16,30*).

6. Major bleeding as an adverse effect of anticoagulant therapy

Anticoagulant therapy carries an increased risk of bleeding complications. In this regard, DOACs

Table 2. Clinical guideline recommendations for anticoagulation therapy in patients with thrombotic antiphospholipid syndrome

Clinical Guidelines	Recommendation
European Alliance of Associations for Rheumatology (EULEAR), 2019 (*5*)	For patients with venous thrombosis, indefinite anticoagulation is recommended. DOACs may be an alternative in patients unable to achieve target INR with VKAs, or intolerant to VKAs. Rivaroxaban should not be used in triple positive patients due to the increased risk of recurrent thrombosis. For arterial events, indefinite anticoagulation is also recommended, avoiding the use of DOACs.
European Society of Cardiology (ESC), 2019 (*35*)	Indefinite treatment with VKAs is recommended. DOACs are not recommended.
American Society of Hematology (ASH), 2020 (*36*)	Indefinite anticoagulation with VKAs is recommended. The use of DOACs is discouraged.
British Society for Haematology (BSH), 2020 (*37*)	For patients with venous thrombosis, indefinite anticoagulation is recommended. DOACs should not be used in triple-positive patients. Evidence is insufficient to establish recommendations in single or double positive patients. In general, it is suggested to avoid them; however, if patients are already being treated with DOACs, they may be continued depending on the clinical profile and patient preferences. In patients with arterial thrombosis, indefinite treatment with VKAs is recommended. DOACs are not recommended.
National Institute for Health and Care Excellence (NICE), 2020 (*38*)	VKAs are recommended in triple positive patients.
International Society on Thrombosis and Haemostasis (ISTH), 2020 (*39*)	In patients with high-risk thrombotic APS*, VKAs are recommended. In patients with APS without high-risk criteria who are already on DOACs therapy, it may be maintained depending on the clinical profile and patient preference.
American College of Cardiology (ACC), 2024 (*40*)	DOACs are not considered standard treatment in patients with APS.

*High-risk thrombotic APS is defined as meeting at least one of the following criteria according to the Sydney Convention (2006): a) triple positivity, b) arterial thrombosis, c) small vessel thrombosis with organ involvement, d) cardiac valvular heart disease. *Abbreviations*: APS, antiphospholipid syndrome; APLAs, antiphospholipid antibodies; DOACs, direct oral anticoagulants; INR, international normalised ratio; VKAs, vitamin K antagonists.

have proven superior to VKAs in patients with atrial fibrillation, particularly in reducing the incidence of intracranial haemorrhage by up to 50% (*31-33*).

In the four RCTs with DOACs, 20 major bleeding events were reported, 10 in each treatment arm. Pooled data showed no difference between therapy with DOACs or VKAs (4.3% versus 4.2%; OR, 1.02; 95% CI, 0.4-2.47; $p = 0.97$) (*15*). There was also no difference for clinically relevant non-major bleeding (6.0% *vs.* 2.9%; OR, 1.90; 95% CI, 0.78-4.66; $p = 0.16$).

All meta-analyses show that the risk of bleeding is similar in patients with APS treated with DOACs or VKAs. The rates of major bleeding were 4.4% and 4.2%, and the rates of clinically relevant non-major bleeding were 5.6% and 5.5%, respectively, during a mean follow-up period of 12-36 months *(19,20,24-26,*34). The incidence of intracranial haemorrhage was also equivalent for both groups (0.37% for DOACs and 0.42% for VKAs).

7. Conclusion

In conclusion, patients with APS are at high risk of recurrent thrombosis; therefore, they require indefinite anticoagulant therapy. Based on the outcomes of RCTs with rivaroxaban and apixaban, DOACs are generally not recommended (Table 2). Nevertheless, the outcomes were poor in high-risk APS patients, including those with previous arterial thrombosis and triple positivity. Furthermore, these studies were small and lacked sufficient power to evaluate thrombotic outcomes robustly. Currently, the clinical guidelines of EULAR (*5*), the British Society for Haematology (*37*) and the International Society on Thrombosis and Haemostasis (*39*) suggest that DOACs could be an alternative in patients unable to achieve the INR target with VKA drugs or who are intolerant to them, as well as in non-high-risk APS patients, including single- or double-positive serology and prior venous thrombosis. In these patients, the use of DOACs appears reasonably safe. We emphasize the need for ongoing research further to optimize anticoagulant therapy for this challenging patient population.

Funding: None.

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

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Received July 3, 2024; Revised August 10, 2024; Accepted August 19, 2024.

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Released online in J-STAGE as advance publication August 28, 2024.

Original Article

DOI: 10.5582/ddt.2024.01037

Prevalence of pregnancy- and lactation-associated osteoporosis in the postpartum period: A systematic review and meta-analysis

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SUMMARY This systematic review and meta-analysis aimed to estimate the prevalence of pregnancy- and lactation-associated osteoporosis in postpartum women within 1 year of delivery. We searched MEDLINE *via* PubMed and Igaku Chuo Zasshi for articles published in English or Japanese from the inception of the database to September 2021. Two researchers independently screened and included observational studies reporting the prevalence of pregnancy- and lactation-associated osteoporosis in postpartum women within 1 year of delivery. Of the 3,425 screened records, 8 articles centered on postpartum women were included in the review. Seven studies used dual-energy X-ray absorptiometry for assessing bone mineral density, while one used a quantitative ultrasound method. In the seven studies that used dual-energy X-ray absorptiometry, the parameters used to define osteoporosis were the T-score (two studies), Z-score (three studies), both T- and Z-scores (one study), and young adult mean (one study). Evaluation timeframes included 1 week (three studies), 1–2 months postpartum (three studies), and 1 week to 12 months postpartum (one study). The estimated prevalence of pregnancy- and lactation-associated osteoporosis defined by dual-energy X-ray absorptiometry was as follows: lumbar spine (six studies), 5% (95% confidence interval [CI], 0–13; heterogeneity [I^2] = 99%) and femoral neck (three studies), 12% (95% CI, 0–30; I^2 = 99%). Pregnancy and lactation were found to elevate the fracture risk in women, underscoring the necessity for a standardized assessment in diagnosing pregnancy- and lactation-associated osteoporosis. This imperative step aims to enable early detection and treatment of bone mineral loss among postpartum women.

Keywords Bone mineral density, breastfeeding, maternal health, postnatal care, dual-energy X-ray absorptiometry

1. Introduction

Pregnancy- and lactation-associated osteoporosis (PLO) is a condition marked by decreased bone mineral density (BMD) and deterioration of bone structure during late pregnancy and postpartum lactation, elevating the vulnerability to fractures. The decline in BMD typically advances without overt symptoms, leading to a subclinical course that may go unnoticed. PLO diagnosis is often prompted by unexpected fractures, including fragility fractures of the vertebral body or fractures resulting from minor external forces during routine activities. Consequently, healthy pregnant or postpartum women may experience sudden fractures, underscoring the covert nature of PLO onset, evident only with the occurrence of fractures (*1*).

Multiple factors are involved in the pathogenesis of PLO, including the pre-existing risk factors for osteoporosis before pregnancy, alongside the characteristics of bone metabolism during pregnancy and lactation (*2*). Pre-existing risk factors for osteoporosis, such as low peak bone mass due to low body weight or poor nutrition, can increase susceptibility to PLO (*3,4*). Hormonal alterations during pregnancy, particularly elevated estrogen levels, promote bone preservation by inhibiting bone resorption (*5*). However, the placental transfer of calcium from mother to fetus may transiently deplete maternal bone calcium (*6*). Subsequently, a sharp postpartum drop in estrogen levels, coupled with prolonged low levels during lactation, prompts

heightened bone resorption and diminished bone formation, ultimately contributing to PLO. Additionally, the production of breast milk necessitates extra calcium; if maternal calcium intake inadequately satisfies both maternal and infant requirements, calcium demand is met through bone resorption to support milk production, consequently eroding maternal bone density (*7,8*). Complete breastfeeding and increased lactation volume or duration precipitate a more pronounced bone loss due to heightened calcium demand (*5*).

Postpartum fractures not only cause distress to the woman, but the treatment associated with the fracture also affects her quality of life and the health of her infant. Typically, women with postpartum fractures discontinue breastfeeding to curtail heightened bone resorption (*2,9-11*). This interruption places postpartum women at risk for mastitis and deprives infants of the infection-protective benefits inherent in breast milk (*12*). Therefore, preventive care for PLO-related fractures during pregnancy and postpartum is imperative. A systematic review by Qian *et al*. (*3*) focusing on patients with PLO associated with vertebral fractures has reported important information on the clinical manifestations of fractures, risk factors, and treatment options. However, while some PLO patients experience fragility fractures (*i.e.*, overt PLO), many others live with decreased BMD without being aware of their condition (*i.e.*, covert PLO). This oversight regarding the potential impact of pregnancy and lactation on bone health results in undiagnosed patients missing necessary care and intervention.

Addressing this issue necessitates establishing the prevalence of PLO, furnishing healthcare providers with a comprehensive understanding of the scope of the problem and its potential impact on maternal health. Although PLO is predominantly identified after a fracture occurs and the prevalence of PLO with vertebral fractures is currently known to be 4-8 per 1 million pregnancies (*2,3*), the exact prevalence of PLO in individuals who have yet to experience fractures remains unknown. A systematic review by Karlsson *et al*. indicates that BMD diminishes by approximately 5% during pregnancy and a further 5% during the postpartum period to 6 months postpartum (*13*). This suggests that many lactating women are prone to osteoporosis without documented fractures.

Defining methods to measure BMD and establishing diagnostic cutoff values for PLO is necessary to establish standardized guidelines (*14*). Some reports have used existing osteoporosis criteria, such as the World Health Organization (WHO) diagnostic criteria for osteoporosis, specifying a T-score < -2.5 standard deviation (SD) using 20-29-year-old Caucasian women as a reference (*15*) or the Z-score recommended for young adults (*16*). The prevalence of PLO could potentially be approximated from such reports (*4,17*). Consequently, this study aimed to determine the prevalence of PLO in the postpartum phase using these reports.

2. Materials and Methods

2.1. Search strategy and selection criteria

We searched MEDLINE *via* PubMed for articles published in English from the inception of the database until September 2021. Additionally, we searched Igaku Chuo Zasshi (Ichushi Web) to include articles written in Japanese. The following search terms were used: "pregnancy," "postpartum," "lactation," "bone mineral density," and "osteoporosis."

The eligibility criteria for the systematic review were: 1) inclusion of postpartum women within 1 year of delivery as participants, 2) use of BMD as an outcome, and 3) cross-sectional or cohort study design, in which prevalence could be calculated. When several articles used the same dataset, the article with the largest number of participants was selected for systematic review. The search results were de-duplicated using Rayyan (*http://rayyan.qcri.org*) before screening by two researchers.

2.2. Quality assessment and data extraction

Two independent researchers (M. F. and M. K.) screened the titles and abstracts of identified articles, followed by full-text reviews to confirm eligibility based on inclusion criteria. The quality of the articles was assessed using the Risk of Bias Assessment Tool for Nonrandomized Studies (RoBANS), focusing on six domains: selection of participants, confounding variables, measurement of exposure, blinding of outcome assessment, incomplete outcome data, and selective outcome reporting (*18*). After independent screening and evaluating bias by individuals, the researchers resolved disagreements through discussions.

2.3. Data synthesis and analysis

We conducted a meta-analysis to estimate the pooled prevalence of PLO. Data analysis was performed using the R statistical software (Version 4.4.0; R Foundation for Statistical Computing, Vienna, Austria) in the Google Colaboratory (Google, Mountain View, CA, USA) environment, using the meta package's metaprop function. Event counts and total participants from each study were used to calculate the proportions and their 95% confidence intervals (CIs).

The DerSimonian–Laird random-effects model was applied with the Freeman–Tukey double arcsine transformation to stabilize the variances of the proportions. Heterogeneity among the studies was assessed using the I^2 statistics and τ^2 variance component. Forest plots were generated to visually assess the extent of heterogeneity across the studies.

For all statistical analyses, statistical significance was

set at $P < 0.05$. All analyses were performed using the R statistical software.

3. Results

3.1. Study selection process

Overall, 3,695 records were identified through electronic database searches, and 67 duplicate articles were excluded (Figure 1). The titles and abstracts were screened based on the inclusion and exclusion criteria, and 3,277 records were excluded. Of the remaining 351 studies, full texts were screened, and 344 studies failing to meet the inclusion criteria concerning population and outcome, study design, language, and the non-utilization of the same data source were excluded. Additionally, studies that did not offer adequate information for calculating the prevalence were excluded. After evaluating the quality of the articles using the RoBANS, eight articles that focused on postpartum women were included in the review (Table 1 and Supplementary Figure 1).

3.2. Characteristics of the studies reviewed

Of the eight studies, six excluded women with complications or obstetric diseases and focused on healthy women (*17,22,19,4,20,21*); four of these studies excluded women with diseases or those taking medications that affect bone metabolism (*17,22,20,21*). Of the remaining two studies, one study did not exclude women with complications or obstetric diseases but did exclude those taking medications that affect bone metabolism (*24*). The other study included all women who delivered at the study hospital (*23*).

Seven studies measured BMD using dual-energy X-ray absorptiometry (DXA) and employed the T-score (*19,20*), Z-score (*4,21,22*) both T and Z scores (*23*), and young adult mean (YAM) (*17*), as parameters for the definition of osteoporosis. One study used quantitative ultrasound (QUS) to measure BMD and stiffness index as a parameter to define osteoporosis (*24*).

Six studies measured BMD at the lumbar spine (*4,17,19,20,22,23*), three at the femoral neck (*4,17,23*), two at the hip (*4,17*), one at the trochanter (*4*), one at the distal radius (*21*), and one at the calcaneus (*24*). The assessment periods were 1 month postpartum (*4,19,20,22,23*), 3 months postpartum (*24*), and 1 week to 12 months postpartum (*21*).

3.3. Prevalence of PLO

The estimated prevalence of PLO, measured by DXA, was as follows (Figure 2): lumbar spine (*4,17,19,20,22,23*) at 5% (95% CI, 0-13; $I^2 = 99\%$, $P < 0.01$) and femoral neck (*4,17,23*) at 12% (95% CI, 1-30; $I^2 = 99\%$, $P < 0.01$).

4. Discussion

We conducted a systematic review and meta-analysis to assess the prevalence of PLO in postpartum women. Eight articles were included in this analysis, with seven using DXA for BMD measurement and one employing QUS. Most measurements were conducted approximately 1 month postpartum. The estimated prevalence of PLO during the postpartum period was 5-12%, drawn from six studies that evaluated lumbar spine BMD *via* DXA and three studies that measured femoral neck BMD.

The prevalence of PLO reported in this study was

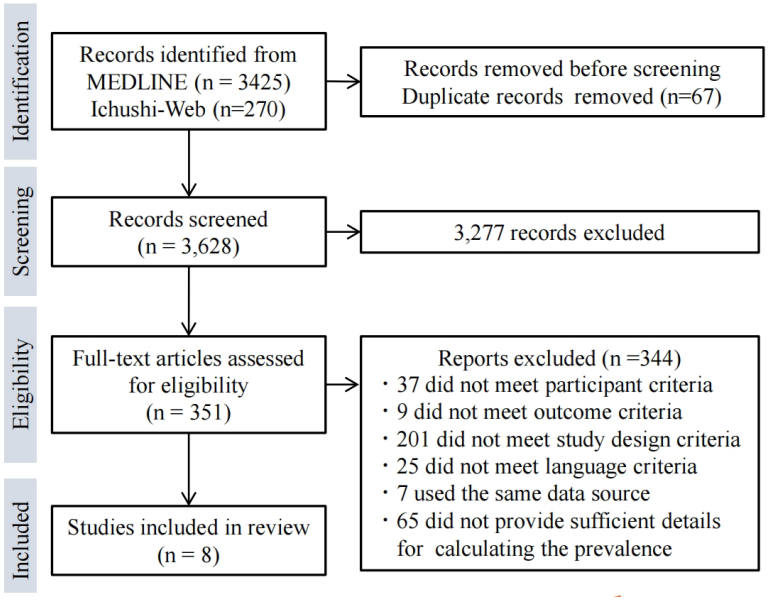

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram for the study selection process.

Table 1. Characteristics of the studies and prevalence of pregnancy and lactation-associated osteoporosis

Study	Participants	Current and past breastfeeding	Evaluation method	Scale	Time	Prevalence (patient/participant)					
						Lumbar spine	Femoral neck	Total hip	Trochanter	Distal radius	Calcaneus
Kurabayashi T *et al.*, 2021 (*17*) Japan	Age (mean ± SD): 33.5 ± 4.5 years BMI before pregnancy (mean ± SD): Normal 22.5 ± 4.3 kg/m^2, Osteopenia 20.3 ± 2.4 kg/m^2, Osteoporosis 19.3 ± 2.5 kg/m^2 Healthy women with no complications No fractures of the spine or femoral bone Not receiving any medications that would affect bone metabolism before or during pregnancy		DXA	YAM <70% or <−2.5 SD	Within 2–3 days	0.6% (7/1079)	4.8% (52/1079)	1.5% (16/1079)			
Eroglu S *et al.*, 2019 (*22*) Germany	Age (mean ± SD): Normal 30.1 ± 7.2 years, Low BMD 29.1 ± 4.1 years BMI before pregnancy (mean ± SD): Normal 28.0 ± 4.6 kg/m^2, Low BMD 27.6 ± 4.8 kg/m^2 Healthy women aged 18–40 years Not using antiresorptive drugs or receiving vitamin D or calcium medications No acute or chronic infections, history of trauma, psychiatric disorders, or any secondary causes of inflammation	Total breastfeeding month mean (range) Normal: 12 (0–24) Low BMD: 11.5 (0–48)	DXA	Z score ≦−2.0	1 month	30.9% (29/93)					
Kajale N *et al.*, 2016 (*19*) UK	Age (mean ± SD): 27.7 ± 3.5 years BMI at study enrolment: 26.3 ± 4.0 kg/m^2 Primiparous women with singleton pregnancies No pre-existing conditions like gestational diabetes or preeclampsia Not diagnosed with intrauterine growth restriction/ retardation or small for gestational age		DXA	T score <−2.0	Within 7 days	10% (12/128)					
Jang DG *et al.*, 2016 (*4*) Korea	Age (mean ± SD): 32.8 ± 3.9 years BMI before pregnancy (mean ± SD): 20.8 ± 2.6 kg/ m^2 Women who gave birth after 32 weeks of gestation Absence of the following diseases: hyperthyroidism, hypothyroidism, systemic lupus erythematosus, rheumatoid arthritis, insulin-dependent diabetes mellitus, kidney disease, epilepsy, depression, schizophrenia, and hematologic disease Not treated for infertility	Exclusive breastfeeding number (%): 407 (26.1%)	DXA	Z score ≦−2.0	4–6 weeks	13.8% (215/1561)	24.8% (387/1561)	16.4% (256/1561)	24.1% (376/1561)		

YAM, young adult mean; DXA, dual-energy X-ray absorptiometry; BMD, bone mineral density; BMI, body mass index.

Table 1. Characteristics of the studies and prevalence of pregnancy and lactation-associated osteoporosis (continued)

Study	Participants	Current and past breastfeeding	Evaluation method	Scale	Time	Prevalence (patient/participant)					
						Lumbar spine	Femoral neck	Total hip	Trochanter	Distal radius	Calcaneus
Lebel E *et al.*, 2014 (*23*) Israel	Age (mean ± SD): 29.9 ± 6.0 years BMI before pregnancy (mean ± SD): 24.2 ± 4.4 kg/m^2 History of fracture: 14 (10.6%) All women who remained in the hospital postpartum (up to 48 h)	Total breastfeeding month Mean ± SD (range): 27.09 ± 31.61 (0–135.0)	DXA	T score and/or Z score <−2.0	Within 48 h	0% (0/132)	9.1% (12/132)				
Kurabayashi T *et al.*, 2009 (*20*) Japan	Age (mean ± SD): 31.3 ± 4.7 years BMI before pregnancy (mean ± SD): Normal 23.0 ±2.9 kg/m^2, Osteopenia 21.6 ± 2.4 kg/m^2, Osteoporosis 20.9 ± 1.8 kg/m^2 Healthy Japanese women with no complications aged 17–46 years No obstetric complications that required prolonged bed rest during pregnancy No history of excessive caffeine intake Not receiving medications that would affect bone metabolism before or during pregnancy		DXA	T score <−2.5 SD	Within 7 days	0.3% (8/2436)					
Costa ML *et al.*, 2012 (*21*) Brazil	Age (mean ± SD): 26.4 ± 6.4 years Weight before pregnancy: (mean ± SD): 62.5 ± 12.3 kg Healthy postpartum women with no complications aged 18–40 years who had a singleton delivery after 37 weeks Intend to delay their next pregnancy for at least 12 months postpartum No history of diseases before or during pregnancy that would affect calcium or bone metabolism Not receiving any of the following medications: corticosteroids, anticoagulants, anticonvulsants, thiazide diuretics, and drugs for the treatment of thyroid disease	Duration of exclusive breastfeeding days mean (SD): 125.9 (±66.6) days 0–3 months; 24 (30.8) 3–6 months; 45 (57.7) >6 months; 9 (11.5)	DXA	Z score <−2.0	7–10 days 3 months 6 months 12 months					1.3% (13/100) 2.6% (2/91) 3.8% (3/84) 3.8% (3/78)	

YAM, young adult mean; DXA, dual-energy X-ray absorptiometry; BMD, bone mineral density; BMI, body mass index.

Table 1. Characteristics of the studies and prevalence of pregnancy and lactation-associated osteoporosis (continued)

Study	Participants	Current and past breastfeeding	Evaluation method	Scale	Time	Prevalence (patient/participant)					
						Lumbar spine	Femoral neck	Total hip	Trochanter	Distal radius	Calcaneus
Hoshino A *et al.*, 2017 (*24*) Japan	Age [median (25th, 75th percentiles)]: 33 (30, 36) years BMI at study enrolment [median (25th, 75th percentiles)]: 20.4 (19.2, 21.9) History of fracture: 268 (22.1%) Women aged <45 years who had singleton deliveries No history of receiving medications affecting bone mass	Mainly breastfeeding number (%): 987 (80.9%)	Quantitative ultrasound	Stiffness score <70.1	3–4 months						10.9% (133/1220)

YAM, young adult mean; DXA, dual-energy X-ray absorptiometry; BMD, bone mineral density; BMI, body mass index.

based on combined results from studies that focused on postpartum women who lacked any pre-existing risk factors for osteoporosis before pregnancy, including medication-induced impacts on bone metabolism or a history of metabolic bone disease (*4,17,19,20,22,23*). While the included studies targeted healthy postpartum women, the prevalence of osteoporosis in this group surpassed that reported in a meta-analysis, which indicated a 3% osteoporosis prevalence in premenopausal women (*25*). This study highlights the heightened fracture risks by pregnancy and lactation compared with women of equivalent age. Recognizing the negative implications of fractures on maternal and infant health renders BMD screening and early detection of decreased BMD or women susceptible to PLO-related fractures during the postpartum phase justifiable.

The pathogenesis of postpartum osteoporosis is linked to the decline in postpartum estrogen and heightened bone resorption to fulfill infant calcium requirements (*26*). Our study aimed to ascertain osteoporosis prevalence in postpartum women within the first year of postpartum, considering the influence of the lactation period. However, six of the eight studies (*4,17,19,20,22,23*) were included in the systematic review, and five of the six studies included in the meta-analysis (*17,19,20,22,23*) were conducted within 2 months postpartum. Therefore, the reported 5-12% prevalence of PLO primarily reflects the early postpartum phase and may not truly represent the entire lactation duration. Sowers *et al.* reported that women lactating for over 6 months experienced a bone loss of 4.8% in the femoral neck and 5.1% in the lumbar spine. In contrast, no significant BMD change was observed for short-term lactation within 1 month or less (*27*). Consequently, the peak prevalence of PLO might be higher during the first postpartum year than that indicated in this study. Given the complexity of postpartum hormonal changes and their potential impact on bone health, further research is needed to comprehensively elucidate the prevalence and trajectory of PLO throughout the entire lactation period.

The estimated prevalence of osteoporosis in postpartum women was 5-12%; however, the I^2 test unveiled a high heterogeneity of 99%. One factor contributing to this heterogeneity is the diversity in definitions of osteoporosis used in the studies included in the meta-analysis. This disparity in definition can be attributed to the absence of standardized diagnostic criteria tailored specifically for PLO. Of the eight articles included in this meta-analysis, two used T-scores, three used Z-scores, one used both T- and Z -scores, one used YAM values, and one used stiffness values.

The absence of consensus on PLO diagnostic criteria and the definition of osteoporosis in young adults contributes to methodological heterogeneity across studies (*14*). The WHO diagnostic criterion for osteoporosis is defined as a T-score < -2.5 SD (using 20–29-year-old Caucasian women as a reference) (*15*).

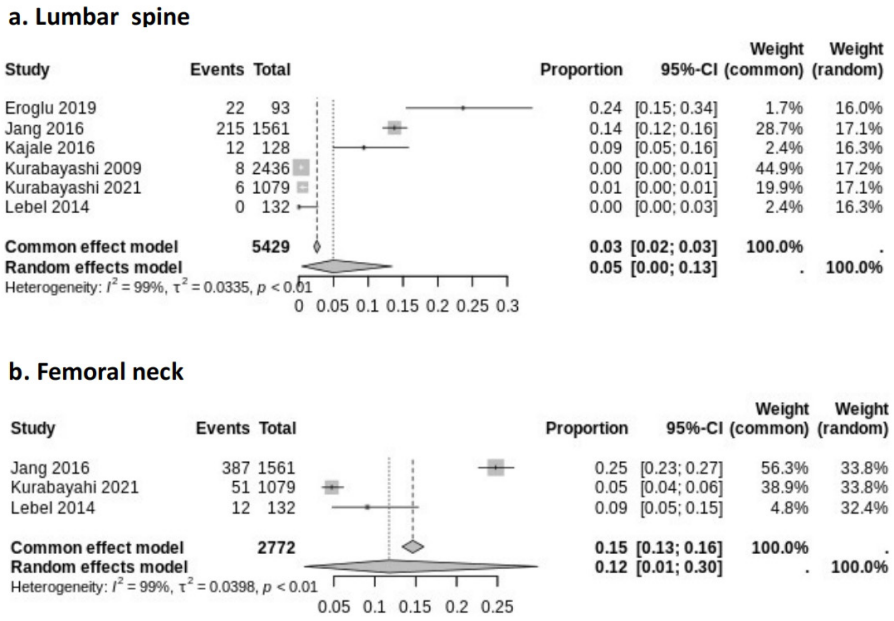

Figure 2. Prevalence of pregnancy- and lactation-associated osteoporosis (a. lumbar spine b. femoral neck). CI, confidence interval.

However, this diagnostic criterion is mainly tailored to postmenopausal osteoporosis. The International Society for Clinical Densitometry suggests diagnosing osteoporosis in young adults with a Z-score < − 2.0 SD in comparison to the mean age-, sex-, and ethnicity-matched reference population (*16*). On the contrary, the International Osteoporosis Foundation recommends using a T-score < − 2.5 SD for osteoporosis diagnosis in young adults, particularly in cases that involve factors affecting bone mass (*28*). The broad spectrum of prevalence estimates for PLO and the substantial heterogeneity across studies underscore the need for consensus and the establishment of standardized diagnostic criteria for PLO. Furthermore, establishing clear criteria for osteoporosis in young adult women would bolster the comparability and reliability of future research in this area.

The high heterogeneity across studies may also stem from the variability in the timing of osteoporosis evaluations, spanning from 48 h to 6 weeks postpartum. This interval coincides with substantial physiological shifts, encompassing modification in breast-milk supply and calcium demands essential for lactation. In cases of complete breastfeeding, the supply of breast milk to the infant increases from approximately 5–30 mL/day in the first few days postpartum to 750–800 mL/day in the first 1–2 months postpartum, with a calcium supply ranging from 280–400 mg/day. Notably, studies conducted 2 months postpartum (*4,22*) yielded a higher osteoporosis prevalence than those conducted within a few days postpartum (*17,19,20,23*). This suggests that the disparity in prevalence could be attributed to fluctuations in calcium loss during the postpartum period. However, because of the limited number of included studies and the absence of lactation information, our review could not perform a stratified analysis based on the postpartum period. Additional reports and further analyses are necessary to better understand the influence of postpartum timing on osteoporosis prevalence.

The considerable prevalence of osteoporosis demonstrated in this study highlights the necessity for BMD screening in postpartum women. Although DXA remains the gold standard for BMD evaluation, limitations related to its lack of portability, cost, and radiation exposure warrant consideration of alternative methods (*15*). Our review identified one report of prevalence assessed using QUS of the calcaneus (*24*). While direct comparisons of prevalence rates among studies must be approached cautiously, the reported PLO prevalence using QUS was higher than that using DXA in our meta-analysis. Thus, QUS might overestimate the risk of PLO; however, it could serve as a useful method for screening women with covert PLO because screening methods should not overlook those at risk. Importantly, compared with DXA, QUS of the calcaneus is radiation-free, more cost-effective, and exhibits potential for osteoporosis screening (*29-31*). Validating the utility of QUS for diagnosing postpartum women may lead to the establishment and widespread adoption of PLO screening methods.

This study had a few limitations. First, it solely included articles that described the number of patients using the osteoporosis definition and were able to calculate prevalence. Most studies that measure BMD in postpartum women provide actual values or changes in BMD but lack patient numbers or prevalence. Second, while this study encompassed women within 1 year postpartum, most studies were conducted in the early postpartum period, with only one being a cohort study. Consequently, variations and changes in

PLO prevalence during the postpartum period remain unknown. Additionally, whether pregnancy and lactation affect BMD and if patients might have had osteoporosis before pregnancy remains uncertain. Third, this study did not explore factors contributing to osteoporosis, such as feeding methods (breastfeeding or formula feeding) or menstrual status. Future studies should include subgroup analysis stratified by the postpartum period and osteoporosis-related factors.

In conclusion, this systematic review and meta-analysis revealed that the prevalence of PLO during the postpartum period ranges from 5 to 12%. Nevertheless, caution must be exercised regarding the accuracy of these estimated figures because of differences in the definition of osteoporosis owing to the lack of standardized diagnostic methods for PLO. Thus, it is imperative to establish a standardized diagnostic method for PLO, conduct further research, and implement early detection and intervention measures through screening in the future.

Funding: This work was supported by JSPS KAKENHI (grant number: 20K23218). The sponsors had no role in the design, execution, interpretation, or writing of the study.

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

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Received May 31, 2024; Revised July 30, 2024; Accepted August 20, 2024.

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Released online in J-STAGE as advance publication August 25, 2024.

Original Article

DOI: 10.5582/ddt.2024.01035

Duzhong Fang ameliorates cognitive impairment of Parkinsonian mice by suppressing neuronal apoptotic pathway

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SUMMARY Parkinson's disease (PD) is a complex multisystem neurodegenerative disease, and cognitive impairment is a common symptom in the trajectory of PD. Duzhong Fang (DZF) consists of *Eucommia ulmoides*, *Dendrobium*, *Rehmanniae Radix*, and *Dried Ginger*. Our previous study showed that DZF improves motor deficits in mice. However, whether DZF can ameliorate cognitive impairment in PD has not been reported. In this study, we established mice models of PD induced by rotenone and examined the effect of DZF on cognitive impairment in Parkinson's disease (PD-CI). The results confirmed that DZF treatment not only significantly improved the motor deficits in PD mice and decreased the loss of dopaminergic neurons, but also had significant effects in improving cognitive impairment. We further integrate serum metabolome and network pharmacology to explore the mechanisms by which DZF improves PD-CI. The results revealed that DZF can treat PD-CI by regulating sphingolipid metabolism to inhibit neuronal apoptotic pathway. In conclusion, preliminary studies confirmed that DZF contributes to the improvement of cognitive ability in PD, and our results provide a potential drug for the clinical treatment of PD and a theoretical foundation for DZF in clinical application.

Keywords Cognitive impairment, Parkinson's disease, Duzhong Fang, sphingolipid metabolism, apoptosis

1. Introduction

Cognitive impairment is one of the most common non-motor symptoms of Parkinson's disease (PD) (*1*). At present, in PD patients with a course of 10 years or more, the cumulative prevalence of cognitive impairment in Parkinson's disease (PD-CI) is 75-90% (*2*). With the increasing life expectancy of PD patients, PD-CI is set to become even more prevalent in the future (*3*). Cognitive impairment greatly worsens life quality in patients, as well as increases caregiver burden and health care costs. However, at present there is no effective drug for the treatment of PD-CI (*4*). For PD-CI, drugs for Alzheimer's disease (AD) are mostly clinically selected. Nevertheless, due to the pathogenesis being different, the treatment effects were not obvious. Therefore, investigating potential drugs for cognitive restoration, and treatment of PD-CI earlier is vital to improved social functioning and quality of life for these patients.

Duzhong Fang (DZF) comes from the Handbook of Prescriptions for Emergencies, which consists of *Eucommia ulmoides*, *Dendrobium*, *Rehmanniae Radix*, and *Dried Ginger*. Our previous study demonstrated that DZF can improve movement disorders and reduce the loss of dopaminergic neurons in PD mice (*5*). Nevertheless, its effect on PD-CI has not been reported. Previous studies have found that *Eucommia ulmoides*, *Dendrobium,* and *Radix rehmanniae* can improve cognitive impairment (*6-8*). It is reasonable to speculate that DZF may not only improve movement disorders but also ameliorate cognitive impairment in PD patients.

In this study, rotenone induced PD model was used to examine the beneficial effects of DZF against PD-CI. Then, the underlying mechanisms of how DZF contributes to the cognitive improvement in PD mice were further explored *via* network pharmacology, metabolomics, immunohistochemical and biochemical analysis. Our data suggested that DZF improved cognitive ability by inhibiting neuronal apoptotic pathway. Figure 1 below illustrates the process and the key findings of the study.

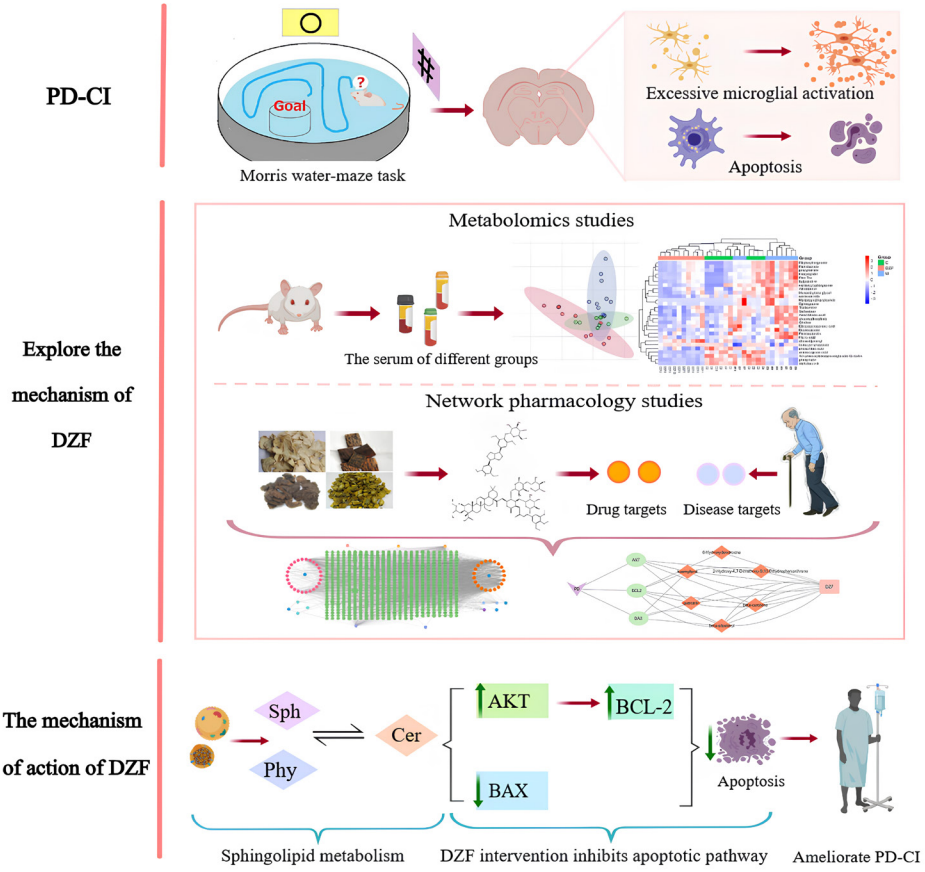

Figure 1. The process and the key findings of the study.

2. Materials and Methods

2.1. Reagents

Eucommia ulmoides, *Dendrobium*, *Rehmanniae Radix*, and *Dried Ginger* were obtained from Beijing Tongrentang (Beijing, China). Rotenone was purchased from Sigma Chemical Co (St. Louis, MO, USA). 4% paraformaldehyde was purchased from Coolaber (Beijing, China). Triton-100 X was supplied by Beijing Suolaibao Technology Co. Ltd. (Beijing, China). Tyrosinehydroxylase (TH)/ Ionized c alcium bindingadaptor molecule-1 (Iba-1) / Neuronal nuclear antigen (Neu-N) rabbit antibodies were supplied from Abcom (Cambridge, MA, USA). Radioimmunoprecipitation assay buffer (RIPA) and protease inhibitors were obtained from Solaibao Biotechnology, Co. Ltd. (Beijing, China). phosphorylated AKT (P-Akt) /Akt/Bax/Bcl-2 rabbit antibodies were purchased from ABclonal Technology Co., Ltd. (Wuhan, China). Goat anti-rabbit secondary antibodies were purchased from Abcom (Cambridge, MA, USA). Acetonitrile and ammonium acetate were purchased from Tianjin Kemiou Chemical Reagent Co. Ltd. (Tianjin, China).

2.2. Animals

The animal study was approved by the Tianjin University of Traditional Chinese Medicine Use Committee (approval number TCM-LACE2022226). Male eight-week-old C57BL/6 mice (weighing 23 ± 0.2 g) were purchased from Beijing Huafukang Biotechnology Co. Ltd. (Beijing, China) and housed at room temperature (RT; 22 ± 2 °C) under a standard 12h light/dark cycle with free access to food and water. The mice were randomly assigned to three groups: the control group (Control; n = 13), the rotenone group (Model; n = 13), the rotenone+ DZF group (Model + DZF; n = 13).

2.3. Experimental design

To establish a suitable PD model, 10 mg/kg of rotenone was suspended in 0.5% carboxymethyl cellulose. Each mouse in the rotenone and rotenone + DZF groups was administrated orally once a day for 3 months. *Dried Eucommia ulmoides*, *Dendrobium*, *Rehmanniae Radix*, and *Dried Ginger* at a ratio of 200:2:3:3 by weight, were refluxed twice in 75% ethanol (reflux for 2 hours each time), and the refluxed supernatant was collected, filtered and concentrated to obtain Duzhong Fang extract (about 11% yield). From the third month, Rotenone + DZF were orally administered 20g/kg/day of DZF for 5 months. The dosages of DZF and rotenone were selected based on previous research (*9*).

2.4. Behavioral tests

2.4.1. Pole test

Place the mouse on top of the vertical pole. Record the time it began to crawl down to the landing of the hind limbs. If the mouse pauses or crawls in the middle, it will be re-measured. If it exceeds 60 s, it will be recorded as 60 s. 3 days before the formal experiment, the mice began to conduct behavioral training once a day for 3 consecutive days. One hour after the last dose, the test was formally started and repeated 3 times. The result of the experiment was the average time.

2.4.2. Rotarod test

The mouse was placed on the rotating rod and moved with the rotating rod at a speed of 25 r/min, and the time of the mouse falling was recorded. If the time exceeds 300 s, record 300 s. The mice were trained before the formal experiment. The formal experiment was repeated 3 times with an interval of 5 min each time, and the average value was calculated.

2.4.3. Grip strength

The mouse was placed on the gripper with its body perpendicular to the metal rod on the dynamometer, and the tail was pulled back at a constant speed to measure the grip. Record the reading and repeat three times to calculate the average value.

2.5. Immunohistochemical staining

The mice were deeply anesthetized and sacrificed by cardiac perfusion with normal saline and 4% paraformaldehyde successively. The whole brain was taken out and fixed with paraformaldehyde and immersed in 30% sucrose for storage at 4°C before sectioning. The brain was sliced into 10 μm coronal sections by frozen sectioning and the sections containing Scripted Non-Playable Character (SNpc) were collected.

Heat-citrate pretreatment was used for antigen retrieval. The tissue sections were treated with 0.25% Triton-100 X to make the tissues permeable, and goat serum was added to block. After blocking, microglia and neurons were detected by immunohistochemical staining with TH antibody, Iba-1 antibody and Neu-N antibody, respectively. Samples were incubated at 4°C overnight with primary antibodies diluted in Bovine Serum Albumin (BSA): anti-TH (1:400), anti-Iba-1(1:400), and anti-Neu-N (1:400). Then the corresponding secondary antibodies, tagged goat anti-rabbit (1:500) were used. Microglia and neuron analysis were captured using a fluorescence microscope.

2.6. Morris water-maze (MWM) test

Spatial learning and memory abilities of were tested with the MWM. The test was carried out using the black circle pool filled with water (22 ± 1°C). The pool was artificially divided into four quadrants (*i.e.*, N, E, S, and W), and the escape platform was located water surface in the quadrant. The mice were released at the farthest position from where the platform had been, if an animal did not find the platform within 60 s of placement in the quadrant, they were guided to the platform and kept there for 30 s. The task consisted of a 6-day acquisition phase with four trials/day. The performances were continuously monitored using an automated tracking system (Noldus Ethovision XT System, the Netherlands). The escape latency to find the platform and the total distance traveled during acquisition were recorded and analyzed.

On the 6th day, spatial memory function was evaluated, the platform was removed and animals were allowed to swim freely for 60 s. The time spent in the target quadrant and the average swim speed were recorded and analyzed.

2.7. The metabolomic assay and data analysis

After a 5-month intervention of DZF, blood was taken by removing the eyeball after fasting for 8 h, centrifuged at 3,000 r/min for 10 min at 4℃ to obtain serum. Chromatographic separations were performed using the ultra-high performance liquid chromatography with quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF/MS) system. The samples were collected on Agilent 1290 Infinity LC. The mobile phase consisted of solvent A (water + 25 mM ammonium acetate + 25 mM ammonia water) and solvent B (acetonitrile), the gradient elution conditions were set as follows: 0-0.5 min, 95% phase B; 0.5-7 min, 95%-65% phase B; 7-8 min, 65%-40% phase B; 8-9 min, 40% phase B, 9-9.1 min, 40%-95% phase B; 9.1-12 min, 95% phase B; 12.1 min, stop. The triple TOF Mass Spectrometer Detector (AB SCIEX) was used to collect data. The ESI source operation parameters were as follows: ion source temperature, 600°C; ion spray voltage, 5500 V; curtain gas, 30.0 psi; One primary mass spectrometry scan range was mass-to-charge ratio 60 to 1,000 Da. The secondary mass spectrometry scan range was a mass-to-charge ratio of 25 to 1,000 Da. QC samples were inserted into the analytical sequence to evaluate the stability of the analytical system during running samples.

The total ion current data were collected by UHPLC-Q-TOF/MS. The data matrix of the sample name and peak intensity was processed by peak matching, peak alignment, control processing, and multivariate statistical analysis. Partial least squares discriminant analysis was used to obtain the score plot by SIMCA. Significant metabolic markers were selected from discriminant analysis and multivariate statistical methods. Variable importance in projection (VIP) values obtained from the orthogonal partial least-squares discriminant analysis

(OPLS-DA) model was used to select variables with the most important impact, and then *t*-tests were performed. The metabolites with VIP > 1 and $P < 0.05$, which is the top 15, were used for pathway enrichment analysis.

2.8. Network target prediction

First of all, compounds in DZF were chosen for the prediction of biological targets using the TCMSP (*http://tcmspw.com/*). Then, these components were put into the SwissTargetPrediction database (*http://www.swisstargetprediction.ch/*) to obtain the Uniprot ID of predicted targets. Next, the biological targets related to PD-CI were selected from the GeneCards (*https://www.genecards.org/*) and OMIM (*https://www.omim.org/*) database. Taking the intersection of the DZF targets and the candidate targets associated with PD-CI as potential therapeutic targets of DZF against PD-CI. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed to identify the enriched top ten pathways of potential therapeutic targets based on the KEGG database.

2.9. Western blotting analysis

The hippocampus, cortex and midbrain ($n = 6$ in each group) were lysed with RIPA lysis buffer. Proteins (25 μg per lane) were separated by 10% or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to the PVDF membrane. Each membrane was blocked in 5% skim milk powder for 1 h at room temperature and then incubated with anti-Akt (1:1,000), anti-p-Akt (1:1,000), anti-Bax (1:1,000), anti-Bcl-2 (1:1,000), anti-tyrosine hydroxylase (1:1,000), at 4°C overnight. The membranes were incubated with goat anti-rabbit IgG or goat anti-mouse IgG (1:1,000) for 1.5h at room temperature. Blots were imaged by the ECL chemiluminescence (Millipore, USA) and a Gel Image System (GE, USA). Band intensities were quantified using Image J software.

2.10. Statistical analysis

SPSS 22.0 software was used for data analysis. Data were expressed as the mean ± standard error of the mean (SEM). Before performing parametric tests, data distributions were tested for normality using the Kolmogorov–Smirnov test. Differences between groups in behavioral tests were analyzed using one-way ANOVA followed by Tukey post-hoc test and $P < 0.05$ is considered statistically significant.

3. Results

3.1. DZF improved locomotor dysfunction and TH levels in the substantia nigra of PD mice

To evaluate the potential protective effect of DZF on locomotor dysfunction in Parkinsonian mice, we performed behavioral tests on mice to evaluate their muscle strength, mobility and balance. Through behavioral testing, compared with the control group, rotenone-induced Parkinsonian mice showed locomotor dysfunction, including longer climbing time, shorter falling time of the rotating rod and lower grip value. However, the DZF treatment used could ameliorate this situation. The results are presented in Figures 2A-2C.

To investigate the protective effects of DZF on dopaminergic neurons in the substantia nigra of PD mice, TH was detected by immunohistochemical staining and Western blot analysis. Immunohistochemical staining revealed a significant loss of TH^+ dopaminergic neurons in model mice compared to the control and DZF group (Figure 2D). Western blot analysis showed that TH expression in the model was lower compared to control and DZF (Figures 2E-2F), the results above indicate that DZF inhibited the reduction of TH expression and protected dopaminergic neurons in PD mice.

3.2. DZF improved the cognitive impairment of PD mice

The escape latency of each group was longest on day 1. From day 5 to day 6, it was observed that the escape latency was decreased in the control and DZF group compared to the model group (Figure 3A). As shown in Figure 3B, the frequency of crossing the platform and platform retention time on day 6 was decreased in the model group compared to the control group, while the DZF treatment used could ameliorate this situation (Figures 3B-3C). Conversely, the latency to reach the platform and the escape passage was increased in the model group compared to the control group, but this effect is still ameliorated significantly following DZF treatment (Figures 3D-3E). The above results suggested that DZF can improve the cognitive impairment of PD mice.

3.3. DZF inhibited microglial overactivation in the hippocampus and cortex

The overactivation of microglia is closely related to the progression of cognitive impairment is well known (*10*). To determine the effect of DZF on microglial cell activation, immunofluorescent staining for Iba-1 in microglia was performed. As shown in Figure 4A, the microglia from the model group with larger soma and thicker but shorter protrusions. Microglia return to normal morphology after DZF treatment. Compared with the model group, microglia in DZF-treated mice displayed an increased number of branches and branch endpoints in the hippocampus (Figures 4D-4E). Like the hippocampus, DZF also inhibited microglial overactivation in the cortex (Figures 4F-4J).

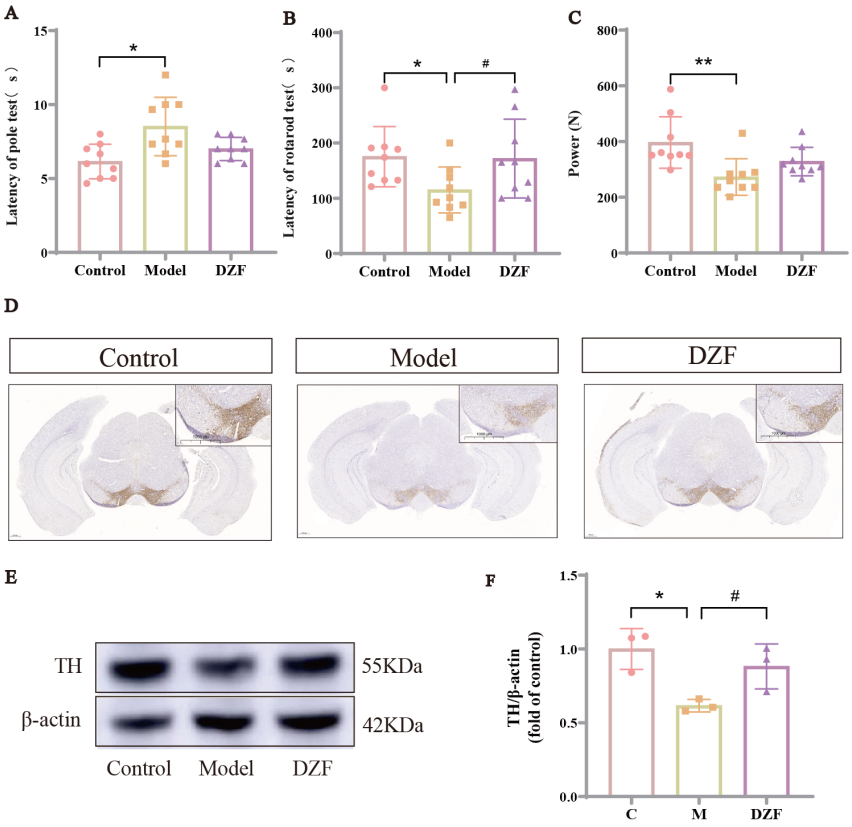

Figure 2. DZF improved rotenone-induced locomotor activity dysfunction including (A) pole, (B) rotarod, (C) grip, (D) TH level in the substantia nigra by immunohistochemistry, (E) TH protein expression in the substantia nigra, and (F) quantitative results of TH level. Data represent the means ± SEM (n = 3 per group); Statistics one-way ANOVA; $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$ versus model group.

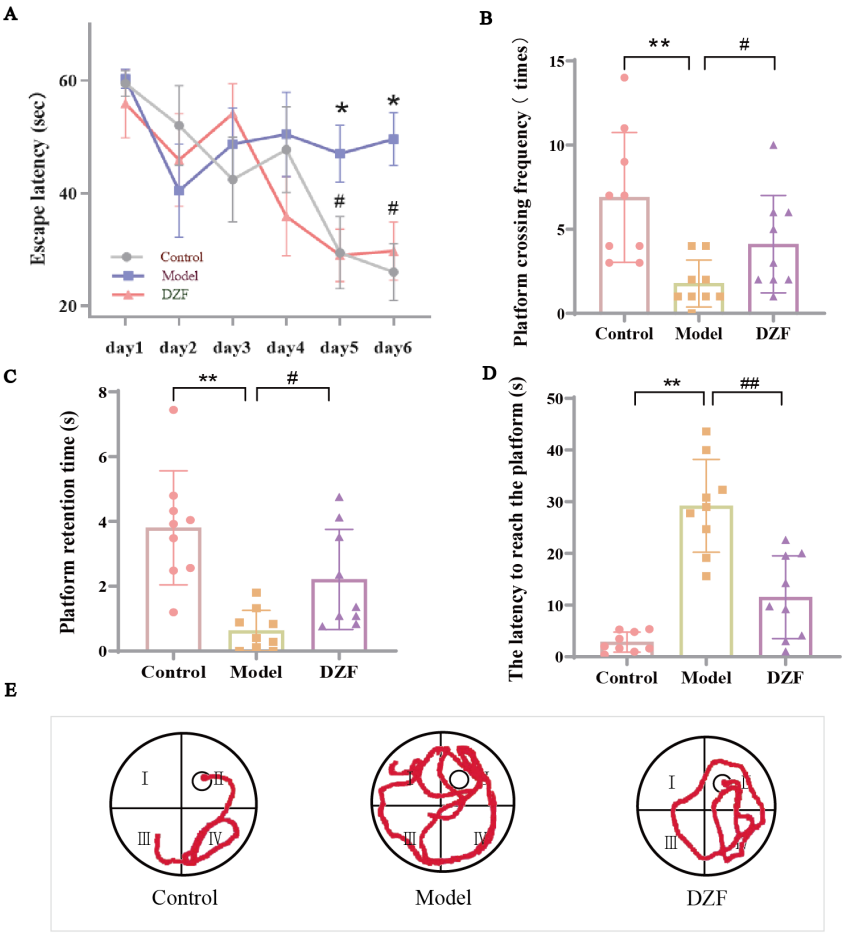

Figure 3. DZF improved the cognitive impairment of PD mice. (**A**) Escape latency from day 1 to day 6. (**B**) The platform crossing frequency on day 6. (**C**) The platform retention time on day 6. (**D**) The latency to reach the platform on day 6. (**E**) Escape passage on day 6. Data represent the means ± SEM (n = 9 per group); Statistics one-way ANOVA; $^{*}P < 0.05$ versus control group, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$ versus model group, $^{\#\#}P < 0.01$ versus model group.

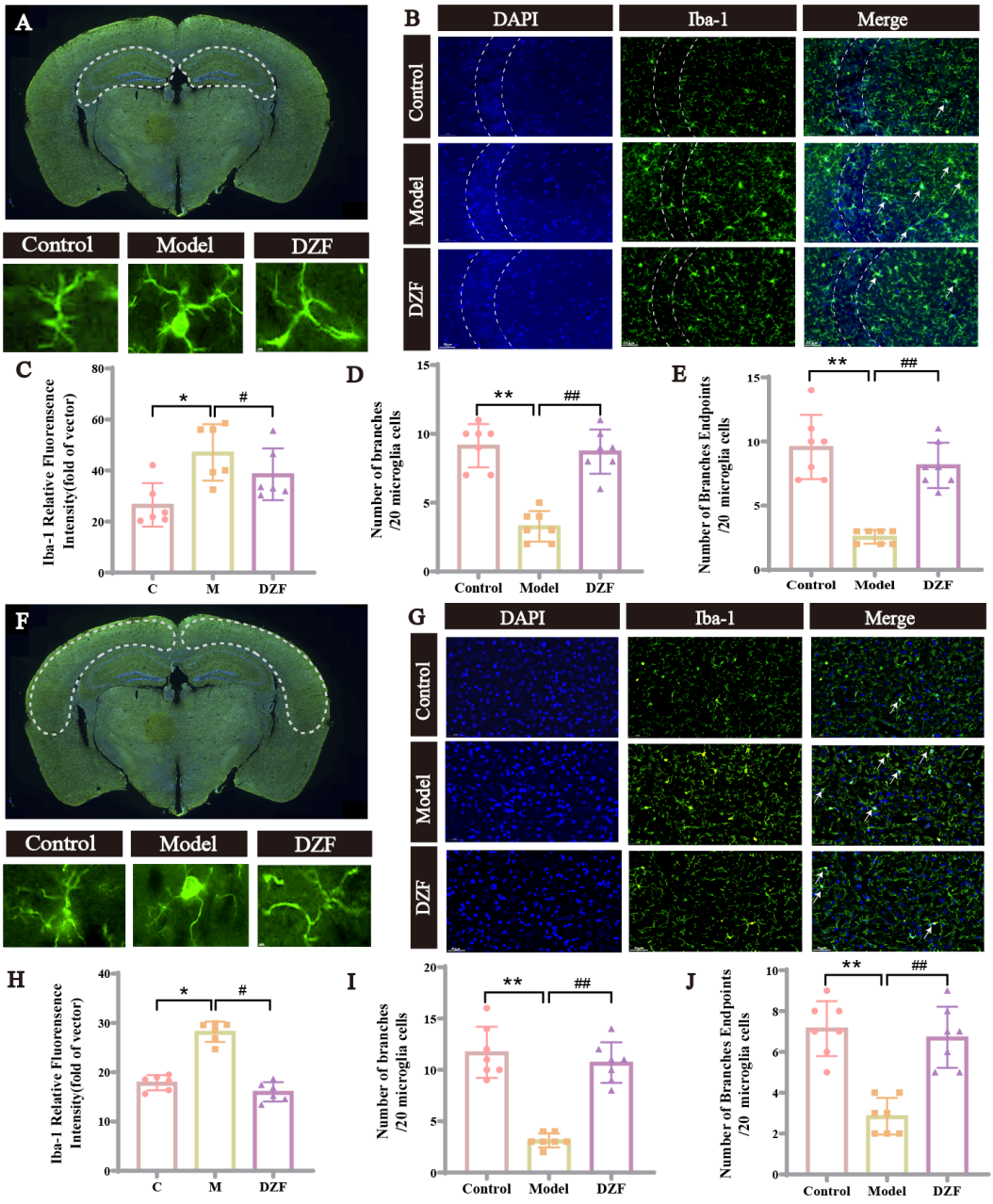

Figure 4. DZF inhibited microglial overactivation in the hippocampus and cortex. (**A**) Microglia in the hippocampus. (**B**) Photomicrographs of immunofluorescence staining for microglial cells in the hippocampus. (**C**) Iba-1 relative fluorescence intensity in the hippocampus. (**D**) Average number of branches in the hippocampus. (**E**) Branches endpoints of microglia in the hippocampus. (**F**) Microglia in the cortex. (**G**) Photomicrographs of immunofluorescence staining for microglial cells in the cortex. (**H**) Iba-1 relative fluorescence intensity in the cortex. (**I**) Average number of branches in the cortex. (**J**) Branches endpoints of microglia in the cortex. Six visual fields were randomly selected and 20 cells in each visual field were counted. Data represent the means ± SEM (n = 6 per group); Statistics one-way ANOVA; *P < 0.05 versus control group, **P < 0.01 versus control group; $^{\#}P$ < 0.05 versus model group, $^{\#\#}P$ < 0.01 versus model group.

3.4. DZF influences sphingolipid metabolism

The OPLS-DA score plot suggested that the inter-groups were well separated, and the DZF group was closer to the control group than the model group, indicating that DZF had a good therapeutic effect (Figure 5A). The differential metabolites between the control group and the model group as well as between the model group and the DZF group were obtained, we take the intersections of the results and there are 28 differential metabolites were obtained (Figure 5B). A cluster heat plot showed different metabolites and their relative increase or decrease in values among different groups, and after treating with DZF, the levels of the metabolites were close to those in the control group (Figure 5C). In addition, we screened the metabolites with VIP top 15 (Figure 5D), and then searched for these metabolites in the Kyoto encyclopedia of genes and genomes (KEGG) database (*http://www.kegg.com*). Their main metabolic pathways including necroptosis signaling pathway and

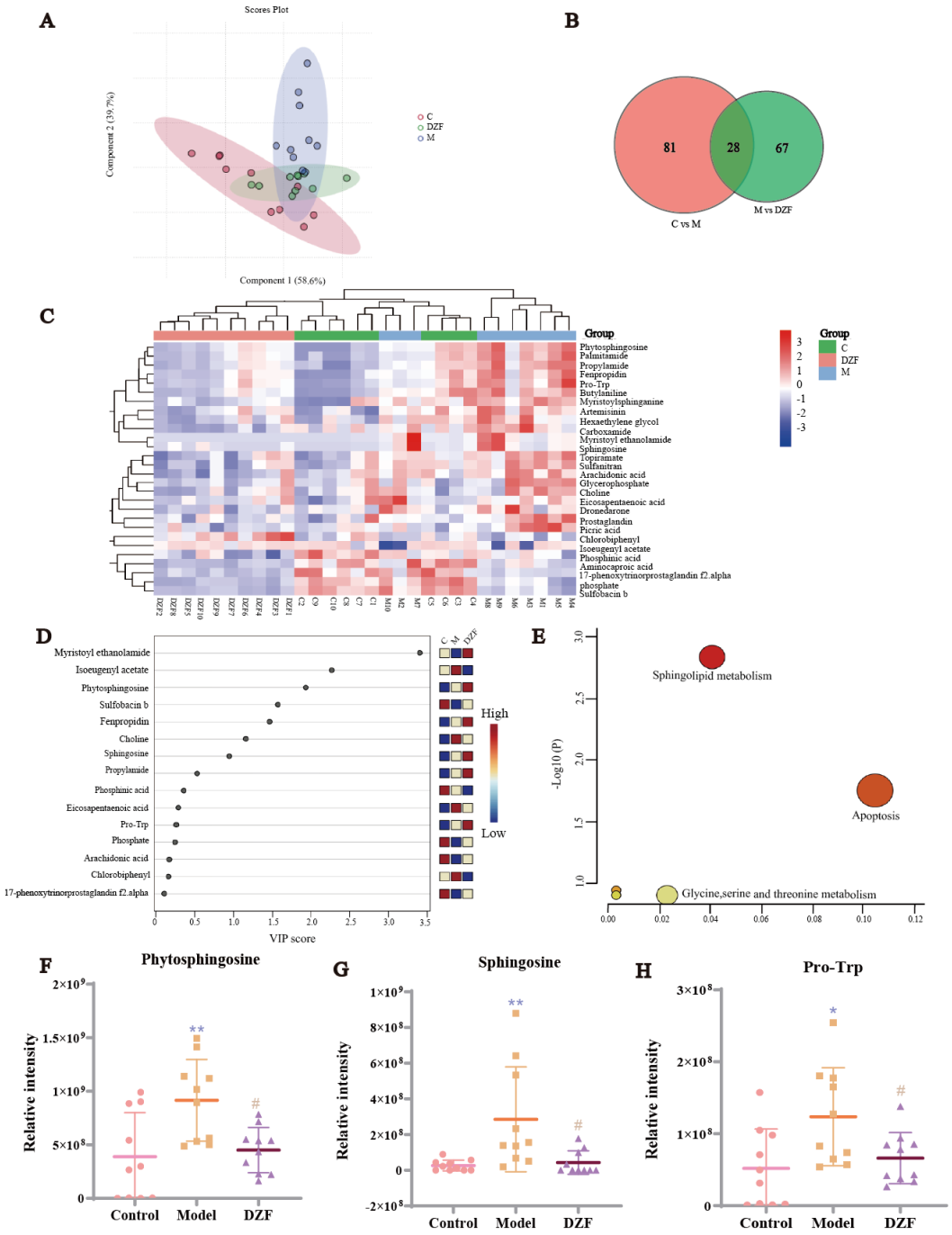

Figure 5. The effect of DZF on serum metabolites. (**A**) The OPLS-DA score plot of each group after. (**B**) The differential metabolites intersections among the control group and the model group as well as between the model group and the DZF group. (**C**) The cluster heat plot of each group after treatment. (**D**) The VIP plot of each group after treatment. (**E**) Main metabolic pathway impact analysis. The relative content changes of potential metabolites. (**F**) Phytosphingosine. (**G**) Sphingosine. (**H**) Pro-Trp. Data represent the means ± SEM (n = 10 per group); Statistics one-way ANOVA; $^{*}P < 0.05$ versus control group, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$ versus model group.

sphingolipid metabolism signaling pathway (Figure 5E). Finally, by comparing the relative content changes in these differential metabolites in sphingolipid signaling pathway, three potential metabolites were identified (Figures 5F-5H). Therefore, results suggested that DZF can regulate sphingolipid signaling pathway and downregulate the potential metabolites such as phytosphingosine, Pro-Trp and sphingosinne.

3.5. DZF treatment suppressed hippocampal and cortex neuronal apoptosis

Active ingredients in DZF with criteria such as oral bioavailability (OB) ≥ 30% and drug-likeness (DL) ≥ 0.18 were selected. We intersected the PD-CI targets with the DZF targets and received 393 common targets (Figure 6A). KEGG pathway enrichment analysis of target genes

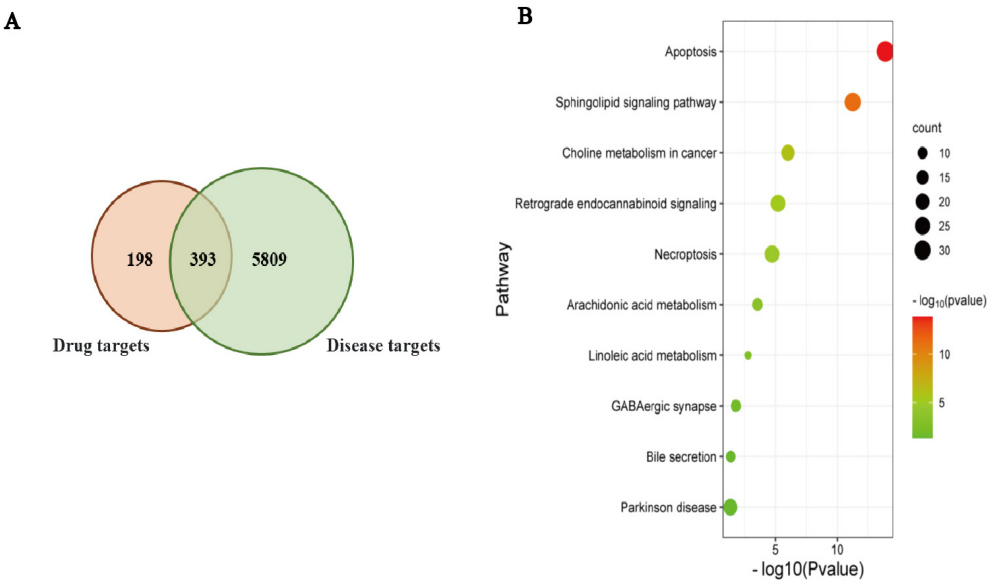

Figure 6. Potential mechanisms of DZF ameliorating cognitive impairment. (**A**) The Venn diagram is the intersection of key disease targets and drug targets. (**B**) Enrichment analysis of common targets pathway.

showed that these target genes were enriched in apoptosis (Figure 6B).

To further determine the mechanism of DZF ameliorate cognitive impairment, neuronal apoptosis and the expression of Bax, Bcl-2 and AKT in the hippocampus and cortex regions were examined by immunofluorescence staining and western blot analysis. In the hippocampus, it could be observed that the number of NeuN expressed positive cells was upregulated in the model group compared with the control group (Figures 7A-7B), which were then decreased by DZF treatment compared with the model group. The expression of apoptosis-related proteins Bax proteins was upregulated, while Bcl-2 and p-Akt protein was downregulated in the model group, which was then reversed in mice that received DZF treatment (Figures 7C-7D). It is noteworthy that we also examined neuronal apoptosis and Bax, Bcl-2 and AKT expression in the cortex, the results indicated that protein expression in the cortex and hippocampus showed the same tendencies (Figures 7E-7H). The above results show that DZF treatment attenuated cognitive impairment *via* inhibition of the Bax/Bcl-2-mediated neuronal apoptosis pathway.

4. Discussion

Currently, PD-CI treatment is based mainly on AD pharmacotherapy and the first-line drugs include acetylcholinesterase inhibitors, but more than 50% of patients produce serious side effects, such as headaches, nausea, fatigue, anorexia, anxiety, and depression (*11,12*). We hope to screen for more targeted and safe alternatives to treat PD-CI. DZF consists of *Eucommia ulmoides*, *Dendrobium*, *Rehmanniae Radix*, and *Dried Ginger*. This study confirmed that DZF could improve motor deficits and reduce the loss of dopaminergic neurons in PD mice, which was consistent with our previous study. Furthermore, shortened escape latency in a Morris water maze test indicated that DZF improved learning and memory in PD mice. Currently, PD-CI is thought to be associated with excessive activation of microglial cells and apoptosis (*13,14*). It has been reported that over-activated microglia can drive neuroinflammation, release a large number of cytokines and induce neuronal apoptosis (*15*). Over-activated microglia can also enhance autophagy, over-pruning and damage neuronal synapses, leading to cognitive impairment (*16*). Therefore, in this study, immunofluorescence was used to detect microglia and neurons in the hippocampus and cortex. The results showed that the microglia in the hippocampus and cortex of PD mice were over-activated, the cell morphology changed obviously, and the number of neurons decreased significantly. However, DZF could inhibit the activation of microglia and increase the number of neurons. To find out the pharmacological mechanism of DZF in improving cognitive impairment, we further analyzed the serum metabolomics of PD mice, metabolomics results showed that DZF can effectively regulate the pathway metabolites, inhibit phytosphingosine, Pro-Trp and sphingosine levels. In cells, sphingolipid metabolites function as second messengers in signal transduction pathways and regulate cell proliferation, migration and apoptosis (*17*). Furthermore, sphingolipids can affect the cell membrane fluidity and permeability to regulate the balance of the internal and external environments and protect cell stability. Araujo *et al*. reported that sphingolipids can regulate cognitive impairment through the glutamine pathway and sphingolipid metabolic

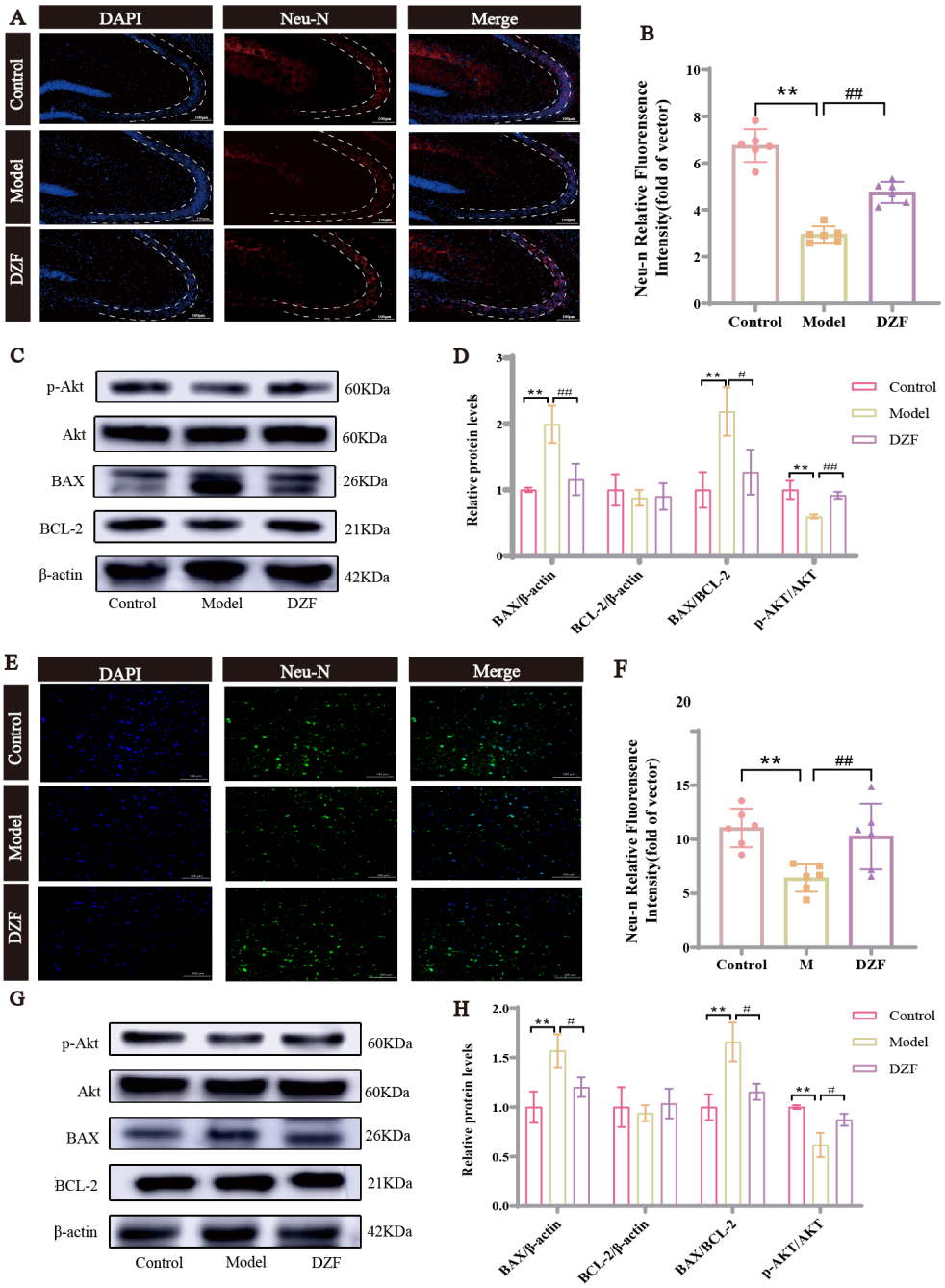

Figure 7. DZF inhibits apoptosis in neuronal. (**A**) Staining of Neu-N (red) in the hippocampus. The scale bar is 100μm. (**B**) Neu-N relative fluorescence intensity in the hippocampus (n=6 mice per group in Figures A-B). (**C**, **D**) Immunoblotting results show the changes in levels of Bax, Bcl-2 and Akt in the hippocampus (n=6 mice per group). (**E**) Staining of Neu-N (green) in the cortex. The scale bar is 100 μm. (**F**) Neu-N relative fluorescence intensity in the cortex (n = 6 mice per group in Figures E-F). (**G**, **H**) Immunoblotting results show the changes in levels of Bax, Bcl-2 and Akt in the cortex (n=6 mice per group). Statistics one-way ANOVA; $^{*}P < 0.05$ versus control group, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$ versus model group, $^{\#\#}P < 0.01$ versus model group.

pathways (*18*).

In addition to serum metabolomics analysis, we also used network pharmacology to predict the targets of the active ingredients of DZF. A total of 393 targets were obtained by crossing the active ingredients of DZF with PD-CI targets, and the pathway enrichment analysis showed that the apoptosis pathway ranked first. It has been reported that the sphingolipid metabolic pathway can regulate apoptosis, and sphingolipid metabolites can be used as signaling molecules to regulate the activity of apoptosis-related protein kinases (*19*). Phytosphingosine caused the release of proapoptotic factors into the cytoplasm and the expression of apoptosis-related proteins Bax, and Bcl-2 in cells (*20*). Moreover, phytosphingosine decreases phosphorylated Akt to induce apoptosis (*21*). Therefore, we further verified the results of network pharmacology, and the results confirmed that DZF could inhibit the apoptosis-related

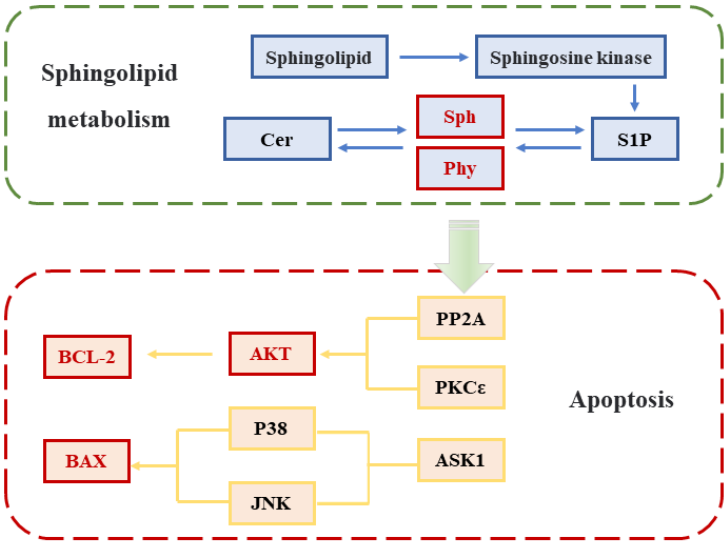

Figure 8. Possible mechanism of DZF for ameliorating the cognitive impairment in PD mice. Key steps affected by DZF are highlighted in red circles.

proteins Bax and Bax/Bcl-2, and increase the level of the upstream apoptosis protein p-Akt. In conclusion, our results suggest that DZF could ameliorate PD-CI by regulating the sphingolipid metabolic pathway, inhibiting apoptosis and protecting microglia and neurons. This study provides potential drugs for improving PD-CI and an experimental basis for the clinical application of DZF.

Many of the components in DZF have been shown to inhibit neuroinflammation and apoptosis, which in turn improves cognitive impairment (*22-25*). The mechanisms by which *Dendrobium nobile* Lindl. alkaloid from *Dendrobium* has been reported to improve cognitive dysfunction may be associated with inhibition of neuroinflammation and neuronal apoptosis, activation of autophagy, and enhanced synaptic connections (*26,27*). *Eucommia ulmoides* can downregulate p38/JNK-Fosl2 gene expression to alleviate neuroinflammation and behavioral impairments in PD mice (*28*). *Rehmanniae Radix* inhibits the p38 MAPK pathways and downstream apoptosis-related molecules to alleviate neural cell loss, reduce oxidative stress and promote functional recovery of PD (*29-31*). Our study revealed that the DZF treatment may trigger the sphingolipid metabolism signaling pathway and in turn, affect the cell apoptosis pathway to ameliorate PD-CI. These putative mechanisms are schematically summarised in Figure 8.

5. Conclusion

Our study confirmed that DZF ameliorates PD-CI by inhibiting neuronal apoptotic pathway. More specifically, DZF could inhibit neuronal apoptosis linked with Bax/Bcl-2 apoptotic signaling pathways by downregulating the sphingolipid metabolites, including phytosphingosine, sphingosine and Pro-Trp. Taken together, our results provide novel evidences that DZF can be a therapeutic agent for PD-CI.

Funding: This work was supported by grants from the National Natural Science Foundation of China (81703827).

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

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Received May 22, 2024; Revised June 20, 2024; Accepted June 23, 2024.

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Released online in J-STAGE as advance publication July 18, 2024.

Brief Report

DOI: 10.5582/ddt.2024.01041

Patient awareness and practices regarding antimicrobial use and drug resistance

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SUMMARY Drug-resistant bacterial infections have become a substantial problem in various communities. Appropriate antimicrobial use is required because reducing antimicrobial use could reduce the number of resistant bacteria. The inappropriate use of antimicrobials can be prevented by improving the knowledge of patients, physicians, and other healthcare professionals; however, no antimicrobial awareness survey specifically aimed at patients has been conducted yet. Therefore, to promote proper antimicrobial use, mainly by patients, we conducted a survey on the attitudes of patients who brought their antimicrobial prescriptions from insurance pharmacies. The results were based on 858 responses. Awareness of the terms "bacteria, viruses, and antimicrobials" was > 80%, whereas that of "drug-resistant bacteria" was only 37.2%. Only 26.5% of respondents understood what the efficacy of antimicrobial drugs meant. Additionally, 31.5% of the respondents had experienced discontinuation antimicrobials, and approximately 70% of the reasons for discontinuation were self-judged symptom improvement. Furthermore, those who had experienced discontinuation were less aware of the various aspects of antimicrobial use than those who had not. In antimicrobial treatment, avoiding the emergence of drug-resistant bacteria is difficult, is detrimental to patients consuming treatment, and presents a major problem in society. Therefore, healthcare professionals should strive to optimize infectious disease treatment by providing appropriate guidance on the proper use of antimicrobials, significance of taking them, and harmful effects of their discontinuation to patients.

Keywords Antimicrobial, drug-resistant bacteria, insurance pharmacy, dose interruption

1. Introduction

Many infectious diseases caused by pathogens, such as bacteria and viruses, can now be treated with antibiotic and antiviral drugs. However, the spread of drug-resistant bacterial infections, for which the available antimicrobials are ineffective, has become a social problem. The increase in drug-resistant bacteria hinders the treatment of patients who were previously curable from drug administration, resulting in the spread of infections and increased mortality. Therefore, the National Action Plan to Antimicrobial Resistance (AMR) 2016–2020 was formulated in Japan in 2016 (*1*). One of the main themes of this action plan is the appropriate use of antimicrobials, with the aim of reducing their use. Indeed, reducing the use of antimicrobials reduces the number of bacteria resistant to those antimicrobials (*2*). Antimicrobials are often used to treat infections; however, the emergence and selection of resistant bacteria is difficult to avoid. Therefore, antimicrobials require appropriate use to balance maximum efficacy, minimal side effects, and the emergence of new resistant bacteria.

Activities to promote the appropriate use of antimicrobials have been centered on hospitals; however, the first place that many patients with infectious diseases, including minor infections, visit is outpatient clinic. Oral antimicrobials are used far more often than injectable antimicrobials in Japan (*3*), and outpatient clinics account for a large proportion of this (*4*). There are reports of antimicrobials being prescribed for acute respiratory tract infections that were not originally necessary (*5*). As clinics are responsible for the majority of outpatient care, it is particularly important to promote the appropriate use of antimicrobials. However, physicians in clinics come from diverse backgrounds and specializations, and trends in infectious disease care and antimicrobial use are expected to vary. Furthermore, as a result of the doctor-patient relationship and patient satisfaction considerations, they often prescribe antimicrobials that are essentially unnecessary (*6-8*). Therefore, it is conceivable that the inappropriate use of antimicrobials

can be prevented by improving the knowledge of physicians and other healthcare professionals, as well as that of patients; however, no antimicrobial awareness survey targeting patients has been conducted.

Therefore, as the division of labor in medicine has progressed and prescriptions from clinics and hospitals are brought to insurance pharmacies, this study conducted a survey on the attitudes of patients who brought their prescriptions to insurance pharmacies regarding antimicrobials to formulate measures aimed at promoting the proper use of antimicrobials, with the patient at the center.

2. Materials and Methods

2.1. Subjects and questionnaire

Patients who brought their prescriptions to Iroha Pharmacy, an insurance pharmacy located in Funabashi City, Chiba Prefecture, between February 19 and April 28, 2024, were included. The study was explained orally at the prescription counter, and patients who provided their consent were given a survey form. The survey form was anonymous and consisted of questions related to patients' awareness and knowledge regarding their demographics (sex and age). The collection was performed using collection boxes placed at medicine delivery counters. This study was approved by the Ethics Committee of the Japanese University of Pharmacy and Life Sciences (Nichiyaku 5-18).

2.2. Evaluation and analytical methods

Questions 1 and 2 were regarding the demographics of the respondents (sex and age), whereas questions 3–5 and questions 7 and 8 were two-choice questions (yes and no). For question 6, efficacy of antimicrobials and for those who selected 'yes' for question 7, ever discontinued taking antimicrobials, the reason was selected (multiple answers allowed) for the relevant item. In addition, for question 7, Have you ever discontinued antimicrobials, those who selected 'Yes' were considered to have discontinued and those who selected 'No' were considered to have never discontinued and were divided into two groups to examine the relationship between each question.

Analysis was performed using simple tabulation and Fisher's exact test. js-STAR XR+ (Release 1.9.7 j) was used for statistical analysis and 5% was considered statistically significant.

3. Results and Discussion

The survey questionnaire covered 869 consenting respondents, of whom 11 persons whose questionnaires were omitted, and 858 were included in the analysis. The respondent characteristics were 431 (50.2%) male and 427 (49.8%) female. The largest age group included 170 respondents (19.8%) in their 40s, followed by 144 respondents in their 30s (16.8%), and 142 respondents in their 50s (16.6%).

The aggregate results for each question by age group are presented in Table 1. The aggregate results were unbiased with regard to sex and age. Awareness of the terms "bacteria, viruses, and antimicrobials" was > 80% (questions 3 and 4). Regarding the efficacy of antimicrobials (questions 6), 536 respondents (62.5%) stated that they were effective against bacteria, but 309 of these also stated that they were effective against viruses and others (*e.g.*, antipyretic and analgesic); therefore, the exact recognition level was 227 (26.5%). The awareness of drug-resistant bacteria was 319 (37.2%) (questions 5). In addition, 270 (31.5%) patients of the respondent had ever interrupted antimicrobials, and 194 (71.9%) of the reasons for discontinuation were self-judged symptom improvement (questions 7). A total of 647 (75.4%) patients were unaware of the presence of the antimicrobial strains (types) (questions 8).

Table 2 shows that association of each question with whether or not the respondent had ever interrupted antimicrobials. Those who had experienced an interruption were significantly less aware than those who had not in terms of awareness of bacteria and viruses, antimicrobials, drug-resistant organisms and strains (types) of antimicrobials (respective p-values: $p = 0.032$, $p = 0.014$, $p = 0.001$, $p < 0.001$).

The survey was conducted in non-general pharmacies near specific healthcare facilities, and the aggregate results were unbiased with regard to sex and age. Regarding the questions, more than 80% of patients were aware of the terms "bacteria, viruses, and antimicrobials," whereas only 37.2% understood drug-resistant bacteria and 26.5% antimicrobial efficacy. In addition, approximately 30% of the patients experienced discontinuation without taking the drug for up to the prescribed number of days, and approximately 70% of the reasons for discontinuation were symptom improvement based on self-judgment. Furthermore, those who had experienced interruptions were less aware of the various aspects of antimicrobial knowledge than those who had not.

Limiting antimicrobial use reduce the number of bacteria that are resistant to these antimicrobials (*2*), and their increased use is associated with an increase in resistant bacteria. The inappropriate use of antimicrobials has been pointed out as a background to the spread of drug resistance, and limiting the use of antimicrobials to cases where they are necessary for the treatment of infectious diseases is crucial. Furthermore, optimizing the amount and duration of use to the minimum necessary while achieving the maximum effect to reduce drug-resistant bacteria. To promote the control of drug resistance, healthcare professionals and patients must understand antimicrobials and drug resistance.

Table 1. Aggregate results by age group for each question

Q1. Age	whole (*n* = 858)	20s or younger (*n* = 98)	30s (*n* = 144)	40s (*n* = 170)	50s (*n* = 142)	60s (*n* = 116)	70s (*n* = 125)	80s or older (*n* = 63)
Q2. Male / Female	431 (50.2%) / 427 (49.8%)	40 (40.8%) / 58 (59.2%)	67 (46.5%) / 77 (53.5%)	77 (45.3%) / 93 (54.7%)	85 (59.9%) / 57 (40.1%)	70 (60.3%) / 46 (39.7%)	61 (48.8%) / 64 (51.2%)	31 (49.2%) / 32 (50.8%)
Q3. I know the words 'bacteria and viruses.'	710 (82.8%)	80 (81.6%)	113 (78.5%)	141 (82.9%)	120 (84.5%)	100 (86.2%)	106 (84.8%)	50 (79.4%)
Q4. I know the word 'antimicrobial.'	752 (87.6%)	84 (85.7%)	124 (86.1%)	157 (92.4%)	127 (89.4%)	104 (89.7%)	107 (85.6%)	49 (77.8%)
Q5. I know the word 'drug-resistant bacteria.'	319 (37.2%)	25 (25.5%)	44 (30.6%)	79 (46.5%)	62 (43.7%)	51 (44.0%)	42 (33.6%)	16 (25.4%)
Q6. What kind of medicine do you think antimicrobials are?								
Drugs that prevent the growth of bacteria.	536 (62.5%)	54 (55.1%)	86 (59.7%)	115 (67.6%)	102 (71.8%)	77 (66.4%)	74 (59.2%)	28 (44.4%)
Medicines that prevent viruses from increasing.	391 (45.6%)	39 (39.8%)	68 (47.2%)	87 (51.2%)	64 (45.1%)	51 (44.0%)	57 (45.6%)	25 (39.7%)
Drugs that have other actions. (*e.g.*, reduce fever, suppress cough and runny nose)	228 (26.9%)	38 (38.8%)	40 (27.8%)	34 (20.0%)	31 (21.8%)	31 (26.7%)	38 (30.4%)	16 (25.4%)
I am not sure about the effect.	126 (14.7%)	18 (18.4%)	16 (11.1%)	17 (10.0%)	13 (9.2%)	15 (12.9%)	25 (20.0%)	22 (34.9%)
Q7. I have ever failed to take the prescribed number of days of antimicrobials.	270 (31.5%)	43 (43.9%)	48 (33.3%)	40 (23.5%)	45 (31.7%)	36 (31.0%)	34 (27.2%)	24 (38.1%)
(Those who answered Yes to Q7). Why did you not take the prescribed number of days?								
Because my symptoms got better.	194 (71.9%)	30 (69.8%)	33 (68.8%)	28 (70.0%)	33 (73.3%)	27 (75.0%)	25 (73.5%)	18 (75.0%)
Because I forgot to take them and had some left over.	91 (33.7%)	20 (46.5%)	14 (29.2%)	19 (47.5%)	14 (31.1%)	8 (22.2%)	7 (20.6%)	9 (37.5%)
Because I decided to take them another time.	3 (1.1%)	0	1 (2.1%)	0	2 (4.4%)	0	0	0
Because I was not told by the doctor or pharmacist to finish taking them.	16 (5.9%)	1 (2.3%)	1 (2.1%)	3 (7.5%)	4 (8.9%)	1 (2.8%)	3 (8.8%)	3 (12.5%)
All other reasons were side effects.	10 (3.7%)	0	2 (4.2%)	1 (2.5%)	2 (4.4%)	3 (8.3%)	2 (5.9%)	0
Q8. I know that there are strains (types) of antimicrobials.	211 (24.6%)	20 (20.4%)	35 (24.3%)	52 (30.6%)	38 (26.8%)	24 (20.7%)	29 (23.2%)	13 (20.6%)

Table 2. Association of each question with whether or not the respondent had 'ever interrupted antimicrobials'

	Experience of discontinuation of medication		*p*-value
	experienced person (*n* = 270)	inexperienced person (*n* = 588)	
Q3. I know the words 'bacteria and viruses.'	212 (78.5%)	498 (84.7%)	0.032
Q4. I know the word 'antimicrobial.'	225 (83.3%)	527 (89.6%)	0.014
Q5. I know the word 'drug-resistant bacteria.'	79 (29.3%)	240 (40.8%)	0.001
Q8. I know that there are strains (types) of antimicrobials.	51 (18.9%)	160 (27.2%)	< 0.001

The problem for patients, which was also mentioned by approximately 30% of the patients in the study, was the discontinuation of antimicrobials owing to self-determination. In a previous survey, one in three parents reported that they had adjusted the dose of a medicine prescribed by a medical institution at their own discretion and given it to their child, and two in three parents had given their child the remaining medicine at their own discretion when their child developed similar symptoms (*9*). Interruption of antimicrobial medication not only allows bacteria in the body to survive because the dose required to kill them is not taken but may also lead to the development of drug-resistant bacteria that are resistant to the drug. In addition, drug-resistant bacteria that develop in the human body can be transmitted to animals (livestock and wildlife) by passing them into the environment, such as through sewage, and the possibility of direct human-to-animal transmission arises. Because approximately 80% of the patients in this study were not aware that there were different strains (types) of antimicrobials, it is possible that if treatment with an antimicrobial is required and the antimicrobial is changed owing to a change in symptoms, the possibility of discontinuation of the drug without being aware of the changed antimicrobial may occur. In addition, the results showed that those who had experienced interruptions were less aware of various aspects of knowledge about antimicrobials than those who had not experienced interruptions, but it can be inferred that the lack of patient knowledge may have led to the discontinuation of the drug. Therefore, it was considered possible to avoid the discontinuation of antimicrobials by providing appropriate guidance to patients when antimicrobials are prescribed and by improving their knowledge of antimicrobials.

Second, although this study was not conducted with healthcare professionals, possible problems with healthcare professionals include overprescription of antimicrobials. Examples of overprescription by healthcare professionals including the prescription of antimicrobials for the common cold and other conditions for which antimicrobials are ineffective, even if the infection is judged to be caused by a virus, fungus, or acid fungus, although normal antimicrobials are ineffective against such infections. For antimicrobial treatment, it is important to determine the causative organisms and their susceptibility to antimicrobials. For example, in empirical treatment, in which multiple possible causative organisms can be considered, it is extremely important to determine the extent of the range of causative organisms to be covered from the beginning of treatment. Given that the use of broad-spectrum antimicrobials generally has the disadvantage of selecting for resistant organisms, it is recommended that, as far as possible, only the main causative organisms are covered; if there is no improvement, it is advisable to switch to broad-spectrum drugs to cover less frequent bacteria. In terms of the educational effect on healthcare professionals, one study found that the education of family doctors reduced antimicrobial prescriptions by 4.5% without worsening patient outcomes (*10*). Therefore, it can be inferred that a link exists between healthcare professionals' awareness of antimicrobials and their use. Based on the above, it is important to use antimicrobials appropriately and to promote and educate healthcare professionals so that they do not use them at inappropriate doses or for inappropriate periods of time to reduce the occurrence of drug-resistant bacteria.

This study has some limitations including the fact that it was conducted in a single insurance pharmacy and that detailed patient backgrounds, such as occupation, were not investigated. To further improve reliability, it is necessary to conduct an ongoing study in several centers with a large number of patients.

In conclusion, patients in this study that experienced antimicrobial treatment interruptions were less aware of various aspects of antimicrobial knowledge than those who had not. In antimicrobial treatment, avoiding the emergence of drug-resistant bacteria is difficult, is detrimental to patients under treatment, and presents a major problem in society. Therefore, healthcare professionals should optimize infectious disease treatment by providing appropriate guidance on the proper use of antimicrobials, significance of taking them, and harmful effects of their discontinuation to patients.

Acknowledgements

We thank all study participants.

Funding: None.

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

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Received June 3, 2024; Revised July 25, 2024; Accepted August 14, 2024.

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Released online in J-STAGE as advance publication August 18, 2024.

Brief Report

DOI: 10.5582/ddt.2024.01040

Evaluation of *in vivo* pharmacokinetic study of the anti-cancer drug imatinib using silkworms as an animal model

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SUMMARY Imatinib is an oral molecular targeted therapy that acts as a tyrosine kinase inhibitor. Silkworms present a promising experimental model for elucidating the pharmacokinetic and toxicity profiles of various compounds. This study aimed to establish an experimental paradigm for investigating the pharmacokinetics of imatinib in silkworms. A comparative analysis of imatinib pharmacokinetic parameters across silkworms, humans, mice, and rats revealed similarities in time to maximum concentration (T_{max}) and apparent clearance values between silkworms and humans. However, differences in elimination half-life ($t_{1/2}$) and apparent volume of distribution between silkworms and humans remained within 5- and 4-fold ranges, respectively. Importantly, mice demonstrated pharmacokinetic parameters closer to those of humans than rats during imatinib studies. Additionally, silkworms and mice exhibit similar T_{max} and $t_{1/2}$ values. This study highlights the potential of silkworms as valuable tools for investigating imatinib metabolism in pharmacokinetic studies. Furthermore, it underscores the applicability of silkworms in elucidating the pharmacokinetic parameters of various molecular-targeted drugs, thus facilitating advancements in drug development and evaluation.

Keywords Tyrosine kinase inhibitor, comparative analysis, *Bombyx mori*

1. Introduction

The tyrosine kinase inhibitor imatinib was the first oral molecular targeted drug developed to target a specific protein kinase and is currently approved as standard care for patients with BCR-ABL-positive chronic myeloid leukemia and gastrointestinal stromal tumors (*1*). Imatinib interacts with several metabolic enzymes that are major sites of drug–drug interactions (DDIs). It is primarily metabolized by cytochrome P450 (CYP) 3A4. Co-administration of imatinib with CYP3A4 and P-glycoprotein modulators alters the pharmacokinetic profile of imatinib (*2*). Intra- and inter-individual variabilities in drug exposure have been extensively documented (*3*). Thus, imatinib is a drug for which therapeutic drug monitoring is recommended due to its exposure–response and exposure–safety relationships (*4*). The feasibility of therapeutic drug monitoring-guided dosing to achieve a minimum blood plasma imatinib concentration of 750-1,500 ng/mL was demonstrated in a prospective randomized controlled trial (*5*).

The silkworm *Bombyx mori* is a valuable experimental animal for evaluating the pharmacokinetic and toxicity of compounds (*6*). Compared to mammals, silkworms offer several advantages, including lower breeding costs, suitability for rearing in smaller spaces, fewer ethical concerns, and easier quantification of injected sample solutions (*7*). Moreover, drug pharmacokinetic and toxicity in silkworms have been studied (*6,8*). Compound absorption from the silkworm intestinal tract is similar to that of mammals (*9,10*). The total clearance, volume of distribution, and half-life values of antimicrobial agents such as chloramphenicol, tetracycline, vancomycin, rifampicin, micafungin, and fluconazole are also comparable in silkworms and mammals (*11*). Therefore, silkworms are suitable experimental animals for evaluating the pharmacokinetic of imatinib. However, the pharmacokinetic of imatinib in silkworms has not yet been studied. Our study aimed to develop an experimental model for studying the pharmacokinetic of imatinib in silkworms.

2. Materials and Methods

2.1. Reagents

Imatinib (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in methanol (Wako, Osaka, Japan),

and stored as a stock solution (10 mg/mL) at −60°C until use. For silkworm injections, imatinib was diluted with physiological saline (0.9% w/v NaCl). High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol (Kanto Chemical Co., Inc., Tokyo, Japan) and KH_2PO_4 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were utilized as HPLC mobile phases.

2.2. Silkworm rearing

Silkworm rearing followed established procedures (*12*). Silkworm eggs, acquired from Ehime-Sanshu Co., Ltd. (Ehime, Japan), were disinfected and incubated at 25-27°C. Silkworms were nourished with an artificial diet, Silkmate 2S, supplemented with antibiotics from Ehime-Sanshu Co., Ltd. Fifth-instar larvae were employed for infection experiments.

2.3. Pharmacokinetic study

To measure the pharmacokinetic parameters of imatinib in the silkworm model, hemolymph samples were collected at 0.1, 1, 2, 4, 8, and 12 h post- imatinib injection (10 mg/kg). Fifth instar silkworm larvae were fasted overnight on Silkmate 2S diet. Imatinib solution (50 µL, 10 mg/kg) was administered into the midgut using a 1 mL tuberculin syringe (Terumo Medical Corporation, Tokyo, Japan). Hemolymph collection followed a previously established method (*13*). Hemolymph was obtained by severing the first leg and centrifuging at 8,000 rpm for 3 min (MX-100; Tomy Seiko Co., Ltd., Tokyo, Japan). The supernatant (50 µL) was mixed with 450 µL of methanol and centrifuged at 15,000 rpm for 15 min. The resulting supernatant was subjected to HPLC analysis.

2.4. HPLC conditions for detecting imatinib

The HPLC system used for detecting imatinib in silkworm hemolymph comprised a pump (PU-4180, Jasco, Tokyo, Japan), UV detector (UV-4075, Jasco, Tokyo, Japan), and autosampler (AS-4550, Jasco, Tokyo, Japan). An octadecylsilyl column (Capcell Pack C18 MG II, 250 mm × 4.6 mm i.d., 5 µm; Osaka Soda, Tokyo, Japan) with a guard column (Capcell Pack C18 MG II guard column, 10 mm × 4.0 mm; Osaka Soda, Tokyo, Japan) served as the analytical column at 25°C (room temperature). Detection wavelength was set at 250 nm. The mobile phase consisted of acetonitrile and 0.5% KH_2PO_4 (pH 4.4; 32:68, v/v), with a flow rate of 1.0 mL/min. A 10 µL sample of silkworm hemolymph, prepared as previously described, was injected. Calibration concentrations for imatinib ranged from 0.25 to 12.5 µg/mL. The retention time for imatinib was 6.0 min. A linear six-point standard calibration curve was established over the concentration range of 0.25-12.5 µg/mL.

2.5. Pharmacokinetic analysis

HPLC was used to measure imatinib concentration in silkworm hemolymph (n = 3 silkworms). Non-compartmental pharmacokinetic analysis of imatinib was conducted using Phoenix WinNonlin 8.3 (Certara, Princeton, NJ, USA).

3. Results and Discussion

The time course of imatinib concentration in silkworm hemolymph following injection of 10 mg/kg imatinib into the midgut is illustrated in Figure 1. The maximum concentration and time to maximum concentration (T_{max}) were 6.5 ± 0.8 µg/mL and one hour, respectively. The elimination half-life ($t_{1/2}$) was 2.9 hours. The apparent volume of distribution (Vz/F) and apparent clearance (CLz/F) were calculated as 1,315 mL/kg and 319 mL/h/kg, respectively.

Table 1 presents a comparison of imatinib pharmacokinetic parameters in silkworms, humans, mice, and rats (*14-17*). The T_{max} and CLz/F values of imatinib showed similarities between silkworms and humans. The differences in $t_{1/2}$ and Vz/F between silkworms and humans were within 5-fold and 4-fold ranges, respectively. Notably, the Vz/F and CLz/F ratios were lowest in rat models. In mice and humans, the Vz/F was within a 2-fold range, while the CLz/F was approximately 10-fold greater in mice than in humans. Interestingly, the imatinib pharmacokinetic parameters of mice exhibited a closer resemblance to those of humans than those of rats. Additionally, both T_{max} and $t_{1/2}$ were comparable between silkworms and mice. Thus, our findings suggest that silkworms hold promise for pharmacokinetic studies aimed at evaluating imatinib metabolism. The results of this study imply the potential for clarifying pharmacokinetic parameters of other molecular targeted drugs using silkworms.

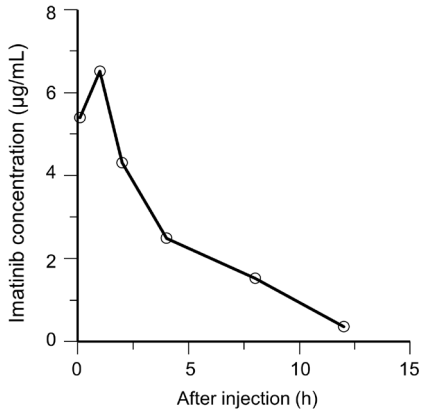

Figure 1. Time course of imatinib concentration changes in silkworm hemolymph. Imatinib injected into the silkworm midgut, followed by hemolymph harvesting at 0.1, 1, 2, 4, 8, and 12 h post-injection. n = 3/group.

Table 1. Imatinib pharmacokinetic parameters in silkworm and mammals

	Silkworm	Human (*14*)	Mice (*15*)	Rat (*16,17*)
T_{max} (h)	1	1.3	0.66	2.4, 4.8
$t_{1/2}$ (h)	2.9	13.5	2.4	3.9, 6.2
V*z/F* (mL/kg)	1,315	4,900	8,100	0.0023, 0.0061
CL*z/F* (mL/h/kg)	319	251	2,310	0.0003, 0.0006

T_{max}, time to maximum concentration; $t_{1/2}$, elimination half-time; V*z/F,* apparent volume of distribution; CL*z/F,* apparent clearance.

The $t_{1/2}$ of imatinib in silkworms was shorter compared to that in humans. Imatinib primarily undergoes metabolism *via* CYP3A4. In silkworms, a total of 79 genes encoding cytochrome P450 have been identified using whole-genome sequencing (*11*). Furthermore, the administration of carbon tetrachloride (CCl_4), a substrate activated by human CYP3A4, to silkworms leads to cytotoxicity. Interestingly, pre-administration of cimetidine, a CYP3A4 inhibitor, significantly attenuates CCl_4-induced cytotoxicity (*18*). These findings suggest the presence of a metabolic mechanism akin to CYP3A4 in humans within silkworms. Hamamoto *et al*. reported that microsomal fractions from the silkworm midgut exhibit metabolic capacities comparable to those of mammals, with a majority of cytochrome P450 enzymes being present in the silkworm midgut (*11*). Consequently, the metabolism of imatinib in silkworms is presumed to be expedited relative to humans, primarily due to the first-pass effect occurring in the silkworm midgut. It is hypothesized that drugs metabolized by CYP3A4 may undergo faster metabolism in silkworms compared to humans; however, future research aims to evaluate the pharmacokinetic parameters of CYP3A substrate drugs, given that a substantial proportion of drugs fall under this category.

The bioavailability of imatinib in humans exceeds 98% (*2*). To examine imatinib absorption in silkworms, the $AUC_{(0–12)}$ of imatinib administered into the hemolymph and midgut was evaluated. The $AUC_{(0–12)}$ of imatinib was 30.95 μg/hr/L when administered into the midgut and 27.44 μg/hr/L when administered into the hemolymph. Thus, in silkworms, as in humans, imatinib was well absorbed and the bioavailability of imatinib was 113%. The absorption of the compound from the intestinal tract of silkworms was similar to that in mammals.

We previously assessed the pharmacokinetic of voriconazole in a silkworm model infected with *Candida*, revealing alterations akin to those observed in human infections (*19*). In this study, we discovered that silkworm pharmacokinetic parameters more closely resemble those of humans compared to other experimental animals, marking a novel observation.

Imatinib is a substrate of various biological pathways, including CYP3A, organic cation transporter 1, organic anion-transporting polypeptide (OATP) 1A2, OATP1B3, breast cancer resistance protein, and P-glycoprotein (*20*). Given that patients on imatinib often use multiple concomitant medications, there is a heightened susceptibility to DDIs. Furthermore, patients with cancer frequently turn to herbal products to ameliorate treatment side effects and enhance quality of life. However, the cumulative impact of these co-administrations on the pharmacokinetic of imatinib remains inadequately explored. Therefore, future investigations are warranted to assess the pharmacokinetic profile of imatinib in the context of DDIs, shedding light on potential interactions with commonly co-prescribed medications in clinical practice. In conducting those studies, we found it useful to examine the use of silkworms as experimental animals.

In conclusion, our study demonstrates the utility of silkworms as an alternative animal model for investigating the single-dose pharmacokinetics of imatinib during the clearance phase.

Acknowledgements

We thank Yuta Shimizu, Mei Nakayama, Sachi Koganesawa, and Hiromi Kanai (Meiji Pharmaceutical University) for their technical assistance in rearing the silkworms. No funds, grants, or other support was received.

Funding: None.

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

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Received June 3, 2024; Revised July 27, 2024; Accepted August 14, 2024.

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Released online in J-STAGE as advance publication August 18, 2024.

Brief Report

DOI: 10.5582/ddt.2024.01043

Development of a silkworm infection model for evaluating the virulence of *Mycobacterium intracellulare* subspecies estimated using phylogenetic tree analysis based on core gene data

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SUMMARY Non-tuberculous mycobacteria (NTM) cause skin infections, respiratory diseases, and disseminated infections. *Mycobacterium avium* and *Mycobacterium intracellulare*, which are slow grown *Mycobacterium*, are main agents of those NTM diseases. A silkworm infection model with *Mycobacterium abscessus*, a rapidly growing *Mycobacterium* species, was established to quantitatively evaluate its virulence within a short period. However, a silkworm infection model to quantitatively evaluate the virulence of *M. intracellulare* has not yet been developed. In this study, we determined the virulence of *M. intracellulare* subspecies within 4 days using a silkworm infection model. The subspecies of *M. intracellulare* strains used in this study were estimated by phylogenetic tree analysis using core gene data. The median lethal dose (LD_{50}) values, which are the dose of a pathogen required to kill half of the silkworms in a group, were determined 4 days after infection. The LD_{50} value of *M. intracellulare* subsp. *chimaera* DSM44623 was higher than that of *M. intracellulare* subsp. *intracellulare* ATCC13950. These results suggest that the virulence of *M. intracellulare* subspecies can be compared using a silkworm model within 4 days.

Keywords *Mycobacterium intracellulare*, subspecies, silkworm, infection, virulence

1. Introduction

Non-tuberculous mycobacteria (NTM) are classified as mycobacteria, excluding *Mycobacterium tuberculosis* and *Mycobacterium leprae* (*1,2*). NTM causes severe chronic diseases such as respiratory infections in immunocompromised humans (*1–4*). *Mycobacterium avium* and *Mycobacterium intracellulare* are mainly isolated from the patients with NTM diseases (*5*). Therefore, *M. avium* and *M. intracellulare* may be highly virulent species in the NTM. Infection experiments using mammals such as mice are useful for evaluating the virulence of these bacteria (*6,7*). However, several weeks are needed for mice to die from infection, and it is difficult to conduct infection experiments using a large number of mice from the viewpoint of animal welfare.

Silkworm, an invertebrate, is useful as an alternative animal for evaluating the virulence of pathogens (*8*). Because silkworms have fewer ethical issues than mammals, a large number of silkworms can be used for infection experiments (*9*). Therefore, silkworm infection models are used to identify virulence-related genes by isolating avirulent mutants from gene-disrupted libraries (*10–13*). Using silkworm infection models, the virulence of pathogens was determined by calculating the median lethal dose (LD_{50}), which is the dose of a pathogen required to kill half of the animals in a group (*14–17*). Moreover, the LD_{50} values of *Mycobacterium abscessus* clinical strains were determined, and the virulence of the *M. abscessus* clinical strains was quantitatively compared (*14*). Furthermore, the silkworm models with mycobacteria such as *M. abscessus*, *M. avium*, or *M. intracellulare*, were developed for evaluating the efficacy of anti-mycobacterial compounds (*14,18,19*). However, quantitative evaluation of the virulence of *M. intracellulare* strains using silkworms has not been performed.

The classification of *Mycobacterium intracellulare* has changed significantly over the past decade. Based on genome information and Average Nucleotide Identity (ANI) analysis, *M. intracellulare* subsp. *yongonense* was integrated into *M. intracellulare* subsp. *chimaera*, and *M. paraintracellulare* was integrated into *M. intracellulare* subsp. *intracellulare*. Currently,

the subspecies of *Mycobacterium intracellulare* are identified as *M. intracellulare* subsp. *intracellulare* and *M. intracellulare* subsp. *chimaera*. Sequencing of the 16S rRNA gene is useful for identifying bacterial species (*20,21*). Because the 16S rRNA gene is highly similar among *Mycobacterium* species, sequencing analysis is insufficient to distinguish closely related subspecies (*22*). *M. avium* subspecies such as *avium*, *hominissuis*, *paratuberculosis*, and *silvaticum* are determined by sequencing the insertion sequences and the internal transcribed spacer 1 region of rRNA genes (*23*). On the other hand, sequencing analysis of the insertion sequences and the internal transcribed spacer 1 region of rRNA genes is not enough to distinguish species/subspecies closely related to *M. intracellulare* (*23*). Even with MALDI TOF-MS, which has been the mainstream method for identification in recent years, it is not possible to distinguish between these two subspecies. Therefore, the development of a novel method for estimating *M. intracellulare* subspecies is desired.

In the present study, we estimated the subspecies of *M. intracellulare* strains by phylogenetic tree analysis based on the core gene data of *M. intracellulare*. The LD_{50} values of the *M. intracellulare* strains were determined using silkworms. These findings suggest that the silkworm infection model is useful for quantitatively calculating the virulence of *M. intracellulare* strains.

2. Materials and Methods

2.1. Phylogenetic tree analysis

Core gene-based phylogeny in *M. intracellulare* strains from the National Center for Biotechnology Information (NCBI) database was generated following the pipeline described by Atxaerandio-Landa *et al*. (*24*). Specifically, 113 assemblies (deposited as *M. intracellulare*, *M. intracellulare* subsp. *chimaera*, *M. intracellulare* subsp. *yongoense*, or *M. paraintracellulare*) were downloaded from the NCBI database with ncbi-genome-download v0.3.1 (accessed on 22 April 2024) and assessed using CheckM2 v1.0.1 (*25*). The 112 *M. intracellulare* sequences were assessed as exhibiting > 99% completeness and < 2% contamination and were annotated using Prokka v1.14.6 (*26*), and general feature format (gff) files were produced. The gff files were analyzed for core genes using Roary v3.13.0 (*27*). The core alignment was trimmed using trimAl v1.4. rev15 with the option '-automated1' (*28*). A maximum likelihood tree was constructed from the alignment composed of 3182 core genes (3,137,717 bp) using the best-fitted nucleotide substitution model (GTR+ F + I + R4) in IQ-TREE v2.2.2.7 (*29*) and visualized using the Interactive Tree of Life (iTOL) (*https://itol.embl.de/*). The core genes were selected based on the criteria that the BLASTp cut-off value was set at 95% according to a previous report (*30*).

2.2. Reagents

Middlebrook 7H9 broth, Middlebrook 7H10 agar, and Middlebrook OADC enrichment were purchased from Becton, Dickinson, and Company (Sparks, MD, USA). Middlebrook 7H9 broth and Middlebrook 7H10 agar were supplemented with 10% Middlebrook OADC Enrichment.

2.3. Bacterial strain and culture condition

M. intracellulare strains were used in this study (Table 1). The *M. intracellulare* strains were grown on Middlebrook 7H10 agar plates at 37°C. A single colony was then inoculated into 5 ml of Middlebrook 7H9 broth and incubated at 37°C for 5 days.

2.4. LD_{50} determination using a silkworm infection model

Silkworm infection experiments with *M. intracellulare* were performed according to a previous study with slight modifications (*14*). Fifth-instar larvae were reared on an artificial diet (Silkmate 2S; Ehime-Sanshu Co., Ltd., Ehime, Japan) for 24 h. *M. intracellulare* cells grown in Middlebrook 7H9 broth were collected by centrifugation and resuspended in sterile saline. A 50-µL sample solution was administered to the silkworm hemolymph by injecting the silkworm dorsally using a 1-ml tuberculin syringe (Terumo Medical Corporation, Tokyo, Japan). The LD_{50} values were determined according to a previous study with slight modifications (*14*). *M. intracellulare* cells grown in Middlebrook 7H9 broth were resuspended in saline. A 2- or 4-fold dilution series of bacterial suspensions was prepared. The bacterial suspension ($2.2 \times 10^5 – 9.6 \times 10^7$ cells/50 µL) was injected into the silkworm hemolymph, and the silkworms were incubated at 37˚C with an artificial diet, Silkmate 2S. The number of surviving silkworms was counted at 4 days after infection. LD_{50} values were determined from the data of three or four experiments using a simple logistic regression model in Prism 9 (GraphPad Software, LLC, San Diego, CA, USA, *https://www.graph pad.com/scientific-software/prism/*).

Table 1. LD_{50} values of *M. intracellulare* strains in a silkworm infection model

Strains	LD_{50} (x 10^7 cells/larva)
M. intracellulare subsp. *intracellulare* ATCC13950	2.1
M. intracellulare subsp. *intracellulare* MOTT64	5.6
M. intracellulare subsp. *chimaera* DSM44623	> 9.6
M. intracellulare subsp. *chimaera* Asan36527	2.1

3. Results and Discussion

We estimated the subspecies of *Mycobacterium* strains (ATCC13950, MOTT64, DSM44623, and Asan36527) used in this study based on phylogenetic tree analysis of core gene sequences. The *M. intracellulare* subsp. *intracellulare* cluster including ATCC13950 and the *M. intracellulare* subsp. *chimaera* cluster including DSM44623 were distinctly separated (Figure 1). Therefore, phylogenetic tree analysis based on core gene

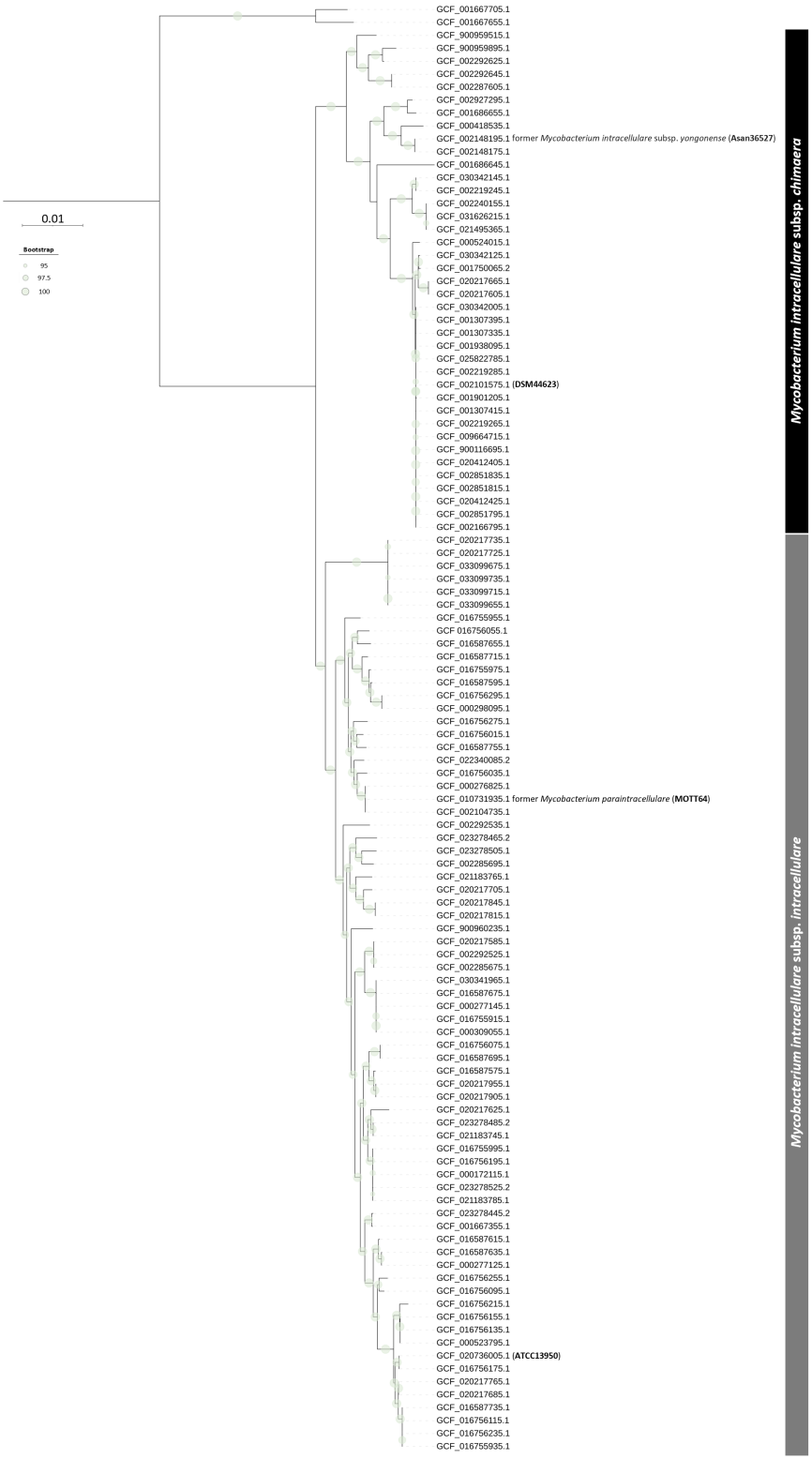

Figure 1. Phylogenetic tree of *M. intracellulare* subspecies strains. Maximum likelihood tree based on 3,182 core genes from 112 strains of *Mycobacterium intracellulare* identified with Roary v3.13.0. The tree was generated using the best-fitted nucleotide substitution model (GTR+ F + I + R4) in IQ-TREE v2.2.2.7, with a 1,000-replicate, ultra-fast bootstrap approximation. Scale bar represents number of nucleotide substitutions per site. The tree is midpoint rooted.

sequences revealed two subspecies; *M. intracellulare* subsp. *intracellulare* and *M. intracellulare* subsp. *chimaera*. MOTT64 is included in the *M. intracellulare* subsp. *intracellulare* cluster (Figure 1). On the other hand, Asan36527 was included in the *M. intracellulare* subsp. *chimaera* cluster (Figure 1). These results suggest that MOTT64 and Asan36527 strains belong to *M. intracellulare* subsp. *intracellulare* and *M. intracellulare* subsp. *chimaera*, respectively.

Next, we established a silkworm infection model to determine the LD_{50} of *M. intracellulare*. ATCC13950 is a type strain of typical *M. intracellulare* subsp. *intracellulare* (*6*). A sample solution of serially diluted ATCC13950 cells ($5.6 \times 10^5 – 3.3 \times 10^7$ cells per larva) was injected into silkworms, and the injection of a large number of bacterial cells led to silkworm death (Figure 2A). The LD_{50} value of ATCC13950 in the silkworm infection model was 2.1×10^7 cells/silkworms (Table 1). These results suggest that a silkworm infection model was established to determine the LD_{50} value of the *M. intracellulare* type strain. We next compared their virulence by determining the LD_{50} values of *M. intracellulare* strains using the silkworm infection model. The LD_{50} values of MOTT64, DSM44623, and Asan36527 were 5.6, > 9.6, and 2.1×10^7 cells/silkworms, respectively (Figures 2B-2D) (Table 1). The LD_{50} values of DSM44623 were higher than those of other strains (Table 1). These results suggest that the virulence of *M. intracellulare* strains was quantitatively calculated based on their LD_{50} values. The silkworm infection model can determine the virulence of the *M. intracellulare* subspecies. In a previously reported silkworm infection model, silkworms were not fed after injection of the sample solution, and saline-injected silkworms died within 4 days (*19*). In this study, silkworms were fed after injection, and the saline-injected silkworms survived for more than 4 days. Therefore, we used the conditions under which the LD_{50} value of the type strain of *M. intracellulare* was calculated after four days of infection. Using these experimental conditions, the LD_{50} values of *M. intracellulare* subspecies were determined. *M. intracellulare* subsp. *chimaera* DSM44623 were lower silkworm killing ability than other strains used in this study. On the other hand, the LD_{50} values for *M. intracellulare* subsp. *intracellulare* ATCC13950 and *M. intracellulare* subsp. *chimaera* Asan36527 were the same. Therefore, the virulence of *M. intracellulare* subsp. *intracellulare* ATCC13950 and *M. intracellulare* subsp. *chimaera* Asan36527 is similar. We assumed that the virulence of each *M. intracellulare* strain was different rather than clear differences in virulence between subspecies of *M. intracellulare*. Revealing the genome structure differences between highly virulent and avirulent strains will be important.

In conclusion, we established a silkworm infection model to compare the virulence of *M. intracellulare* subspecies estimated by phylogenetic tree analysis using core gene data. Using the silkworm infection model, the virulence of *M. intracellulare* strains can be determined within 4 days. We assumed that these experimental methods might contribute to the comparison of the virulence of *M. intracellulare* strains.

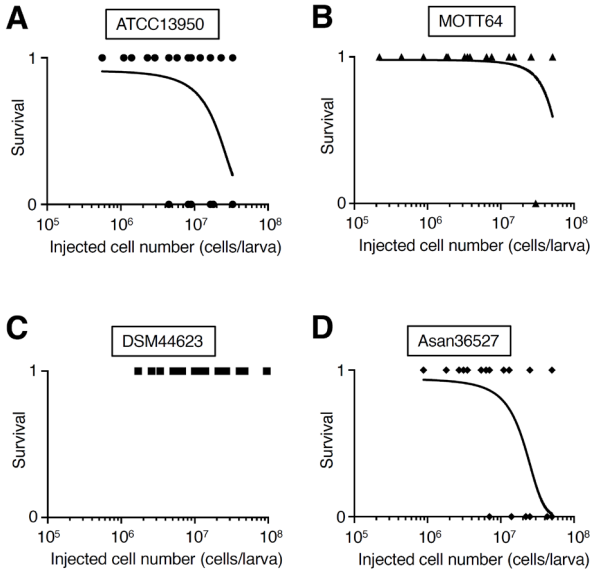

Figure 2. Infection experiments of *M. intracellulare* strains against silkworms. Silkworms were injected with saline or *M. intracellulare* cells ($2.2 \times 10^5 – 9.6 \times 10^7$ cells/silkworm). ATCC13950 strain: $5.6 \times 10^5 – 3.3 \times 10^7$ cells/silkworm. MOTT64 strain: $2.2 \times 10^5 – 5.1 \times 10^7$ cells per silkworm. DSM44623 strain: $1.7 \times 10^6 – 9.6 \times 10^7$ cells/silkworm. Asan36527 strain: $8.8 \times 10^5 – 5.0 \times 10^7$ cells/silkworm. The silkworms were incubated at 37°C for 4 days with an artificial diet. The number of surviving silkworms was counted at 4 days after infection.

Acknowledgements

We thank Sachi Koganesawa, Hiromi Kanai, Yuta Shimizu, and Mei Nakayama (Meiji Pharmaceutical University) for their technical assistance in rearing the silkworms. We also thank Akiko Yamashita, Yukari Nogi, and Ginko Kaneda (National Institute of Infectious Diseases) for their assistance.

Funding: This study was supported in part by grants from the Japan Agency for Medical Research and Development/Japan International Cooperation Agency to Y.H. (JP22jm0510004, JP22wm0225004, JP22wm0325003, JP22fk0108553, JP22fk0108558, JP23fk0108608, JP23fk0108673, JP24gm1610003, JP24gm1610007, JP24wm0125007, JP24wm0225022, JP24wm0325054, and JP24fk0108701), to H.F. (JP22fk0108558 and JP24wm0325054, and JP24fk0108701), and to Y.M. (JP22fk0108553 and JP24wm0325054, and JP24fk0108701). This study was also supported in part by grants from the Japan Society for the Promotion of Science (JSPS) for International Collaborative Research to Y.H. (JP63KK0138-A), and for Scientific Research (A) to Y.H. (JP24H00331),

and for Scientific Research (C) to Y.H. (JP23K07665 and JP23K07958) and to Y.M. (JP23K06141), and for Early-Career Scientists to H.F. (JP22K16382) and to T.K. (JP24K19189).

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

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Received June 4, 2024; Revised July 29, 2024; Accepted August 16, 2024.

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Released online in J-STAGE as advance publication August 25, 2024.

Brief Report

DOI: 10.5582/ddt.2024.01048

The BDNF-ERK/MAPK axis reduces *phosphatase and actin regulator1*, *2* and *3* (*PHACTR1*, *2* and *3*) mRNA expressions in cortical neurons

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SUMMARY Actin rearrangement and phosphorylation-dephosphorylation in the nervous system contribute to plastic alteration of neuronal structure and function. Phosphatase and actin regulator (PHACTR) family members are actin- and protein phosphatase 1 (PP1)-binding proteins. Because some family members act as regulators of neuronal morphology, studying the regulatory mechanisms of *PHACTR* is valuable for understanding the basis of neuronal circuit formation. Although expression patterns of PHACTR family molecules (PHACTR1–4) vary across distinct brain areas, little is known about the extracellular ligands that influence their mRNA levels. In this study, we focused on an important neurotrophin, brain-derived neurotrophic factor (BDNF), and examined its effect on mRNA expression of *PHACTR* family member in cortical neurons. *PHACTR1-3*, but not *PHACTR4*, were affected by stimulation of primary cultured cortical neurons with BDNF; namely, sustained downregulation of their mRNA levels was observed. The observed downregulation was blocked by an inhibitor of the extracellular signal-regulated protein kinase/mitogen-activated protein kinase (ERK/MAPK) pathway, U0126, suggesting that ERK/MAPK plays an inhibitory role for gene induction of *PHACTR1-3*. These findings aid the elucidation of how BDNF regulates actin- and PP1-related neuronal functions.

Keywords BDNF, PHACTR, megakaryoblastic leukemia, myocardin-related transcription factor, extracellular signal-regulated protein kinase, serum response factor

1. Introduction

Actin dynamics and phosphorylation events in the nervous system play essential roles in brain circuit formation and functions (*1-3*). A series of proteins containing the amino acid sequence RPXXXEL (RPEL motif) have been identified as important regulators of cell motility, cell shape, and gene expression (*4-7*). One of these RPEL-containing proteins, megakaryoblastic leukemia/myocardin-related transcription factor, is a serum response factor transcriptional coactivator that regulates immediate early and cytoskeletal genes, as well as neuronal morphology (*8,9*). The phosphatase and actin regulator (PHACTR) family is an RPEL-containing protein family that binds to actin and protein phosphatase 1 (PP1) (*10,11*). There are four PHACTRs (PHACTR1–4) that are spatiotemporally expressed in the brain (*10,12*). Overexpression of PHACTR3 in neurons results in decreased axonal elongation *via* RPEL motifs (*13*) and increased dendritic complexity *via* the PP1-binding domain (*14*), suggesting that PHACTR proteins may contribute to the construction of brain structure. Although such regulation of neuronal morphology is thought to require modulation of PHACTRs mediated by extracellular stimuli at the protein level, only a few studies have identified ligands that control PHACTR functions [*e.g.*, serum-induced nuclear translocation of PHACTR1 (*15*) and Slack channel stimulation-induced dissociation of PHACTR1 from the channel (*16*)]. Moreover, with the exception of vascular endothelial growth factor (*17*), it is largely unknown what ligands control *PHACTRs* at the mRNA level. Here, we investigate whether brain-derived neurotrophic factor (BDNF), a representative neurotrophin involved in neuronal plasticity and development (*18*), influences *PHACTR* mRNA expression in cortical neurons. In this study, we demonstrate differential expression profiles of *PHACTR1–4* after BDNF stimulation and identify the signaling route required for BDNF-mediated alteration of mRNA levels.

2. Materials and Methods

2.1. Animals

For primary cultures of rat cortical neurons, we used rat embryos from pregnant female Sprague-Dawley rats (Japan SLC, Hamamatsu, Shizuoka, Japan) in compliance with guidelines of the Animal Care and Experimentation Committee of University of Toyama, Sugitani Campus (Approval Nos. A2022PHA-6, A2019PHA-7, A2016PHA-8, A2013PHA-4, A2012PHA-1, and A2011PHA-5) and the ARRIVE guidelines.

2.2. Reagents

Human recombinant BDNF protein was a gift from Sumitomo Pharma Co., Ltd. (Osaka, Japan). We used U0126 from Calbiochem (La Jolla, CA, USA, 662005) as a MEK inhibitor. According to previously established concentrations of each inhibitor, U0126 (*19*), LY294002 [L9908, Sigma-Aldrich (St. Louis, MO, USA)] (*20*), and U73122 (U6756, Sigma-Aldrich) (*21*) were added to the medium before BDNF stimulation.

2.3. Cell culture

Rat cortical neurons were prepared from rats aged embryonic day 17, seeded at a density of 8×10^5 cells/well in poly-D-lysine (P6407, Sigma)-coated 12-well plates, and maintained in Neurobasal medium (21103-049, Invitrogen, Carlsbad, CA, USA) including 1× B27 supplement (17504-044, Invitrogen), 2 μg/mL gentamicin (15750-060, Invitrogen), and 0.5 mM glutamine (25030-081, Invitrogen), as previously described (*22*). Medium exchange was carried out by replacing half of the conditioned medium with fresh medium every 3 days.

2.4. RNA preparation and quantitative (q)PCR

RNA preparation followed by reverse-transcription (RT)-qPCR was executed by previously reported methods (*22*). TRIsure (BIO-38032; Bioline, London, UK) was used for RNA extraction and, subsequently, the isolated total RNA was reverse-transcribed for complementary DNA (cDNA) synthesis using SuperScript II (18064-014; Invitrogen). To detect *PHACTR1–4* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), SYBR Select Master Mix (4472908; Thermo Fisher Scientific, Waltham, MA, USA) was used for qPCR according to the manufacturer's experimental conditions. The PCR program was as follows: initial preheating to 50°C for 2 min; subsequent denaturing at 95°C for 2 min; and 40 cycles of denaturing at 95°C for 15 s, annealing at 57°C for 15 s, and extending at 72°C for 1 min. qPCR primer sequences were as follows:

GAPDH-sense: 5′-ATCGTGGAAGGGCTCATGAC-3′,
GAPDH-antisense: 5′-TAGCCCAGGATGCCCTTTAGT-3′,
PHACTR1-sense: 5′-GAGCTCTCCCTGGCATCCTACAC-3′,
PHACTR1-antisense: 5′-CTGCATGGTCATAGCAAGTGTC-3′,
PHACTR2-sense: 5′-TGTCCCCCAACACAGTCACTTC-3′,
PHACTR2-antisense: 5′-GTCCATCACTGACTAGGACCATG-3′,
PHACTR3-sense: 5′-CACCCAAGGCCAGCTCTA-3′,
PHACTR3-antisense: 5′-TTGAAAACTGTCCTGACGGTGC-3′,
PHACTR4-sense: 5′-GCTGAACTGTCCCAAGCAATG-3′,
PHACTR4-antisense: 5′-TTGTCAGCGGTGGTTCCAAAC-3′.

PCR reactions were performed using standard vectors carrying DNA fragments of interest and cDNA samples simultaneously to acquire the standard curve and levels of mRNA expression. Standard vectors containing *PHACTR1*, *PHACTR2*, and *PHACTR4* were made by PCR and subsequent ligation of partial cDNA into the vector. The HA-rat *PHACTR3* vector, which was generated and reported previously (*14*), was also used as a standard vector. The internal control in this experiment was *GAPDH* mRNA expression.

2.5. Statistical analysis

All data are expressed as the mean ± S.E. ($n = 4$, n indicates the number of animals). Statistics were executed using Microsoft Excel 2013 (version 15.0.5127.1000). Data were analyzed by paired *t*-tests with Bonferroni's correction were applied; $p < 0.05/x$ (where x was the number of tests) was regarded as significant (see figure legends).

3. Results and Discussion

Initially, we investigated whether BDNF influenced *PHACTR1–4* mRNA levels in cortical neurons. We found that mRNA expression of *PHACTR1–3* was downregulated by BDNF stimulation (Figure 1). Specifically, downregulation was observed at 1 h for *PHACTR1* and *PHACTR3*, at 3 h for *PHACTR1–3*, at 6 h for *PHACTR3*, and at 12 h for *PHACTR2* and *PHACTR3*. *PHACTR2* displayed the lowest mRNA level among all *PHACTRs*. *PHACTR4* mRNA expression was unchanged at the indicated times (Figure 1D), suggesting that the responsiveness of *PHACTR4* gene to BDNF is less or too minute to be detected. Previously, Allen et al. studied the expression patterns of *PHACTRs* in the brain using *in situ* hybridization (*10*) and Kim et al. reported differential mRNA expression patterns of *PHACTR* family members in the developing and injured brain following traumatic brain injury (*12*). Our findings demonstrating high expression of *PHACTR1* and *PHACTR3* are consistent with these previous studies (*10,12*).

There are three intracellular signaling routes activated by BDNF (*18*). Therefore, we subsequently investigated which signaling pathway is involved in the reduction of *PHACTR1–3* mRNA by using three inhibitors to block

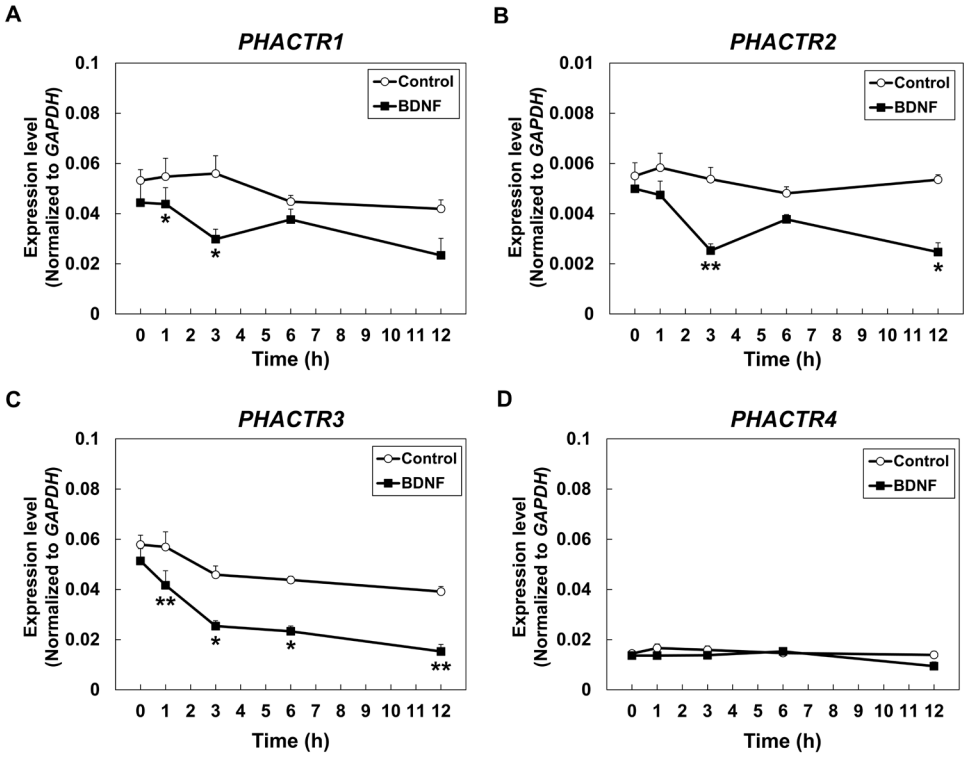

Figure 1. Downregulation of *PHACTRs1*, *2* and *3*, but not *PHACTR4* genes by BDNF stimulation. Primary cultured cortical neurons (9 days *in vitro*) were stimulated with vehicle or 100 ng/mL BDNF. At the time indicated, RNA was isolated and subjected to RT-qPCR for measuring mRNA levels. Time course of *PHACTR1* **(A)**, *PHACTR2* **(B)**, *PHACTR3* **(C)**, and *PHACTR4* **(D)** $^{*}p < 0.05/5$ and $^{**}p < 0.01/5$ (*vs*. vehicle control at the same time point).

the main signals propagated by BDNF. Administration of U0126, a Ras–Raf–mitogen-activated protein kinase (MEK) inhibitor for extracellular signal-regulated protein kinase (ERK)/mitogen-activated protein kinase (MAPK), significantly blocked the decreases of *PHACTR1–3* mRNA levels induced by BDNF (Figures 2A–2C, closed bars). In contrast, the phosphatidylinositol-3 kinase (PI3K) inhibitor LY294002 did not block BDNF-induced reductions of *PHACTR1–3* mRNA levels (Figures 2D–2F, closed bars). Similarly, the phospholipase C inhibitor U73122 did not inhibit the observed reductions (Figures 2G–2I, closed bars). These findings suggest that the MEK–ERK/MAPK cascade is deeply involved in BDNF-mediated attenuation of *PHACTR1–3* mRNA levels. Because transcription of a few genes is reportedly downregulated by ERK/MAPK-mediated C-terminal binding protein corepressor activation (*23*), such a similar mechanism might occur in this study.

The treatment with U0126 alone in the absence of BDNF altered the mRNA level of *PHACTR3* (Figure 2C). Unlike *PHACTR1* (Figure 2A, open bars) and *PHACTR2* (Figure 2B, open bars), *PHACTR3* mRNA level was upregulated by U0126 alone (the third bar from the left) compared with the untreated control sample (the first bar from the left) (Figure 2C). This finding suggests that the MEK–ERK/MAPK suppresses *PHACTR3* gene induction at the resting state of cortical neurons.

Although PHACTRs are expected to play versatile roles in health and disease of the brain, primarily because of their abundant expression (*10*), only a few reports describe the possible involvement of PHACTRs in function or dysfunction of the nervous system, such as roles in neuronal morphology (*13,14*) and epilepsy (*24*). In terms of neuronal morphology, the regulatory roles of PHACTR3 have been examined by its overexpression in primary cultured neurons, whereby it disrupts axonal elongation (*13*), increases dendritic complexity, upregulates percentages of matured spines, and decreases spine densities (*14*). As it remains largely unknown how endogenously expressed PHACTRs influence the nervous system, future studies applying knockout or knockdown technologies to *PHACTR1–4* expression are needed. In this study, we show downregulation of *PHACTR1–3* gene expression caused by BDNF. As described above, the regulatory ligands that regulate *PHACTR1–4* gene expression and role of endogenous PHACTRs remain to be fully elucidated. This study provides valuable insights into the contribution of endogenous PHACTRs and upstream signaling regulating their expression to brain structure and function.

Acknowledgements

The authors would like to thank Sumitomo Pharma Co.,

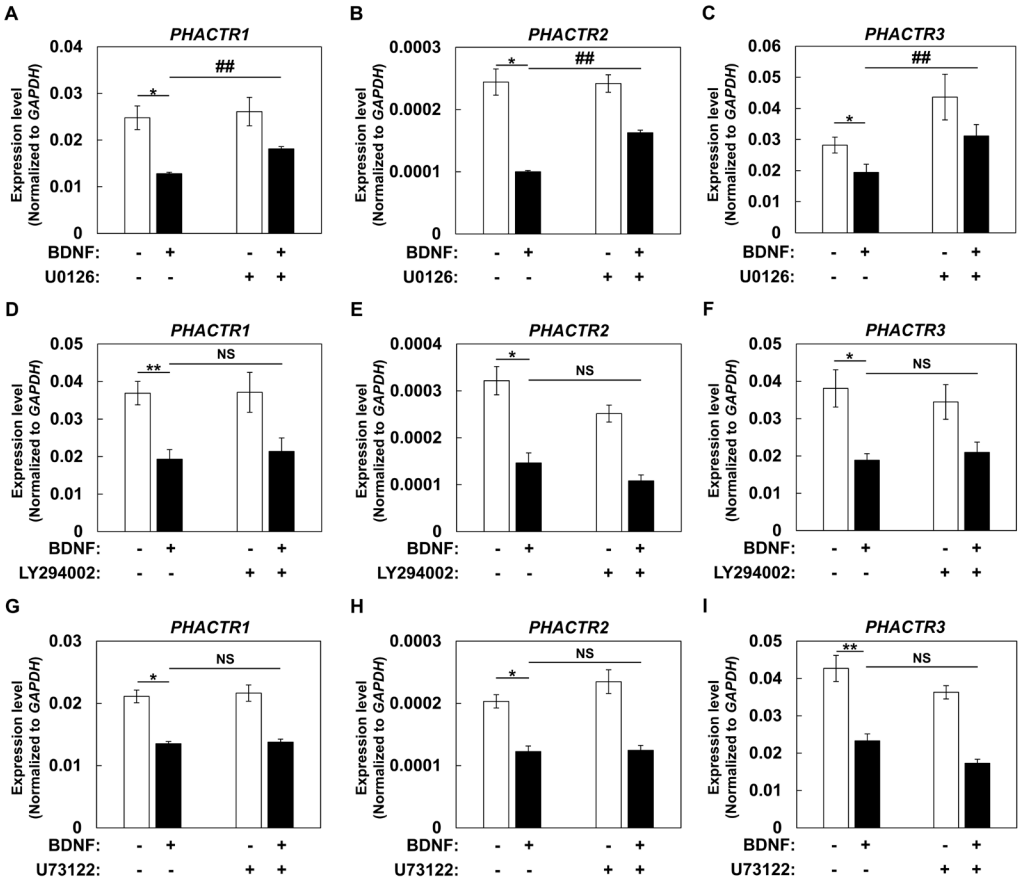

Figure 2. Contribution of ERK/MAPK pathway to BDNF-induced decrease of *PHACTRs1*, *2* and *3* mRNA. The mRNA levels in terms of *PHACTR1* **(A, D, G)**, *PHACTR2* **(B, E, H)** and *PHACTR3* **(C, F, I)**. Three inhibitors, U0126 (20 μM), LY294002 (10 μM) or U73122 (10 μM), were administered into cortical neurons (9 days *in vitro*) 30 min before BDNF stimulation (100 ng/mL). Three hours later, RNA was isolated and subjected to RT-qPCR for measuring mRNA levels. $^{*}p < 0.05/2$ and $^{**}p < 0.01/2$ (*vs*. vehicle control), $^{\#\#}p < 0.01/2$ and NS, not significant (*vs*. BDNF alone).

Ltd. for their provision of BDNF.

Funding: This work was supported by the Smoking Research Foundation (to A.T.), the Uehara Memorial Foundation (to A.T.), and KAKENHI (Grant Nos. JP26460064, JP18K06625, and JP23H03305 to A.T.; and JP20K15989 to D.I.).

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

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Received June 19, 2024; Revised August 5, 2024; Accepted August 16, 2024.

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Released online in J-STAGE as advance publication August 25, 2024.

Brief Report

DOI: 10.5582/ddt.2024.01049

Determination of anamorelin concentration in human plasma using a simple high-performance liquid chromatography-ultraviolet detection method

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SUMMARY Anamorelin, a non-peptide ghrelin analog and growth hormone secretagogue, is a novel oral drug used to treat cancer cachexia. Patients with cancer cachexia frequently use several drugs and anamorelin is a substrate of cytochrome P450 (CYP) 3A4; therefore, drug-drug interactions with CYP3A4 inhibitors and inducers pose a clinical problem. In this study, we aimed to determine the concentration of anamorelin in human plasma using a simple high-performance liquid chromatography-ultraviolet (HPLC-UV)-based method. The analysis involved extracting a 200-μL plasma sample and protein precipitation using solid-phase extraction. Anamorelin was isocratically separated using a mobile phase consisting of 0.5% potassium dihydrogen phosphate (pH 4.5) and acetonitrile (61:39, v/v) on a Capcell Pack C18 MG II column (250 mm × 4.6 mm) at a flow rate of 1.0 mL/min and monitored at a detection wavelength of 220 nm. The calibration curve was linear within a plasma concentration range of 12.5-1,500 ng/mL, with a coefficient of determination of 0.9999. The intra- and inter-day coefficients of variation were 0.37-6.71% and 2.05-4.77%, respectively. The accuracy of the assay and recovery were 85.25-112.94% and > 86.58%, respectively. This proposed HPLC-UV method is simple and can be applied in clinical settings.

Keywords cancer cachexia, drug-drug interactions, clinical settings

1. Introduction

Anamorelin, a non-peptide ghrelin analog and growth hormone secretagogue, is a novel oral drug used to treat cancer cachexia (*1*). Approximately 80% of patients with advanced cancer develop cancer cachexia, which is characterized by involuntary weight loss frequently accompanied by anorexia and altered metabolism (*2*). Anamorelin has been reported to increase appetite, overall body weight, lean body mass, and muscle strength in clinical studies involving patients with cancer experiencing cancer cachexia (*3,4*). However, this therapy for cancer cachexia is approved and marketed only in Japan. Anamorelin is a substrate of cytochrome P450 (CYP) 3A4; therefore, drug-drug interactions with CYP3A4 inhibitors and inducers pose a clinical problem. A three-fold increase in the plasma values for the area under the plasma concentration-time curve over a 24-h dosing interval was observed with ketoconazole, a CYP3A4 inhibitor (*5*). Patients with cancer cachexia use several medications and herbs more frequently than the general population to improve cancer-associated symptoms and quality of life, decrease cancer progression, and reduce the side effects of chemotherapy (*6*). However, such concomitant use and its effects on the pharmacokinetics of anamorelin have not been fully studied. Hyperglycemia, hepatotoxicity, and depression of the stimulant conduction system have been reported as common adverse events of anamorelin (*7*). Furthermore, the exposure-response and exposure-safety relationships of anamorelin levels in plasma have not been reported. We hypothesize that monitoring anamorelin levels in the blood will aid in determining the relationship between its blood levels and efficacy

or safety. Following this, anamorelin treatment can be effectively optimized in patients with cancer cachexia in clinical settings. To date, liquid chromatography coupled with tandem mass spectrometry and high-performance liquid chromatography-ultraviolet (HPLC-UV) have not been used to determine anamorelin concentration in human plasma. HPLC-UV offers many advantages over other detection systems, such as low operational cost, versatility, and simple operation. In this study, we aimed to measure anamorelin levels in human plasma using a HPLC-UV-based method that can be applied in clinical practice.

2. Materials and Methods

2.1. Standards and reagents

Anamorelin and ketoconazole were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Tokyo Chemical Industry Co. (Tokyo, Japan), respectively. HPLC-grade acetonitrile, methanol, and water were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan) and potassium dihydrogen phosphate (KH_2PO_4) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). An Oasis hydrophilic-lipophilic balance (HLB) extraction cartridge was purchased from Waters Corp. (Milford, MA, USA). Human plasma and EDTA-2Na were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan).

2.2. HPLC apparatus and analytical conditions

The HPLC apparatus consisted of a pump (PU-4180; Jasco, Tokyo, Japan), UV detector (UV-4075; Jasco, Tokyo, Japan), and autosampler (AS-4550, Jasco, Tokyo, Japan). Separation was performed on a octadecylsilyl analytical column (Capcell Pack C18 MG II, 250 mm × 4.6 mm i.d., 5 µm, Osaka Soda, Tokyo, Japan). The detection wavelength was 220 nm and the mobile phase consisted of 0.5% KH_2PO_4 (pH 4.5) and acetonitrile (61:39, v/v) with a flow rate of 1.0 mL/min.

2.3. Preparation of stock and working solutions

Anamorelin and ketoconazole stock or working solutions were diluted in methanol and then stored at −60°C in the dark until use.

2.4. Sample preparation

A 10-µL anamorelin working solution was vortexed with 200 µL plasma for 1 min. Anamorelin-spiked plasma (200 µL) was mixed with 10 µL of the internal standard (IS; 10 µg/mL ketoconazole) and 800 µL water, vortexed for 30 s, and applied to the pre-conditioned Oasis HLB cartridges. The cartridges were washed with 1 mL of water and 60% methanol in water (v/v). The analytes were eluted with 1 mL of 100% methanol and vacuum-evaporated to dryness at 80°C using a rotary evaporator. The dried residues were reconstituted with 50 µL methanol, and a 20-µL aliquot was injected into the HPLC system.

2.5. Calibration and Method validation

The calibration concentrations of anamorelin were 12.5, 25, 100, 500, 1,000, and 1,500 ng/mL. Method validation was based on the Guidelines for the Validation of Methods for the Quantitative Analysis of Biological Samples by the US Food and Drug Administration (FDA) (*8*). The recovery and accuracy of the assay were determined at the calibration concentrations of anamorelin (12.5–1,500 ng/mL). The assay precision was evaluated by analyzing five sets of control samples on the same day (intra-day) and five different days (inter-day) at concentrations of 12.5, 25, 100, 500, 1,000, and 1,500 ng/mL.

2.6. Sample stability

Anamorelin stability in plasma samples was evaluated at three different concentrations (12.5, 100, and 1,500 ng/mL). Bench-top stability was evaluated using samples stored at 20°C for 6 h (n = 5). The stability of the processed samples was evaluated after storage at 4°C for 24 h (n = 5). Long-term stability was evaluated using samples stored at −60°C for 4 weeks (n = 5). The freeze and thaw stability were evaluated using samples thawed after storage at −60°C after three freeze-thaw cycles (n = 5).

2.7. Clinical application

Blood samples were collected after obtaining written informed consent from a patient with non-small-cell lung cancer (NSCLC) receiving anamorelin. Plasma samples were obtained by centrifuging the blood samples at 3,000× g for 5.5 min. Plasma and serum were stored at −80°C until analysis. This study was approved by the Institutional Review Board of Tokyo Metropolitan Bokutoh Hospital (approval number: #05–101) and conducted in accordance with the Declaration of Helsinki. Concomitant medications used by the patient were acetaminophen, loxoprofen, rabeprazole, naldemedine, magnesium oxide, oxycodone, prochlorperazine, trichlormethiazide, amlodipine, and insulin.

3. Results and Discussion

In the present study, we developed a sensitive HPLC-UV-based method to determine anamorelin concentrations in human plasma in a clinical setting.

The chromatograms of blank plasma and anamorelin in plasma (100 ng/mL) are provided in Figure 1A and 1B, respectively. The retention times for anamorelin and the IS were 10.2 and 17.4 min, respectively, and there were no interfering peaks (Figure 1B). The six-point standard calibration curve for anamorelin was linear over the 12.5–1500 ng/mL concentration range. The equation for the calibration curve was y = 0.0041x + 0.0026 (r^2 = 0.9999), where y and x are the peak height ratio and anamorelin concentration in plasma (ng/mL), respectively. The lower limit of quantification for anamorelin and the recovery were 6.25 ng/mL and > 86.58%, respectively. At these concentrations, the ranges of intra- and inter-day coefficients of variation were 0.37–6.71% and 2.05–4.77%, respectively (Table 1). The stability of anamorelin in plasma was assessed under various conditions (Table 2). Anamorelin was not significantly degraded, and the final concentration was within 85.25–112.94% of the theoretical values. The matrix effects were assessed by comparing the peaks of the quality control sample in the mobile phase with those of the supernatant from the extracted blank plasma. No significant peak suppression or enhancement was observed. Therefore, the intra- and inter-assay variations, accuracy, recovery, and stability under various conditions complied with the FDA guideline recommendations (*8*). In Japanese patients with NSCLC, the median maximum concentration (C_{max}) and trough concentration (C_{trough}) of 100 mg anamorelin on days 7, 21, and 42 at multiple doses were 1,120 ± 922 and 26.1 ± 25.2 ng/mL, respectively (*9*). In the present study, a concentration range of 12.5–

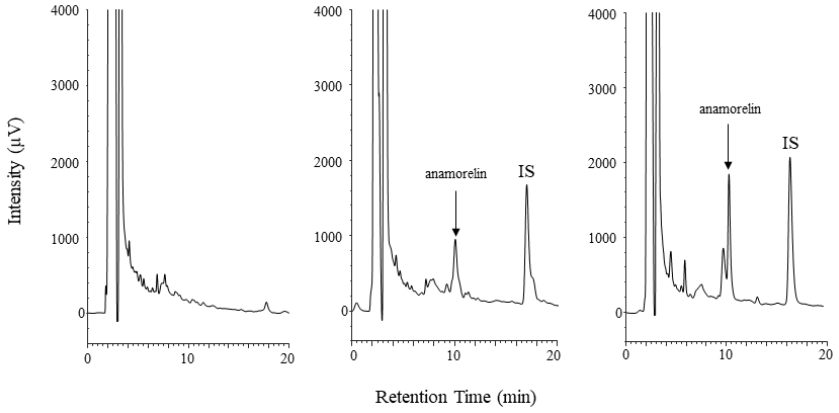

Figure 1. Chromatograms of the (A) blank plasma sample, (B) plasma sample containing 100 ng/mL anamorelin, and (C) plasma sample from a patient treated with 100 mg anamorelin (294.9 ng/mL).

Table 1. Intra- and inter-day accuracy and precision results for anamorelin samples

Anamorelin added (ng/mL)	Intra-day (n = 5)			Inter-day (n = 5)			Recovery (%)
	Mean ± SD (ng/mL)	CV (%)	Accuracy (%)	Mean ± SD (ng/mL)	CV (%)	Accuracy (%)	
12.5	12.51 ± 0.37	0.37	100.11	13.34 ± 0.64	4.77	106.73	89.49
25	26.75 ± 1.61	6.02	107.00	27.13 ± 0.87	3.20	108.51	86.58
100	96.51 ± 5.31	5.50	96.51	100.68 ± 3.54	3.51	100.68	98.14
500	474.05 ± 22.65	4.78	94.81	493.29 ± 10.10	2.05	98.66	91.46
1000	975.71 ± 65.45	6.71	97.57	991.27 ± 38.61	3.90	99.13	94.31
1500	1395.42 ± 58.66	4.20	93.03	1468.47 ± 35.57	2.42	97.90	98.62

CV, coefficient of variation; SD, standard deviation.

Table 2. Stability analyses under various conditions (n = 5)

Anamorelin added (ng/mL)	Stability condition (%)			
	Benchtop mean ± SD	Processed sample mean ± SD	4 weeks mean ± SD	Freeze-and-thaw mean ± SD
12.5	97.69 ± 7.95	112.94 ± 4.90	99.26 ± 5.88	108.55 ± 5.09
100	101.69 ± 3.44	95.40 ± 2.58	87.32 ± 3.07	98.93 ± 1.90
1500	108.28 ± 6.36	104.19 ± 6.03	85.25 ± 1.06	103.92 ± 1.29

SD, standard deviation.

1,500 ng/mL was covered using our method, indicating its suitability for the evaluation of C_{max} and C_{trough} of anamorelin in a clinical setting.

A 76-year-old man with NSCLC who received carboplatin + nab-paclitaxel + atezolizumab was started on anamorelin (100 mg/day) for anorexia. The loss of appetite improved after 5 days of anamorelin treatment, and body weight increased from 60.8 kg to 62.1 kg after 7 days of anamorelin treatment, indicating the effectiveness of anamorelin. At the initiation of anamorelin administration, the patient's aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were 28 and 34 IU/L, respectively. However, blood tests showed AST and ALT levels of 98 IU/L (grade 2 based on the Common Terminology Criteria for Adverse Events [ver.5.0]) and 187 IU/L (grade 3), respectively, on day 28 (Figure 2). The patient was admitted to the hospital for close examination of liver damage. Anamorelin-induced hepatotoxicity was suspected; therefore, anamorelin was discontinued after admission. Seven days after anamorelin discontinuation, the patient's AST and ALT levels improved to normal values, and the patient was diagnosed with anamorelin-induced hepatotoxicity. A plasma sample of anamorelin obtained on day 28 showed a value of 294.9 ng/mL 4 h after administration. The time for anamorelin to reach the maximum serum concentration ranges from 0.5 to 2.0 h (*10*), indicating its presence (half-life of 1–2 h) in the blood. In the ONO-7643-04 study by Ono Pharmaceutical, the mean level of anamorelin in the blood 4–6 h after multiple 100 mg doses was 385 ± 324 ng/mL in Japanese patients with NSCLC (*9*). Compared with the patient in the present study, those in the ONO-7643-04 study had higher blood anamorelin levels 4–6 hours after administration. There are no reports on the relationship between anamorelin levels in the blood and hepatotoxicity; however, there have been reports of moderately elevated but normalized AST and ALT levels in patients after anamorelin discontinuation (*11*), as in the patient in this study. Notably, only one case was studied; therefore, there may be no relationship between anamorelin blood levels and hepatotoxicity. In two phase III trials, the most frequent drug-related adverse events were hyperglycemia (4.2–5.3%). Only a few drug-related Grade ≥ 3 AEs were observed in both studies (0.9% and 2.7%, respectively) (*3,4*). In a phase 1 study, nine healthy male volunteers received 10, 25, and 50 mg oral doses of anamorelin, and serum growth hormone levels increased significantly in a dose-dependent manner (*12*). Therefore, hyperglycemia may be associated with anamorelin levels in the blood. In the future, we aim to evaluate the exposure-response and exposure-safety relationships in cases of hyperglycemia, a characteristic adverse event of anamorelin, in a large sample size using our assay. Nevertheless, the therapeutic range of anamorelin remains unclear. The number of older patients with cancer cachexia has increased with population aging, resulting in increased anamorelin use. Our proposed method can be applied to patients receiving anamorelin and can be used to explore the therapeutic target range.

Our reported method has a limitation, as it was applied to samples from only one patient. During full validation in blank human plasma, amamorelin and biogenic components were completely separated. Furthermore, no interferential peak near anamorelin was observed in six different lots of blank human plasma. However, in this one patient's plasma, with separation between anamorelin and the biogenic component being 1.0 (Figure 1C). Therefore, it may be necessary to reconsider the measurement conditions. Nevertheless, an interference peak near anamorelin in this case did not affect the quantification of anamorelin. It is therefore necessary to measure samples from a large number of patients in the future and assess foreign substances, such as concomitant medications and individual patient biogenic components.

In conclusion, we developed a simple HPLC-UV-based method to determine anamorelin concentration in human plasma. This method is simple and can be used to evaluate interactions between anamorelin and concomitant drugs in clinical settings. Further

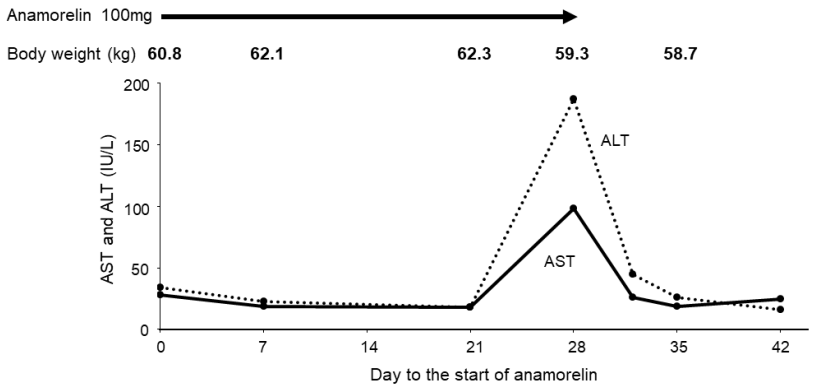

Figure 2. Clinical course of anamorelin for cancer cachexia in a patient with non-small cell lung cancer. The straight line indicates aspartate transaminase (AST), whereas the dashed line indicates alanine transaminase (ALT).

studies using this assay are required to elucidate the relationship between anamorelin blood levels and its clinical efficacy and safety.

Acknowledgements

The authors thank the staff of the Department of Clinical Laboratory, Tokyo Metropolitan Bokutoh Hospital, for sample management.

Funding: None.

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

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Received June 29, 2024; Revised July 30, 2024; Accepted August 19, 2024.

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Released online in J-STAGE as advance publication August 25, 2024.

Brief Report

DOI: 10.5582/ddt.2024.01051

Differences in fluidity and viscosity of brand-name and generic injectable ointment

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SUMMARY Generic medications contain the identical active ingredient in the same concentration as their branded counterparts and are administered in the same manner, aiming to deliver comparable efficacy, dosage, and clinical outcomes. Nevertheless, variations in additives and formulation processes, particularly noticeable in topical medications, can influence factors like ease of use and patient adherence. Therefore, in this study, we aimed to compare the rheological attributes of branded and generic injectable ointments, assessing disparities in formulation performance and their impact on patient care. Posterisan® Forte and Hemoporison® ointments were used as the branded and generic versions, respectively, and their viscosity, ductility, and viscoelastic properties were evaluated. Posterisan® Forte showcased enhanced spread ability, maintaining uniform flow characteristics across varying temperatures, whereas Hemoporison® displayed pronounced thixotropic properties and stiffness, suggesting potential benefits for applications necessitating reversible viscosity adjustments and heightened rigidity. Despite sharing identical additives, observable differences in physical characteristics highlight the necessity of understanding formulation traits, which could influence ointment behavior. Alterations in fluidity and viscosity may affect how patients perceive and apply the medication, potentially influencing treatment outcomes and the occurrence of adverse effects.

Keywords Generic drugs, brand-name drugs, injectable ointments, rheological properties, patient treatment

1. Introduction

Generic drugs, which contain the same active ingredient in the same amount as their brand-name counterparts, are administered *via* the same route, and are designed to provide equivalent efficacy, dosage, and clinical effect. Because they bypass the need for extensive drug discovery research and clinical trials (except for bioequivalence studies), generic drugs are typically more affordable. As such, their utilization is advocated for the dual purpose of reducing national healthcare expenditures and alleviating patient financial burdens (*1*).

In Japan, revisions in reimbursement policies have led to a significant expansion in the utilization of generic drugs, with approximately 80% of drugs by sales volume being generics as of September 2023. However, in terms of value, generics account for only 56.7% of drug usage. This discrepancy is partly attributed to the slow adoption of generic alternatives for expensive medications. Moreover, differences in additives and formulation processes between generic and brand-name drugs, particularly in topical formulations, can impact factors such as usability and patient adherence to treatment. For instance, variations in properties like viscoelasticity, even in common additives like white Vaseline, can influence the quality and user experience of topical medications (*2*). Although pharmaceutical formulation guidelines emphasize the importance of ensuring efficacy, safety, quality, and stability, post-marketing considerations such as supply stability and promoting proper medication adherence are equally crucial (*3,4*). Regardless of a drug's efficacy, therapeutic outcomes are contingent upon patients adhering to correct dosage and administration instructions. In the case of topical medications, which are directly applied to the site of action, factors such as texture and usability play a pivotal role. Furthermore, in injectable ointments, the amount of medication dispensed depends on the force applied during administration. Elderly patients, whose physical abilities may decline, may encounter difficulties in administering ointments

effectively, impacting treatment outcomes (*5*).

Therefore, in this study, we aimed to compare the rheological characteristics of brand-name and generic injectable ointments to evaluate potential differences in formulation performance and their implications for patient treatment. Although both brand-name and generic injectable ointments used in this study shared the same additives, discrepancies in physical properties were observed. This emphasizes the importance of understanding formulation characteristics, which may affect ointment behavior.

2. Materials and Methods

2.1. Materials

Posterisan® forte ointment (lot: 7A187, Maruho Co., Ltd., Osaka, Japan) was utilized as the brand-name drug, while Hemoporison® ointment (lot: G142, J-Dorph Pharmaceutical Co., Ltd., Shiga, Japan) served as the generic drug. In terms of composition, both ointments contained identical ingredients: dead Escherichia coli flotation fluid and hydrocortisone, with purified lanolin, white petroleum jelly, and phenol as additives (Table 1).

2.2. Ductility test of ointments

The viscosity and ductility of the injectable ointments was evaluated using a parallel spread meter (Rigo, Tokyo, Japan). The diameter spread of the ointment sandwiched between two glass plates was recorded for up to 300 s. The relationship of the sample diameter to the logarithmic value of the elapsed time was plotted. Viscosity was compared based on the intercept value of this straight line, while the ease of ductility of each formulation was compared based on the slope of the straight line. Ointment ductility tests were conducted three times at 25°C and 37°C for each formulation.

2.3. Evaluation of viscoelasticity of ointments using a rheometer

Rheological measurements of the ointments were performed using a HAAKE MARS rheometer (Thermo Fisher Scientific K.K., Tokyo, Japan) equipped with a parallel plate PP35 (diameter 35 mm, gap 0.3 mm) to measure stress value (Pa), storage modulus (G') and loss modulus (G"). For rate-dependent measurements, stress values were recorded while varying the shear rate from 0 to 500 s^{-1} and then from 500 to 0 s^{-1}, and the area values surrounding the outward and return curves were presented as thixotropy. Stress-dependent measurements were conducted at 25°C and 37°C at a constant frequency (1 Hz) to determine the storage modulus (G'), indicating solid-like properties, and the loss modulus (G"), indicating liquid-like properties.

3. Results and Discussion

Ductility refers to the ability of a material to deform under stress, which in the context of ointments relates to their spread ability and ease of application. We conducted experiments using a spread meter under varied temperature conditions to assess both the reference and generic products. The ductility of Posterisan® forte and Hemoporison® ointments were compared at two different temperatures: room temperature (25°C) and body temperature (37°C) (Figure 1). At 25°C, both ointments differed in their ductility levels. At 37°C, ductility generally increased for both ointments, suggesting easier application on the skin. Posterisan® Forte ointment demonstrated a more facile spread compared to the Hemoporison® ointment, with a significant difference observed under both temperature conditions. To quantitatively com-pare the flow and spread, we utilized the following equation:

$$S = (D2 - D1) / \log10 (T2 / T1) + IC \quad [eq. 1]$$

where S and IC indicate slope and intercept of the equation 1, respectively; D1 and D2 indicate diameter of spread (mm) after time duration T1 and T2 (s).

T1, T2: Measurement time (s) $T2 > T1$, $5 \leq T1$ and $T2 \leq 100$,

$$\Delta T = (T2 - T1) > 40 \quad [eq. 2]$$

The slope of the graph in Figure 1 indicates greater

Table 1. Composition of the ointments used in the study

	Name	Company	Active pharmaceutical ingredients	Additive 1	Additive 2	Additive 3
Brand-name	Posterisan® forte ointment	Maruho Co., Ltd., Osaka, Japan	0.163 ml of a suspension solution of dead *E. coli* bacteria (containing approximately 259 million bacteria) and 2.5 mg of hydrocortisone per Japan Pharmacopoeia	Refined lanolin	White Vaseline	Phenol
Generic-drug	Hemoporison® ointment	J-Dorph Pharmaceutical Co., Ltd., Shiga, Japan	0.163 ml of a suspension solution of dead *E. coli* bacteria (containing approximately 259 million bacteria) and 2.5 mg of hydrocortisone per Japan Pharmacopoeia	Refined lanolin	White Vaseline	Phenol

sample flow with a larger value. The sample flow (S) of Posterisan® Forte ointment differed between the two temperatures, with S = 0.1184 at 25°C and S = 0.0908 at 37°C. Conversely, for the Hemoporison® ointment, the sample flow values were almost identical, with S = 0.0863 at 25°C and S = 0.0841 at 37°C. The spread was determined from the intersetion of the vertical axes as depicted in Figure 1 (T = 1). A higher IC value indicates lower viscosity and greater flow of the sample. For the Posterisan® Forte ointment, the IC values were almost equal, with IC = 2.6784 at 25°C and IC = 2.7434 at 37°C. Conversely, differences were observed for the Hemoporison® ointment, with IC = 2.5005 at 25°C and IC = 2.6567 at 37°C (Table 2). These results indicate that for the Posterisan® Forte ointment, sample flow decreases as the temperature increases, indicated by the decrease in S from 25°C to 37°C. However, for Hemoporison® ointment, the difference in sample flow between the two temperatures was very small, indicating minimal change in sample flow with temperature increase. Overall, this suggests that the sample flow of Posterisan® Forte ointment is more temperature-sensitive compared to Hemoporison® ointment.

The characterization of thixotropic behavior entails examining the area enclosed by the shear rate versus shear stress curves for both the forward and reverse directions, which delineates the yield history following the application of shear. The shear rate-dependent properties of Posterisan® Forte and Hemoporison® ointments at 25°C are illustrated in Figure 2. Upon increasing the shear rate (dg/dt) from 0 to 500 s^{-1}, the corresponding shear stress values (τ) approximately doubled, measuring 1,200 Pa and 2,100 Pa for the Posterisan® Forte and Hemoporison® ointments, respectively. Notably, the area beneath the return curve, indicative of thixotropy, exhibited notable discrepancies between the two formulations. Specifically, the area for Hemoporison® ointment surpassed that of Posterisan® Forte ointment.

Figure 3 shows the relationship between shear stress on the horizontal axis and the storage modulus (G') and loss modulus (G") on the vertical axis, both expressed on a logarithmic scale. For both ointments, the storage modulus (G') at 25°C exhibited a linear region unaffected by increasing shear stress and thus maintaining a solid-like structure. The G' value within this linear region reflects the

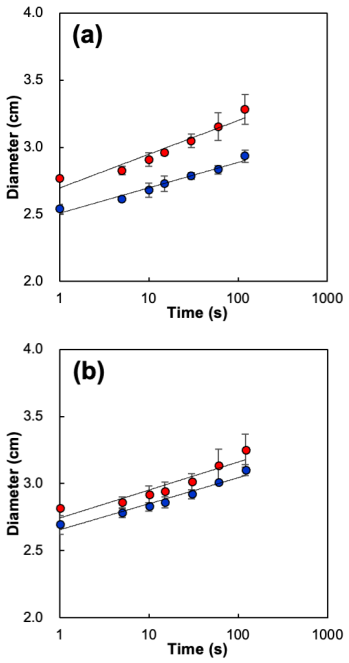

Figure 1. Ductility of Posterisan® forte (red circles) and Hemoporison® (blue circles) ointments at 25°C (a) and 37°C (b).

Table 2. The slope and intercept values of Posterisan® forte and Hemoporison® ointments at 25°C and 37°C

Ointment	Temperature (°C)	slope value	Intercept value
Posterisan® Forte	25	0.1184	2.6784
	37	0.0908	2.7434
Hemoporison®	25	0.0863	2.5005
	37	0.0841	2.6567

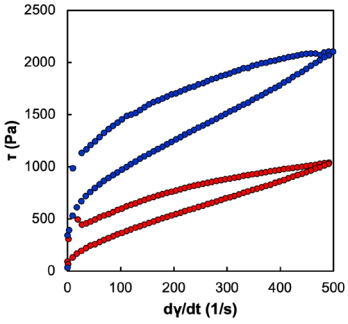

Figure 2. The rate-dependent thixotropic properties of Posterisan® forte (red circles) and Hemoporison® (blue circles) ointments at 25°C.

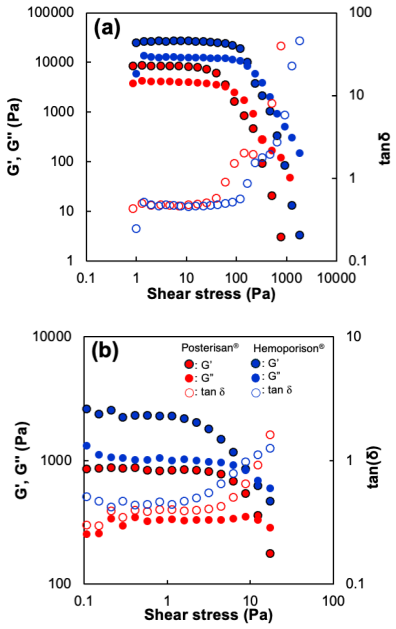

Figure 3. The rate-dependent properties of Posterisan® forte and Hemoporison® ointments at 25°C (a) and 37°C (b).

hardness of the ointment, with Hemoporison® ointment (27,000 Pa) demonstrating higher hardness compared to Posterisan® Forte ointment (8,500 Pa). With increasing shear stress, G' gradually decreased, followed by a rapid decline with additional stress application. The comparison by temperature revealed a notable disparity between the formulations at 25°C; however, at 37°C, both ointments exhibited lower values than those at 25°C, suggesting a temperature-dependent alteration in their rheological properties. At 37°C, the viscosity of Posterisan® Forte ointment measured 800 Pa, whereas that of Hemoporison® ointment was 2,200 Pa. Despite a decrease in viscosity relative to 25°C, Hemoporison® maintained a higher viscosity, hinting at inherent differences in composition or formulation. Regarding the loss modulus (G'), Posterisan® Forte ointment exhibited a higher value at 25°C, indicative of greater energy dissipation during deformation, potentially influencing its flow and deformation characteristics. The behavior at 37°C indicated a convergence between the two formulations, with the loss modulus (G'') closely mirroring the storage modulus (G'). This convergence suggests a mitigated distinction between the formulations at elevated temperatures compared to those at 25°C. These findings underscore the significance of temperature in modulating the rheological properties of the formulations, revealing differential responses under varying conditions.

The study provides valuable insights into the rheological behavior of Posterisan® Forte and Hemoporison® ointments, highlighting differences in spread ability, thixotropic properties, and stiffness between the two formulations. Injectable ointments are preferred to be easily extrudable from the tube at the intended usage temperature of 25°C, indicating good spread ability. Additionally, it is desirable for the ointment to maintain its shape after injection at body temperature (37°C). Using a spread meter, we compared the elongation of the ointments at 25°C and 37°C, assuming the temperature during use and after injection, respectively (Figure 1). At 25°C, the elongation of Posterisan® Forte ointment was significantly higher than that of the Hemoporison® ointment. In contrast, at 37°C, Posterisan® Forte ointment tended to elongate more, but not significantly more than the Hemoporison® ointment. Posterisan® Forte demonstrated superior spread ability, maintaining consistent flow values across different temperatures, whereas Hemoporison® exhibited greater thixotropic behavior and stiffness, indicating potential advantages in applications requiring reversible viscosity changes and increased rigidity. These variations in rheological properties between brand-name and generic ointments could impact patient experience and treatment efficacy. Changes in fluidity and viscosity may influence patient perception and application behavior, potentially affecting treatment outcomes and the risk of side effects. Therefore, precise guidance on application amounts is essential to ensure appropriate use and minimize potential adverse effects. Although generic drugs are increasingly popular, concerns about their quality persist among patients, particularly regarding differences in additives and formulation properties (*6*). Although both Posterisan® Forte and Hemoporison® ointments used in this study share the same additives, discrepancies in physical properties were observed. This emphasizes the importance of understanding formulation characteristics, including container hardness and injection diameter, which may affect ointment behavior. Further research is needed to explore the implications of these findings in clinical practice, considering individual differences in usability and therapeutic efficacy. Additionally, ongoing investigation into other drugs and their rheological properties is warranted to optimize their use and enhance patient outcomes in pharmaceutical and cosmetic applications.

Funding: None.

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

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Received July 8, 2024; Revised August 9, 2024; Accepted August 20, 2024.

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Released online in J-STAGE as advance publication August 29, 2024.

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(As of December 2022)

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