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CONTENTS

Volume 16, Number 2, 2022

Editorial

Review

Original Article

Communication

Commentary

Letter to the Editor

Editorial

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Challenges in the screening and treatment of latent multidrug-resistant tuberculosis infection

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SUMMARY Individuals in close contact with multidrug-resistant tuberculosis (MDR-TB) patients are subject to an elevated risk of infection, and may develop latent MDR-TB infection. Numerous studies have described latent tuberculosis infection (LTBI) as a reservoir of new TB disease. The screening and treatment of latent MDR-TB infection are challenging. Hereby, we reviewed the epidemiology, current management and prevention approach of LTBI in MDR-TB close contacts, to provide additional information for future research direction and policy design formulation to reduce the LTBI reservoir.

Keywords multidrug-resistant tuberculosis, latent tuberculosis infection, close contact, diagnosis, treatment

1. Introduction

The World Health Organization (WHO) global tuberculosis report reveals that 9.87 million people were newly diagnosed with tuberculosis in 2020, of which 490,000 were new multidrug-resistant TB (MDR-TB) cases. Meanwhile, 20.1% of these new cases were resistant to fluoroquinolones, a class of antibiotics which are key regimen components for patients with drug-resistant TB (*1*). China is in the second place in the global list of high burden countries for MDR-TB. Treatment success rate of MDR-TB is reported to be 54% only (*1*). The treatment of MDR-TB is difficult and is a huge challenge for disease control in China and many other high burden countries in the world (*2*).

Latent tuberculosis infection (LTBI) is defined as the presence of immune responses to *Mycobacterium tuberculosis* without clinical evidence of active tuberculosis (TB). Around 5-15% of people with LTBI will progress to active TB over their lifetime (*3*). The WHO recently advocated the urgency to address the problem of LTBI in order to contain and eliminate the tuberculosis epidemic, especially MDR-TB (*4*). Evidence-based studies have reported the effectiveness of LTBI treatment in preventing progression to active TB (*5*). However, only a small portion of population at risk have received preventive treatment (*6*). It is more complicated to assess and tailor an optimal regimen for individuals who have developed LTBI from being a close contact to an MDR-TB patient. Therefore, we reviewed the studies relevant to care and prevention of MDR-TB in close contacts and describe the problems faced by people at risk of MDR-TB disease.

2. Global burden of LTBI in MDR-TB close contacts

A study by Knight *et al*. (*7*) estimated that three out of 1,000 in the world population are latent MDR-TB infected, and the infection rate among individuals under the age of 15 is about 10 times higher than those over 15 years. If the current trend continues, the proportion of LTBI caused by MDR-TB will increase and become a serious challenge to TB management. We speculate that about 2 million people in the global population were already infected by the year 2015 (*i.e*. infection in 2013 or 2014) and this population is at an increased risk of progressing to active MDR-TB. The mathematical model developed by Mehra *et al*. (*8*) showed the prevalence of LTBI caused by MDR-TB was growing at a faster pace than those caused by drug-susceptible tuberculosis (DS-TB) in China. Moreover, compared with adults, children with LTBI are at an elevated risk of progressing to MDR-TB and their timely diagnosis and treatment is also more challenging (*9*). With the ongoing MDR-TB epidemic, the global burden of latent MDR-TB infection has become an obstacle to the End TB Strategy.

3. Diagnosis of LTBI in MDR-TB close contacts

There are no definite tests to accurately diagnose LTBI until now. Currently, the tuberculin skin test (TST) and tuberculosis interferon-gamma release assay (IGRA)

recommended by WHO are in use for the diagnosis of LTBI in close contacts (*4*). Nonetheless, both methods cannot assess the drug susceptibility profile of LTBI. Some studies suggested the use of prediction models combining epidemiological and clinical risk factors of the index cases and TST results of their contacts as effective index like biomarkers to predict and stratify risk of active TB progression (*10,11*). But the use of these tools to screen LTBI in practice is limited by different clinical settings. Before the introduction of novel diagnostic testing, TST and IGRA still are considered to be acceptable in contact tracing and LTBI management. Optimization of both tests may play a key role in the contact investigation strategy (*4*).

4. Treatment of LTBI in MDR-TB close contacts

The clinical trials of preventive treatment of LTBI in MDR-TB close contacts have begun in 2015. With emerging evidence from observational studies and the introduction of new drugs, treatment regimen design has evolved over the years (*4*). At present, levofloxacin is the key medicine for treatment of LTBI caused by MDR-TB and shows a good tolerability profile (*12*). However, 16.3% of multidrug-resistant strains were reported to be resistant to fluoroquinolone (*1*), which raised the concern of the feasibility of fluoroquinolone in the treatment of LTBI caused by a fluoroquinolone-resistant MDR-TB strain. A treatment regimen modelling by Holland *et al*. showed that fluoroquinolone prophylaxis treatments for latent MDR-TB infection could significantly save health care payments, lower mortality and improve the quality of patients' life (*13*). Although the pre-existing fluoroquinolone-resistance rate is as high as 85% in the total number of fluoroquinolone-resistant MDR-TB first-episode cases, the regimen still shows prospective results in lowering incidence of latent-to-active MDR-TB progression in close contacts (*13*). Therefore, prophylactic treatment of fluoroquinolones may be a cost-effective strategy. In 2020, Huang *et al*. had demonstrated good results of using isoniazid in reducing incidence of latent-to-active MDR-TB progression in patients under 19 years old (especially under 5 years old) (*14*). The main controversy of these studies is the lack of a clear and credible mechanism to explain the results. Still, the rigor of observation and detailed analysis are noteworthy and lays a foundation for further research and national strategies (*15*).

In recent years, several randomized clinical trials (RCT) of prophylactic drug treatment for latent MDR-TB infection showed that later-generation fluoroquinolones can be used either in a monotherapy or with a second drug. But an increasing incidents in its associated toxicity, adverse events, and discontinuation suggest that pyrazinamide should not be routinely used as a second drug (*9,16*). A systematic review from Marks *et al*. found that the most cost-effective regimen is fluoroquinolone combined with ethambutol, followed by fluoroquinolone alone, then pyrazinamide combined with ethambutol (*17*). Based on current evidence of treatment outcome for latent MDR-TB infection, the WHO recommends fluoroquinolones, such as levofloxacin or moxifloxacin, for the treatment of latent MDR-TB infection (*4*), but as there is a lack of RCT or cohort study, the recommendation is not based on robust evidence (*18*). Another important subject in debate is whether preventive treatment is necessary for all latent MDR-TB infections (*19*). More evidence from stratified studies in MDR-TB close contacts in different immune states are needed to find out prevalence and risk of progression from LTBI to active TB. The high cost and toxicity associated with active MDR-TB treatment should be taken into account in identifying an optimal preventive treatment design for latent MDR-TB infection to lower the incident rate of active TB.

5. Management of LTBI in MDR-TB close contacts

Contact tracing and LTBI management in MDR-TB close contacts should be monitored closely and constantly for 24 months to enable early detection of active MDR-TB (*20*). In addition, the vast majority of close contacts without evidence of latent MDR-TB infection should be followed up for possible onset of active TB within 24 months. Preventive treatment involving drugs with high intolerability profile should be avoided (*19*).

6. Conclusion

In conclusion, current studies have provided evidence on the importance of preventive treatment for LTBI in MDR-TB close contacts, which is also an essential step in containing the MDR-TB epidemic. However, it is necessary to explore new strategies for the diagnosis, treatment and management of LTBI in MDR-TB close contacts. More prospective studies in the area are needed to generate a larger data set and provide validation to formulate updated clinical guidelines and public health advice. Together we are moving in the direction of WHO's End TB goal by 2035.

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References

1. World Health Organization. Global tuberculosis report 2021. Geneva. World Health Organization.
2. Antimicrobial Resistance Collaborators. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. Lancet. 2022; 399:629-655.
3. Comstock GW, Livesay VT, Woolpert SF. The prognosis of a positive tuberculin reaction in childhood and

adolescence. Am J Epidemiol. 1974; 99:131-138.
4. World Health Organization. Latent tuberculosis infection: Updated and consolidated guidelines for programmatic management Background document on the 2019 revision. Geneva, WHO Global TB Programme.
5. Zenner D, Beer N, Harris RJ, Lipman MC, Stagg HR, van der Werf MJ. Treatment of latent tuberculosis infection: An updated network meta-analysis. Ann Intern Med. 2017; 167:248-255.
6. World Health Organization. Latent tuberculosis infection, updated and consolidated guidelines for programmatic management. Geneva; 2018.
7. Knight GM, McQuaid CF, Dodd PJ, Houben R. Global burden of latent multidrug-resistant tuberculosis: trends and estimates based on mathematical modelling. Lancet Infect Dis. 2019; 19:903-912.
8. Mehra M, Cossrow N, Kambili C, Underwood R, Makkar R, Potluri R. Assessment of tuberculosis burden in China using a dynamic disease simulation model. Int J Tuberc Lung Dis. 2013; 17:1186-1194.
9. Migliori GB, Tiberi S, Zumla A, *et al*. MDR/XDR-TB management of patients and contacts: Challenges facing the new decade. The 2020 clinical update by the Global Tuberculosis Network. Int J Infect Dis. 2020; 92s:S15-s25.
10. Li R, Nordio F, Huang CC, Contreras C, Calderon R, Yataco R, Galea JT, Zhang Z, Becerra MC, Lecca L, Murray MB. Two clinical prediction tools to improve tuberculosis contact investigation. Clin Infect Dis. 2020; 71:e338-e350.
11. Saunders MJ, Wingfield T, Tovar MA, Baldwin MR, Datta S, Zevallos K, Montoya R, Valencia TR, Friedland JS, Moulton LH, Gilman RH, Evans CA. A score to predict and stratify risk of tuberculosis in adult contacts of tuberculosis index cases: a prospective derivation and external validation cohort study. Lancet Infect Dis. 2017; 17:1190-1199.
12. Bamrah S, Brostrom R, Dorina F, Setik L, Song R, Kawamura LM, Heetderks A, Mase S. Treatment for LTBI in contacts of MDR-TB patients, Federated States of Micronesia, 2009-2012. Int J Tuberc Lung Dis. 2014; 18:912-918.
13. Holland DP, Sanders GD, Hamilton CD, Stout JE. Strategies for treating latent multiple-drug resistant tuberculosis: a decision analysis. PLoS One. 2012; 7:e30194.
14. Huang CC, Becerra MC, Calderon R, Contreras C, Galea J, Grandjean L, Lecca L, Yataco R, Zhang Z, Murray M. Isoniazid preventive therapy in contacts of multidrug-resistant tuberculosis. Am J Respir Crit Care Med. 2020; 202:1159-1168.
15. Schluger NW. Prevention of multidrug-resistant tuberculosis in close contacts. Back to the future? Am J Respir Crit Care Med. 2020; 202:1077-1078.
16. Nahid P, Mase SR, Migliori GB, *et al*. Treatment of drug-resistant tuberculosis. An official ATS/CDC/ERS/IDSA clinical practice guideline. Am J Respir Crit Care Med. 2019; 200:e93-e142.
17. Marks SM, Mase SR, Morris SB. Systematic review, meta-analysis, and cost-effectiveness of treatment of latent tuberculosis to reduce progression to multidrug-resistant tuberculosis. Clin Infect Dis. 2017; 64:1670-1677.
18. World Health Organization, WHO operational handbook on tuberculosis (Module 1 – Prevention): Tuberculosis preventive treatment. Geneva.
19. Moore DA. What can we offer to 3 million MDRTB household contacts in 2016? BMC Med. 2016; 14:64.
20. Shah NS, Yuen CM, Heo M, Tolman AW, Becerra MC. Yield of contact investigations in households of patients with drug-resistant tuberculosis: systematic review and meta-analysis. Clin Infect Dis. 2014; 58:381-391.

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Review

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A review on characterization, applications and structure-activity relationships of *Bacillus* species-produced bacteriocins

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SUMMARY Antimicrobial peptides (AMPs) are inherently occurring proteins that are produced by microorganisms as secondary metabolites. Members of genus *Bacillus* produce many types of AMPs by ribosomal (bacteriocins) and non-ribosomal (polymyxins and iturins) mechanisms. Bacteriocins are ribosomally synthesized peptides that inhibit the growth of closely related bacterial strains. Moreover, bacteriocins produced by *Bacillus* species have been widely used in pharmaceutical, food industry, fishery, livestock as well as in agriculture sector. The objective of this review is to assess the characterization of the *Bacillus*-derived bacteriocins, their potential use in different sectors and structure-activity relationships.

Keywords Antimicrobial agents, lantibiotics, bacteriocin-like inhibitory substance (BLIS), probiotics

1. Introduction

Microorganisms are good source of antimicrobial agents. The production of antibiotics by microorganisms and use of antimicrobial peptides (AMPs) as therapeutics has been one of the major achievements in medicine (*1*). *Bacillus* is Gram-positive, rod shaped and spore-forming bacteria. They are aerobic and catalase producing bacteria and found in different natural environments such as soil, rocks, dust, marine, agricultural produce, and the gastrointestinal tract of animals (*2*). Among the strain for producing antimicrobial compounds, *Bacillus subtilis* is the major producer followed by other Bacilli such as *Bacillus brevis* (brevistin, edeines, gramicidines, tyrocidin), or *Bacillus amyloliquefaciens* (*3*). The bacteriocin is one of a heterogenous subgroup of ribosomally synthesized antimicrobial peptides that have bacteriocinogenic plasmids which are lethal to closely related bacteria (*4*). Both Gram-positive and Gram-negative bacteria produce bacteriocins.

Members of the *Bacillus* group are known to be a major producer of antimicrobial substances (*4*). Some of its members, such as *B. subtilis*, devote more than 4% of its genome for the synthesis of polyketides (PKs), non-ribosomal peptides (NRPs), bacteriocins as well as other uncommon antibiotics (*5*). The antimicrobial agents produced by various strains of *Bacillus* are found to exhibit antibacterial as well as antifungal activity against many pathogenic microorganisms including phytopathogens (*6*).

Because of potency of bacteriocins in different sectors, their study is important notion. This review illustrates an overview of bacteriocins produced by *Bacillus* including its classification as well as their applications in different sectors such as human health, food industry, fishery, agriculture, and environment.

2. Bacteriocins produced by *Bacillus* species

Bacillus genus strains produce large number of antimicrobial peptides with different chemical structures. Specifically, they produce antimicrobial substances including peptides, lipopeptides and bacteriocins (*4*). Similarly, *Bacillus* species produce major antibiotics that are made by ribosomal (bacteriocins) or non-ribosomal (polymyxins and iturins) pathway according to their mechanism of action. Among them, high number was produced by *B. subtilis*, followed by *B. brevis* and few by other *Bacillus* species (*3*). Different strains of *B. subtilis* produce variety of bacteriocins. For example, *B. subtilis*, *B. subtilis* A1/3, *B. subtilis* 168, *B. subtilis* strain HILY-85 produces subtilin, ericin S and ericin A, sublancin 168, mersacidin, respectively. Other *Bacillus* species like *B. licheniformis*, *B. cereus*, *B. thuringiensis*, and *B. pseudomycoides*, *etc.* also produce bacteriocins like bacillocin 490, cerein 8A, thuricin 7, and pseudomycoicidin respectively (*7-11*). Recently, a new bacteriocin was reported namely amylocyclicin which was produced by *B. amyloliquefaciens* FZB42 (*12*). Sonorensin is a new peptide belonging to

heterocycloanthracin, subfamily of bacteriocin isolated from marine bacteria *B. sonorensis* MT 93. This peptide showed activity against broad spectrum bacteria including *B. subtilis*, *E. coli*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Vibrio vulnificus* (*13*). Other class iii bacteriocins produced by *B. subtilis* group are baciamin and Bac 14 B which have antibacterial as well as antifungal activity (*5*) Abriouel *et al.* in 2011 reported a long list of bacteriocins/BLIS produced by *Bacillus* species (*14*). Some of the reported *Bacillus* produced bacteriocins are summarized in Table 1.

2.1. Classification of *Bacillus* bacteriocins

The classification of bacteriocins was initially done by Klaenhammer in 1993, Nes and colleagues in 2007, and by Abriouel and his coworkers in 2011 (*14,20*). They classified bacteriocins into three classes, the first class (I) include antimicrobial peptides that undergo different forms of post-translational modifications; the second class (II) presents nonmodified and linear peptides, and the last class (III), which includes large proteins (> 30 kDa). Recently, Soltani *et al.* (2021) reclassified bacteriocins into two large classes. Class I resembles peptides group with molecular masses < 5 kDa and that contain post translationally modified bacteriocin. Class II bacteriocins contain unmodified peptides with molecular masses of 6-10 kDa including peptides with unstable disulfide bridges (*21*).

Class I. Post-translationally modified peptides

The class I bacteriocins include small (< 5 kDa) heat stable peptides. This class can be further divided into 4 subclasses (subclasses I.1, I.2, I.3, and I.4). Subclasses I.1-I.3 includes lantibiotic peptides, containing lanthionine and methyllanthionine residues. While the subclass I.4 includes peptide with unique modifications (*14*).

Subclass I.1. Single peptide, elongated lantibiotics

This group is represented by lantibiotics, are small peptides (*22,23*) (19-38 aminoacids) and contains dehydrated amino acids (lanthionine and methyllanthionine) introduced by posttranslational modifications (*24,25*). Unusual amino acids lanthionine and 3-methyllanthionine are in the form of ring structures that make lantibiotics more stable against heat, wide range of pH and proteolytic enzymes, thus these properties differentiate them from other antimicrobial peptides *(25*). Subtilin is a lantibiotic produced by *B. subtilis*, one of the extensively studied peptide that belongs to type A lantibiotics. It is active against most of the Gram-positive and some Gram-negative bacteria (*26*). Ericin S (3,442 Da) and ericin A (2,986 Da) are two related lantibiotics produced by *B. subtilis* A1/3 with strong resemblances to subtilin (*27*).

Subclass I.2. Other single peptide lantibiotics

Subclass I.2 includes globular lantibiotics mersacidin and other lantibiotics namely sublancin 168 and paenibacillin. Mersacidin which is produced by *Bacillus* sp. strain HIL Y-85.54728 shows more globular structure due to the presence of four intermolecular thioether bridges. It inhibits the growth of Gram-positive bacteria including methicillin resistant *Staphylococcus aureus* (MRSA) (*22*). Sublancin 168 is produced by *B. subtilis* 168, consists of one lanthionine linkage and two unusual disulfide bonds. It shows activity against Gram-positive bacteria, including *B. cereus*, *Streptococcus pyogenes* and *S. aureus*. Since a lot is known about sublancin, it has potential for novel biomaterial engineering (*28*). In 2013, Arias and colleagues identified amylolysin from *B. amyloliquefaciens* GA 1, a type B lantibiotic which exhibits antibacterial activity against Gram-positive bacteria including MRSA and *Listeria monocytogenes* (*19*).

Subclass I.3. Two-peptide lantibiotics

This subclass includes lantibiotics containing two components. The two peptide lantibiotics produced by *Bacillus* species are haloduracin and lichenicidin produced by *B. halodurans* C-125, *B. licheniformis* DSM 13 respectively (*29,30*). These peptides are closely related to two peptide lantibiotics produced by other bacteria such as, cytolysin from *Enterococci*, lacticin 3147 from *Lactococcus lactis* DPC3147, staphylococcin C55 produced by *S. aureus* C55, plantaricin W from

Table 1. Bacteriocins and BLIS produced by *Bacillus species*

Bacillus species	Bacteriocin/BLIS	Study reports
Bacillus subtilis GAS101	GAS101	Sharma *et al.* (2018) (*15*)
Bacillus subtilis KIBGE-17	Bac-IB17	Ansari *et al.* (2012) (*16*)
Bacillus subtilis SN7	Mejucin	Lee *et al.* (2018) (*73*)
Bacillus subtilis L-Q11	Subtilin L-Q11	Qin *et al.* (2019) (*17*)
Bacillus subtilis EMD4	Subtilosin A. BacEMD4	Liu *et al.* (2015) (*18*)
Bacillus amylolequefaciens FZB42	Amylocyclicin	Scholz *et al.* (2014) (*12*)
Bacillus amylolequefaciens GA1	Amylolysin	Arias *et al.* (2013) (*19*)
Bacillus sonorensis MT 93	Sonorensin	Chopra *et al.* (2014) (*13*)

Lactobacillus plantarum, and Smb produced by *Streptococcus mutans* GS5 (*31-35*). In two peptide lantibiotics group, the antimicrobial activity is exhibited due to the synergistic activities of two lanthionine containing peptides (A1 and A2) (*14*). Haloduracin consist of two post translationally modified peptides Halα/A1 and Halβ/A2, both of which act synergistically to produce bactericidal activity. Similarly, lichenicidin also consist of two prepeptides Bliα/A1 and Bliβ/A2 that has 38% and 52% similarity to HalA1 and Hal A2 respectively (*29*).

Class II. Non-modified peptides

The class II bacteriocins are heterogenous group of small peptides having size of less than 10 kDa (*25*). These are heat stable, non-modified cationic peptides that are hydrophobic in nature. Klaenhammer *et al.* (1993) had sub divided these peptides under three subgroups: class IIa pediocin like, class IIb two-component peptide and class IIc thiol activated peptides. In 1996, Nes and colleagues have suggested that, based on some common characters, class II bacteriocins can be divided as pediocin like and anti- listeria bacteriocins, two peptide bacteriocins and bacteriocins with *sec*-dependent signal sequence (*20,36*). Later, Cotter *et al.* (2005) have suggested 4 subdivisions, retaining class IIa and IIb with two new subdivisions (class IIc and IId). The class IIc included cyclic bacteriocins while class IId has non-pediocin single linear peptides (*37*). Nissen-Meyer *et al.* (2009) have maintained this classification scheme in their review about the structure and function relationship of class II bacteriocins (*38*). In 2011, Belkam and coworkers suggested that circular bacteriocins as a separate class of bacteriocin (*39*).

Class III. Large proteins

This class includes large proteins (30 kDa), which have phospholipase activity such as megacins A-216 and A-19213 produced by *Bacillus megaterium* ATCC 19213. Megacin A-216 contains 293 amino acid residues and shows a native molecular weight of c. 66 kDa (*40*). Other proteins like colicins, klebicin (from *Klebsiella pneumoniae*), helveticin I (from *Lactobacillus helveticus*), and enterolysin (from *Enterococcus faecalis*) are the members of this group (*41*).

2.2. Application of *Bacillus* bacteriocin

The *Bacillus* species are industrially important because of their excellent safety record, rapid growth rates, short fermentation cycles and their high capacity for protein secretion into the extracellular medium (*42*). Peptides derived from *Bacillus* species have shown antibacterial, antifungal, antiviral, antitumor, antiamebocytic, and antimycoplasmic activities (*4,42*). Similarly, many *Bacillus* species, such as *B. subtilis*, *B. clausii*, *B. cereus*, *B. coagulans*, *and B. licheniformis*, have been used as probiotic supplements in both animals and humans (*23,43*). Some of the applications of bacillus bacteriocin are discussed below (Figure 1).

2.2.1. Application in human health

Bacteriocins are considered as alternative antimicrobials for treatment of human infections, as there is increasing bacterial resistance to conventional antibiotics (*14*). Bacteriocins or bacteriocin like substances (BLIS) produced by bacillus have shown antimicrobial activity against multi drug resistant bacteria such as MRSA, vancomycin resistant enterococci (VRE), *etc.* In addition to bacteriocins, lantibiotics has recently been the peptide of interest (*14*). Bacitracin is one of the important polypeptides, which is effective against *Streptococcus pyogenes* and *Staphylococcus aureus*. Bacitracin has been used clinically in combination with other antimicrobial agents (*44*). Others have reported that oral administration of bacitracin daily for 7-10 days was successful in the treatment of antibiotic-associated colitis and diarrhea caused by *Clostridium difficile* (*45*). Amylolysin, a novel bacteriocin produced by the *B. amyloliquefaciens* GA1 strain, has been reported to exhibit activity against *L. monocytogenes* strains, which are responsible for food-born listeriosis. Mersacidin, a lantibiotic shows strong antimicrobial activity against *S. aureus* both *in vitro* and *in vivo* studies (*19,46*). The lantibiotic subtilosin A shows antimicrobial activity against pathogens such as *L.*

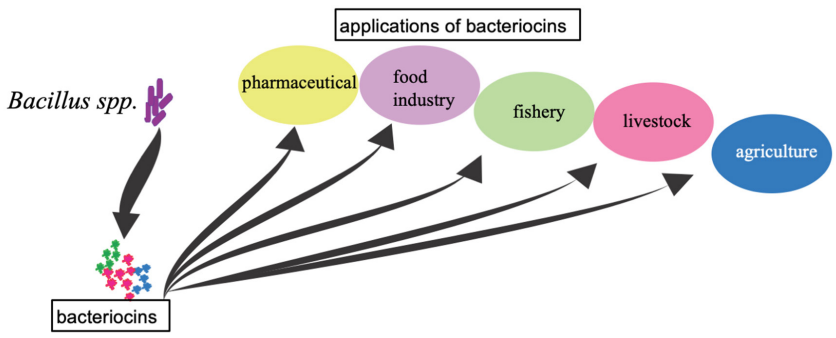

Figure 1. Illustration showing applications of bacteriocins in different sectors.

monocytogenes, *Gardnerella vaginalis* and *Streptococcus agalactiae*. Similarly, Pep5 and epidermin prevent the adhesion of coagulase-negative staphylococci, specifically *Staphylococcus epidermidis*, to siliconisated catheters (*47*). Besides antibacterial activity of bacteriocin, it has been found to have antifungal activity. For example, baciamin, an antifungal protein produced by *B. amyloliquefaciens* was reported to be active against various fungi like *Botrytis cinerea*, *Helminthosporium turcicum*, *Harpophora maydis*, *Valsa mali*, *Mycosphaerella arachidicola*, *Pythium aphanidermatum*, *Rhizoctonia solani*, and *Fusarium oxysporum* (*14,48*). Bacteriocin producing bacterial strains can be used as probiotic supplement for human and animals as they can inhibit the intestinal pathogens such as *Clostridium perfringens*, *Clostridium difficile* and others (*49,50*). For example, *B. clausii* produces inhibitory substances against *S. aureus*, *Enterococcus faecium* and *C. difficile* (*50*). *Bacillus polyfermenticus* SCD (polyfermenticin SCD producer) is a probiotic, commercially used for the treatment of long-term intestinal disorders as it inhibits the growth of *C. perfringens* (*51*).

2.2.2. Application in food industry

LAB derived bacteriocins are promising food preservatives and they are safe for human use because they are non-toxic compound. Nisin (as nispalin) and pediocin PA-1 (as ALTA 2341) are commercially available food additives (*38*). There are two bacteriocins from *Bacillus* that have potential preservative application in dairy products (*14*). For example, bacillocin 490 showed activity against closely related *Bacillus* spp. The bactericidal activity was found to be stable at 4°C, wide pH range and high temperature (*8*). Another is cerein 8A produced by *B. cereus* 8A, was used to control cheese surface contamination by *L. monocytogenes*. In 2008, Bizani and colleagues showed that cerein 8A only caused a delay in the start of exponential growth phase in soft cheese (*10*). Furthermore, BLIS produced by *B. amyloliquefaciens* GA1 was used as biopreservatives in poultry meat (*14*) . These days there is a trend of discouraging the use of chemical preservatives which has increased the interest in the application of natural preservatives. Recently, the antimicrobial lipopeptide microcapsules made from *B. amyloliquefaciens* ES2 was tested as food additives (*52*).

2.2.3. Application in livestock

Bacteriocin producing *Bacillus* strain could be used as probiotics in livestock to improve the health of animals (*53*). For example, a lichenin derived from *B. licheniformis* was found to exhibit antibacterial effect against *Eubacterium ruminantium* and *Streptococcus bovis* and it also possessed the hydrolytic activity against polysaccharides (*54*). Therefore, Pattnaik and colleagues (2001) postulated that lichenin have potential applications to improve rumen fermentation due to its role as a digestive aid and due to its antimicrobial properties (*54*). Amylolysin at concentration of 5-10 µg/g has shown to inhibit the growth of *L. monocytogenes* in poultry meat (*19*). The bacteriocin-producing strains *Paenibacillus polymyxa* NRRL B-30507, NRRL B-30508, NRRL B-30509 and *Bacillus circulans* NRRL B-30644 were used to control *Campylobacter jejuni* for treating animals carrying zoonoses. Spores of the *B. amyloliquefaciens* CECT 5940 are used as a probiotic in poultry feeds (Ecobiols, Norel & Nature Nutrition) to reduce the effect of pathogenic bacteria such as *C. perfringens*, *E. coli* and *Yersinia* (*14*).

2.2.4. Application in fishery

Probiotics produced by *Bacillus* strain could be used in two ways: as preservative to improve the storage in fish procession industry and as an antimicrobial to improve fish health in aquaculture. Bacteriocin, an antimicrobial peptide, possess antagonistic activity against other bacteria showing immunoprotective effects against fish bacterial infections (*55*). Probiotics or bioactive molecules isolated from fish gut-derived *Bacillus* spp., are found promising source of natural antimicrobial compounds against fish bacterial diseases. Similarly, bacteriocin TSU4 isolated from fish inhabited *Lactobacillus* showed a wide range of antimicrobial activity against *Aeromonas hydrophila* (MTCC 646) and *Pseudomonas aeruginosa* (MTCC 1688) and showed pH and thermal stability as well. Thus, bacteriocin "TSU4" has potentiality for using as preservative in fish processing industry (*56*). Likewise, nisin was found as effective bio-preservative agent to increase shelf life of rainbow trout (*Oncorhynchus mykiss*) storage. Analogously, nisin, which is produced by *Lactobacillus lactis* subsp. *lactis*, is allowed to use as food additive. Nisin-treated vacuum packaged rainbow trout increased the self-life from 12 days to 16 days at 4°C (*57*). These evidences show the usefulness of bacteriocins in fishery industry.

2.2.5. Application in environment

Bacillus species is naturally found in soil and plants. Thus, the bacteriocins or BLIS produced by *Bacillus* could be acquiescent to be used as biocontrol agent (*14*). For example, ericin S is active against *Clavibacter michiganensis*, the causative agent of tomato bacterial canker. Therefore, purified ericin or its producer strain could be developed as a bioprotectant on tomato cultivation against bacterial canker disease. As, the bioactivity of BLIS produced by *B. subtilis* 14B, Bac 14B, is active against *Agrobacterium tumefaciens*, thus it could be used as a biocontrol agent against *A. tumefaciens* associated infections. Moreover, some

of the BLIS are effective against fungal strains, thus there is potential of using those BLIS as biocontrol agent to preserve plants decay and postharvest control of fruits and vegetables (*14,18,27*). Likewise, a BLIS produced by *B. amyloliquefaciens* AC 2 is bioactive against *Colletotrichum dematium*, mulberry anthracnose fungus and several other phytopathogenic fungi as well as bacteria, such as *Rosellinia necatrix*, *Pyricularia oryzae, A. tumefaciens* and *Xanthomonas campestris* pv. *campestris* (*14*). Furthermore, lipopeptides such as fengycin and iturins have antifungal activity (*58*). Moreover, surfactin has surfactant activity and emulsification properties, indicating that these peptides might be applied in bioremediation. The surfactin lipopeptide has also demonstrated activity as antitumor, antiviral, antibacterial activities and hypocholesterolemic agent (*59*). Many *Bacillus*-derived antimicrobial peptides can be used to inhibit plant pathogens and preserve grain. The *B. subtilis* species is widely used in the biocontrol of plant diseases. Recently, Guo and colleagues (2014) discovered that the *B. subtilis* NCD-2 strain secretes fengycin-type lipopeptides that exhibited antifungal activity against *Rhizoctonia solani*, the causative agent of cotton damping-off disease (*58*).

BLIS producing bacilli also have other environmental applications. The antimicrobial substances (AMS) produced by strains *B. licheniformis* T6-5 and *Bacillus firmus* H(2)O-1 prevented the formation of *Bacillus pumilus* LF4 biofilm and eliminated pre-established LF4 biofilm (*60*). In addition, Korenblum and coworkers reported that the presence of AMS produced by *B. firmus* H(2)O-1 reduced the viability and attachment of the SRB consortium biofilm thus, suggested that the AMS produced by *Bacillus* strains T6-5 and H(2)O-1 may have a potential for pipeline-cleaning technologies to inhibit biofilm formation and consequently reduce biocorrosion (*60*).

3. Structure-activity relationships (SARs) of bacteriocins

The molecular structure of antimicrobial peptides (AMPs) affects their mechanism of action and therapeutic effects. Etayash and his colleagues reported the structure-activity relationships (SARs) of seven bacteriocins (nisin, microcin J25, microcin B17, microcin C, leucocin A, sakacin P and pediocin PA-1 (*61*). It is relevant to discuss some examples of structure-activity relationships of nisin, a type of bacteriocins.

Nisin is one of the most well studied peptides. This highly potent peptide inhibits food-spoilage bacteria and used to treat drug-resistant bacterial infections. Nisin has two most common forms; they are nisin A and nisin Z. Many analogues of nisin were synthesized using site directed mutagenesis and chemical synthesis. After the mutations at rings A and B of nisin, the mutants retained the biological activity of the peptide (*62*). The hinge region of nisin was mutated in many ways and the mutants N20P, M21V and K22P showed activity greater than the native type nisin A against *S. aureus, L. monocytogenes* and *S. agalactiae respectively* (*63*). Field *et al.* isolated novel nisin variant with increased activity against clinical and foodborne pathogens by bioengineering process (*64*). They identified a variant with a serine to glycine change at position 29 (S29G). Moreover, they made three nisin A derivatives (S29A, S29D and S29E) which are active against Gram-positive drug resistant bacteria, by site-directed mutagenesis.

Similarly, Cotter and coworkers changed the three amino acids at the hinge region (N20, M21 and K22) of nisin to increase its bioactivity against many target strains (*65*). Likewise, Evelyn *et al.* created a bank of nisin A derivatives in which K 12 was substituted with all other standard amino acid residues to make more antibacterial peptide analogue, using bioengineering technology (Table 2) and the site-directed mutagenesis (*66*). Furthermore, Arnusch *et al.* (2008) conjugated nisin to vancomycin and the conjugate has 40-fold increase in antibacterial activity. The nisin fragment (1-12) was made by enzymatic cleavage and then it was conjugated to vancomycin by the click chemistry and the activity increased to wide strains of bacteria (*67*) (*66*).

Pediocin PA-1 is a class II a bacteriocin that shows activity against *Listeria monocytogenes* (*69*). There are several studies regarding structure functional relationship of pediocin. Several mutants of pediocin were generated by chemical synthesis or by site directed mutagenesis (*38*). In the study done by Tomigana *et al.* (2007) showed some of the residues were essential for retaining activity of pediocin (*70*). They replaced each residue of the native codon with the NNK triplet Oligonucleotide by using NNK scanning method and generated 35 peptide mutants (Table 3). They found that, the bioactivity of pediocin was retained by almost all mutants having mutations at K1, T8, G10, S13, G19, N28 and N41, whereas the activity was completely lost in analogues with mutations at residues Y2, G6, C9, C14, C24, W33, G37, and C44, implying the importance of these residues for the bioactivity of pediocin (*70*).

Analogously, Song *et al.* made nine mutants of

Table 2. Nisin A mutants obtained by bioengineering technology. Mutation at Lysine (K)12 with various amino acid substitutions exhibited increased, decreased and no activity as shown by Etayash *et al.* (2015) (*61*).

Original residue	Mutant residue (s)	Activity (%)
K12	P,A,T,S	> 125
K12	Q,M,C,N,V	100
K12	R,H,W,F,Y,I,G	50-70
K12	D,E	no activity

P- Proline, A- Alanine, T- Threonine, S- Serine, Q- Glutamine, M- Methionine, C- Cisteine, N- Asparagine, V- Valine, R- Arginine, H- Histidine, W-Tryptophan, F- Phenylalanine, Y- Tyrosine, I- Isoleucine, G- Glycine, D-Aspartic acid, E-Glutamic acid.

Table 3. Pediocin mutants obtained by NNK scanning where, N = A/C/G/T, K= G/T. Mutations at Y2, G6, C9, C14, W33, G37 and C44 showed > 90% activity; while substitution at K1, T8, G10, S13, G19, N28, and N41 had < 10% relative activity described by Tomigana and Hatakeyama, *et al.* (2006) (*68*).

Original residue	Mutant residue (s)	Activity (%)
K1	ND*	< 10
Y2	Y	> 90
Y2	H	30-50
G6	G	> 90
T8	D,P	<10
C9	C	> 90
G10	C,P,Q	< 10
S13	C,P	< 10
C14	C	> 90
G19	C,H	< 10
C24	C	> 90
N28	ND*	< 10
W33	W	> 90
W33	L	50-70
G37	G	> 90
G37	A	50-70
N41	ND*	< 10
C44	C	> 90

activity = (diameter of the circle formed by the wild type/diameter of the circle formed by the wild mutant)*100%. P- Proline, A- Alanine, T- Threonine, S- Serine, Q- Glutamine, M- Methionine, C- Cisteine, N- Asparagine, V- Valine, R- Arginine, H- Histidine, W-Tryptophan, F- Phenylalanine, Y- Tyrosine, I- Isoleucine, G- Glycine, D-Aspartic acid. ND*, residues where the stop codon was introduced, or peptide was damaged by PCR error.

pediocin with different substitution and increasing positively charged residues (*71*). They found two-fold increase in the activity of some of the mutants. The mutant S13K was found to be more potent than the native pediocin PA-1, which also indicated that charged residues at position 13 had a positive effect on the activity (*71*). In addition, other study by Fimland *et al.* (1998), explained that a 15-mer peptide fragment made from pediocin PA-1had ability to inhibit the activity of pediocin (*72*). However, many aspects are yet to be investigated since there are several studies done on pediocin PA-1.

4. Conclusion

Susceptibility toward antimicrobials has been a major challenge due to increasing number of resistant pathogens. This review demonstrates potentiality of the genus *Bacillus* as an important source of bacteriocins that has applied benefits in various fields of human and animal health, food industry, fishery, environment, and agriculture. Not only as a preservative but also as a potential antimicrobial agent in the manufacturing industry level, the genus *Bacillus* has potentiality as a source of new antimicrobials against resistant strains. In this review, we have explored the sources of bacteriocins, characterized and classified them and discussed bacteriocins structure-activity relationships. This review provides valuable information about multifarious use of bacteriocins.

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References

1. Karbalaei-Heidari HR, Partovifar M, Memarpoor-Yazdi M. Evaluation of the bioactive potential of secondary metabolites produced by a new marine micrococcus species isolated from the Persian Gulf. Avicenna J Med Biotechnol. 2020; 12:61-65.
2. Zhao X, Kuipers OP. Identification and classification of known and putative antimicrobial compounds produced by a wide variety of Bacillales species. BMC Genomics. 2016; 17:882.
3. Koumoutsi A, Chen XH, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol. 2004; 186:1084-1096.
4. Stein T, Heinzmann S, Dusterhus S, Borchert S, Entian KD. Expression and functional analysis of the subtilin immunity genes spaIFEG in the subtilin-sensitive host *Bacillus subtilis* MO1099. J Bacteriol. 2005; 187:822-828.
5. Caulier S, Nannan C, Gillis A, Licciardi F, Bragard C, Mahillon J. Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. Front Microbiol. 2019; 10:302.
6. Beric T, Stankovic S, Draganic V, Kojic M, Lozo J, Fira D. Novel antilisterial bacteriocin licheniocin 50.2 from *Bacillus licheniformis* VPS50.2 isolated from soil sample. J Appl Microbiol. 2014; 116:502-510.
7. Cherif A, Ouzari H, Daffonchio D, Cherif H, Ben Slama K, Hassen A, Jaoua S, Boudabous A. Thuricin 7: a novel bacteriocin produced by *Bacillus thuringiensis* BMG1.7, a new strain isolated from soil. Lett Appl Microbiol. 2001; 32:243-247.
8. Martirani L, Varcamonti M, Naclerio G, De Felice M. Purification and partial characterization of bacillocin 490, a novel bacteriocin produced by a thermophilic strain of *Bacillus licheniformis*. Microb Cell Fact. 2002; 1:1.
9. Bizani D, Brandelli A. Characterization of a bacteriocin produced by a newly isolated *Bacillus sp.* Strain 8 A. J Appl Microbiol. 2002; 93:512-519.
10. Bizani D, Dominguez AP, Brandelli A. Purification and partial chemical characterization of the antimicrobial peptide cerein 8A. Lett Appl Microbiol. 2005; 41:269-273.
11. Basi-Chipalu S, Dischinger J, Josten M, Szekat C, Zweynert A, Sahl HG, Bierbaum G. Pseudomycoicidin, a class II lantibiotic from *Bacillus pseudomycoides*. Appl Environ Microbiol. 2015; 81:3419-3429.
12. Scholz R, Vater J, Budiharjo A, Wang Z, He Y, Dietel K, Schwecke T, Herfort S, Lasch P, Borriss R. Amylocyclicin, a novel circular bacteriocin produced by *Bacillus amyloliquefaciens* FZB42. J Bacteriol. 2014;

196:1842-1852.
13. Chopra L, Singh G, Choudhary V, Sahoo DK. Sonorensin: an antimicrobial peptide, belonging to the heterocycloanthracin subfamily of bacteriocins, from a new marine isolate, *Bacillus sonorensis* MT93. Appl Environ Microbiol. 2014; 80:2981-2990.
14. Abriouel H, Franz CM, Ben Omar N, Galvez A. Diversity and applications of *Bacillus bacteriocins*. FEMS Microbiol Rev. 2011; 35:201-232.
15. Sharma G, Dang S, Gupta S, Gabrani R. Antibacterial activity, cytotoxicity, and the mechanism of action of bacteriocin from *Bacillus subtilis* GAS101. Med Princ Pract. 2018; 27:186-192.
16. Ansari A, Aman A, Siddiqui NN, Iqbal S, Ali ul Qader S. Bacteriocin (BAC-IB17): screening, isolation and production from *Bacillus subtilis* KIBGE IB-17. Pak J Pharm Sci. 2012; 25:195-201.
17. Qin Y, Wang Y, He Y, Zhang Y, She Q, Chai Y, Li P, Shang Q. Characterization of subtilin L-Q11, a novel class I bacteriocin synthesized by *Bacillus subtilis* L-Q11 isolated from orchard soil. Front Microbiol. 2019; 10:484.
18. Liu X, Lee JY, Jeong SJ, Cho KM, Kim GM, Shin JH, Kim JS, Kim JH. Properties of a bacteriocin produced by *Bacillus subtilis* EMD4 isolated from Ganjang (Soy Sauce). J Microbiol Biotechnol. 2015; 25:1493-1501.
19. Arias AA, Ongena M, Devreese B, Terrak M, Joris B, Fickers P. Characterization of amylolysin, a novel lantibiotic from *Bacillus amyloliquefaciens* GA1. Plos One. 2013; 8:e83037.
20. Moineau S, Pandian S, Klaenhammer TR. Restriction/ Modification systems and restriction endonucleases are more effective on lactococcal bacteriophages that have emerged recently in the dairy industry. Appl Environ Microbiol. 1993; 59:197-202.
21. Soltani S, Hammami R, Cotter PD, Rebuffat S, Said LB, Gaudreau H, Bedard F, Biron E, Drider D, Fliss I. Bacteriocins as a new generation of antimicrobials: toxicity aspects and regulations. FEMS Microbiol Rev. 2021; 45:fuaa039.
22. Chatterjee S, Chatterjee DK, Jani RH, Blumbach J, Ganguli BN, Klesel N, Limbert M, Seibert G. Mersacidin, a new antibiotic from *Bacillus*. *In vitro* and *in vivo* antibacterial activity. J Antibiot (Tokyo). 1992; 45:839-845.
23. Cutting SM. *Bacillus* probiotics. Food Microbiol. 2011; 28:214-220.
24. Field D, Hill C, Cotter PD, Ross RP. The dawning of a 'Golden era' in lantibiotic bioengineering. Mol Microbiol. 2010; 78:1077-1087.
25. Nishie M, Sasaki M, Nagao J, Zendo T, Nakayama J, Sonomoto K. Lantibiotic transporter requires cooperative functioning of the peptidase domain and the ATP binding domain. J Biol Chem. 2011; 286:11163-11169.
26. Klein C, Kaletta C, Schnell N, Entian KD. Analysis of genes involved in biosynthesis of the lantibiotic subtilin. Appl Environ Microbiol. 1992; 58:132-142.
27. Stein T, Borchert S, Conrad B, Feesche J, Hofemeister B, Hofemeister J, Entian KD. Two different lantibiotic-like peptides originate from the ericin gene cluster of *Bacillus subtilis* A1/3. J Bacteriol. 2002; 184:1703-1711.
28. Paik SH, Chakicherla A, Hansen JN. Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. J Biol Chem. 1998; 273:23134-23142.
29. McClerren AL, Cooper LE, Quan C, Thomas PM, Kelleher NL, van der Donk WA. Discovery and *in vitro* biosynthesis of haloduracin, a two-component lantibiotic. Proc Natl Acad Sci U S A. 2006; 103:17243-17248.
30. Dischinger J, Josten M, Szekat C, Sahl HG, Bierbaum G. Production of the novel two-peptide lantibiotic lichenicidin by *Bacillus licheniformis* DSM 13. Plos One. 2009; 4:e6788.
31. Dougherty BA, Hill C, Weidman JF, Richardson DR, Venter JC, Ross RP. Sequence and analysis of the 60 kb conjugative, bacteriocin-producing plasmid pMRC01 from *Lactococcus lactis* DPC3147. Mol Microbiol. 1998; 29:1029-1038.
32. Gilmore MS, Segarra RA, Booth MC, Bogie CP, Hall LR, Clewell DB. Genetic structure of the *Enterococcus faecalis* plasmid pAD1-encoded cytolytic toxin system and its relationship to lantibiotic determinants. J Bacteriol. 1994; 176:7335-7344.
33. Holo H, Jeknic Z, Daeschel M, Stevanovic S, Nes IF. Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. Microbiology (Reading). 2001; 147:643-651.
34. Navaratna MA, Sahl HG, Tagg JR. Two-component anti-*Staphylococcus aureus* lantibiotic activity produced by *Staphylococcus aureus* C55. Appl Environ Microbiol. 1998; 64:4803-4808.
35. Yonezawa H, Kuramitsu HK. Genetic analysis of a unique bacteriocin, Smb, produced by *Streptococcus mutans* GS5. Antimicrob Agents Chemother. 2005; 49:541-548.
36. Nes IF, Diep DB, Havarstein LS, Brurberg MB, Eijsink V, Holo H. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek. 1996; 70:113-128.
37. Cotter PD, Hill C, Ross RP. Bacterial lantibiotics: strategies to improve therapeutic potential. Curr Protein Pept Sci. 2005; 6:61-75.
38. Nissen-Meyer J, Rogne P, Oppegard C, Haugen HS, Kristiansen PE. Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by gram-positive bacteria. Curr Pharm Biotechnol. 2009; 10:19-37.
39. van Belkum MJ, Martin-Visscher LA, Vederas JC. Structure and genetics of circular bacteriocins. Trends Microbiol. 2011; 19:411-418.
40. Kiss A, Baliko G, Csorba A, Chuluunbaatar T, Medzihradszky KF, Alfoldi L. Cloning and characterization of the DNA region responsible for Megacin A-216 production in *Bacillus megaterium* 216. J Bacteriol. 2008; 190:6448-6457.
41. Kaur S, Kaur S. Bacteriocins as potential anticancer agents. Front Pharmacol. 2015; 6:272.
42. Benitez LB, Velho RV, Lisboa MP, Medina LF, Brandelli A. Isolation and characterization of antifungal peptides produced by *Bacillus amyloliquefaciens* LBM5006. J Microbiol. 2010; 48:791-797.
43. Elshaghabee FMF, Rokana N, Gulhane RD, Sharma C, Panwar H. Bacillus as potential probiotics: Status, concerns, and future perspectives. Front Microbiol. 2017; 8:1490.
44. Haddar HO, Aziz GM, Al-Gelawi MH. Optimization of bacitracin production by *Bacillus licheniformis* B5. Pak J Biol Sci. 2007; 10:972-976.
45. Chang TW, Gorbach SL, Bartlett JG, Saginur R. Bacitracin treatment of antibiotic-associated colitis and diarrhea caused by *Clostridium difficile* toxin. Gastroenterology. 1980; 78:1584-1586.

46. Kruszewska D, Sahl HG, Bierbaum G, Pag U, Hynes SO, Ljungh A. Mersacidin eradicates methicillin-resistant *Staphylococcus aureus* (MRSA) in a mouse rhinitis model. J Antimicrob Chemother. 2004; 54:648-653.
47. Fontana MB, de Bastos Mdo C, Brandelli A. Bacteriocins Pep5 and epidermin inhibit *Staphylococcus epidermidis* adhesion to catheters. Curr Microbiol. 2006; 52:350-353.
48. Wong JH, Hao J, Cao Z, Qiao M, Xu H, Bai Y, Ng TB. An antifungal protein from *Bacillus amyloliquefaciens*. J Appl Microbiol. 2008; 105:1888-1898.
49. Chen H, Wang L, Su CX, Gong GH, Wang P, Yu ZL. Isolation and characterization of lipopeptide antibiotics produced by *Bacillus subtilis*. Lett Appl Microbiol. 2008; 47:180-186.
50. Urdaci MC, Bressollier P, Pinchuk I. *Bacillus* clausii probiotic strains: antimicrobial and immunomodulatory activities. J Clin Gastroenterol. 2004; 38:S86-90.
51. Lee KH, Jun KD, Kim WS, Paik HD. Partial characterization of polyfermenticin SCD, a newly identified bacteriocin of *Bacillus polyfermenticus*. Lett Appl Microbiol. 2001; 32:146-151.
52. Wang J, Ma H, Ge X, Zhang J, Teng K, Sun Z, Zhong J. Bovicin HJ50-like lantibiotics, a novel subgroup of lantibiotics featured by an indispensable disulfide bridge. Plos One. 2014; 9:e97121.
53. Ben Lagha A, Haas B, Gottschalk M, Grenier D. Antimicrobial potential of bacteriocins in poultry and swine production. Vet Res. 2017; 48:22.
54. Pattnaik P, Kaushik JK, Grover S, Batish VK. Purification and characterization of a bacteriocin-like compound (Lichenin) produced anaerobically by *Bacillus licheniformis* isolated from water buffalo. J Appl Microbiol. 2001; 91:636-645.
55. Sahoo TK, Jena PK, Patel AK, Seshadri S. Purification and molecular characterization of the novel highly potent bacteriocin TSU4 produced by *Lactobacillus animalis* TSU4. Appl Biochem Biotechnol. 2015; 177:90-104.
56. Santos RA, Oliva-Teles A, Pousao-Ferreira P, Jerusik R, Saavedra MJ, Enes P, Serra CR. Isolation and characterization of fish-gut *Bacillus* spp. as source of natural antimicrobial compounds to fight aquaculture bacterial diseases. Mar Biotechnol (NY). 2021; 23:276-293.
57. Behnam S, Anvari M, Rezaei M, Soltanian S, Safari R. Effect of nisin as a biopreservative agent on quality and shelf life of vacuum packaged rainbow trout (*Oncorhynchus mykiss*) stored at 4 degrees C. J Food Sci Technol. 2015; 52:2184-2192.
58. Guo Q, Dong W, Li S, Lu X, Wang P, Zhang X, Wang Y, Ma P. Fengycin produced by *Bacillus subtilis* NCD-2 plays a major role in biocontrol of cotton seedling damping-off disease. Microbiol Res. 2014; 169:533-540.
59. Schallmey M, Singh A, Ward OP. Developments in the use of *Bacillus* species for industrial production. Can J Microbiol. 2004; 50:1-17.
60. Korenblum E, Sebastian GV, Paiva MM, Coutinho CM, Magalhaes FC, Peyton BM, Seldin L. Action of antimicrobial substances produced by different oil reservoir *Bacillus* strains against biofilm formation. Appl Microbiol Biotechnol. 2008; 79:97-103.
61. Etayash H, Azmi S, Dangeti R, Kaur K. Peptide Bacteriocins – structure activity relationships. Curr Top Med Chem. 2015; 16:220-241.
62. Rink R, Wierenga J, Kuipers A, Kluskens LD, Driessen AJ, Kuipers OP, Moll GN. Dissection and modulation of the four distinct activities of nisin by mutagenesis of rings A and B and by C-terminal truncation. Appl Environ Microbiol. 2007; 73:5809-5816.
63. Field D, Connor PM, Cotter PD, Hill C, Ross RP. The generation of nisin variants with enhanced activity against specific gram-positive pathogens. Mol Microbiol. 2008; 69:218-230.
64. Field D, Begley M, O'Connor PM, Daly KM, Hugenholtz F, Cotter PD, Hill C, Ross RP. Bioengineered nisin A derivatives with enhanced activity against both Gram positive and Gram negative pathogens. Plos One. 2012; 7:e46884.
65. Healy B, Field D, O'Connor PM, Hill C, Cotter PD, Ross RP. Intensive mutagenesis of the nisin hinge leads to the rational design of enhanced derivatives. Plos One. 2013; 8:e79563.
66. Molloy EM, Field D, PM OC, Cotter PD, Hill C, Ross RP. Saturation mutagenesis of lysine 12 leads to the identification of derivatives of nisin A with enhanced antimicrobial activity. Plos One. 2013; 8:e58530.
67. Arnusch CJ, Bonvin AM, Verel AM, Jansen WT, Liskamp RM, de Kruijff B, Pieters RJ, Breukink E. The vancomycin-nisin(1-12) hybrid restores activity against vancomycin resistant *Enterococci*. Biochemistry. 2008; 47:12661-12663.
68. Tominaga T, Hatakeyama Y. Determination of essential and variable residues in pediocin PA-1 by NNK scanning. Appl Environ Microbiol. 2006; 72:1141-1147.
69. Papagianni M, Anastasiadou S. Pediocins: The bacteriocins of *Pediococci*. sources, production, properties and applications. Microb Cell Fact. 2009; 8:3.
70. Tominaga T, Hatakeyama Y. Development of innovative pediocin PA-1 by DNA shuffling among class IIa bacteriocins. Appl Environ Microbiol. 2007; 73:5292-5299.
71. Song DF, Li X, Zhang YH, Zhu MY, Gu Q. Mutational analysis of positively charged residues in the N-terminal region of the class IIa bacteriocin pediocin PA-1. Lett Appl Microbiol. 2014; 58:356-361.
72. Fimland G, Jack R, Jung G, Nes IF, Nissen-Meyer J. The bactericidal activity of pediocin PA-1 is specifically inhibited by a 15-mer fragment that spans the bacteriocin from the center toward the C terminus. Appl Environ Microbiol. 1998; 64:5057-5060.
73. Lee SG, Chang HC. Purification & characterization of mejucin, a new bacteriocin produced by *Bacillus subtilis* SN7. LWT. 2018; 87:8-15.

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Review

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Real-world evidence of tofacitinib in rheumatoid arthritis patients in Spain

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SUMMARY The purpose of this narrative review is to provide an overview of the real-world data on the use of tofacitinib in patients with active rheumatoid arthritis (RA) in Spain. Sixteen retrospective studies carried out in Spain between 2019 and 2021 have been analyzed, considering patients' characteristics, and treatment patterns, effectiveness, and safety. In those studies, approximately 511 patients received tofacitinib during the study period. They were predominantly women (mean age: 48-61 years). The percentage of patients receiving tofacitinib as monotherapy ranged between 20.0% and 67.9%. Only five studies reported the combined use of corticosteroids (42.0-84.5% of patients), with a mean dose varying from 1.8 to 7.2 mg. A wide range of patients (36.0-85.7%) had failed a previous biological disease-modifying anti-rheumatic drug. The most frequent reason for treatment discontinuation was the lack of efficacy, and the most common adverse event described was herpes zoster infection. Real-world studies complement clinical trials by adding efficacy and safety data in real-world settings to the benefit/risk profile of the drug. The profile of RA patients receiving tofacitinib in Spain has similarities with other real-world studies conducted in other countries.

Keywords Tofacitinib, real-world data, rheumatoid arthritis, DMARD, JAK inhibitor

1. Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory autoimmune disease characterized by persistent inflammation of the joints (*1*). Its worldwide prevalence is approximately 1%, and there is a higher incidence in women (*2*). In the case of Spain, the prevalence is 0.9% (*3*). Therapeutic resources for RA have increased considerably in the last 30 years and are used to control the devastating effects of its progression which include the destruction of the joints, the reduction in life expectancy, early unemployment, disability and cardiovascular (CV) damage (*4*). Among current pharmacological approaches for the treatment of RA are nonsteroidal anti-inflammatory drugs, glucocorticoids, and disease-modifying anti-rheumatic drugs (DMARDs), with the latter dividing into conventional synthetic DMARDs (csDMARDs) such as methotrexate (MTX); biological DMARDs (bDMARDs) such as tumor necrosis factor (TNF) inhibitors; or targeted synthetic DMARDs (tsDMARDs) such as Janus kinase (JAK) inhibitors (*5*). The American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) recommend csDMARDs, usually MTX, as the first-line therapy in patients with RA. However, sometimes it is not sufficient, and therefore patients with csDMARD inadequate response are recommended bDMARDs or tsDMARDs either alone or in combination with other csDMARDs (*6,7*). Tofacitinib is an orally bioavailable small molecule that inhibits by blocking the adenosine triphosphate (ATP) binding site. In human cells, tofacitinib preferentially inhibits signaling by heterodimeric cytokine receptors associated with JAK3 and/or JAK1 with functional selectivity over cytokine receptors that signal *via* pairs of JAK2. Inhibition of JAK1 and JAK3 by tofacitinib attenuates signaling of interleukins (IL-2, -4, -6, -7, -9, -15, -21) and type I and type II interferons, which results in modulation of the immune and inflammatory response (*8,9*). As specified in the European Medicines Agency (EMA) summary of product characteristics (SmPC), it is indicated for moderate to severe active RA in adult patients who have not responded adequately or are intolerant to DMARDs (*9*). It can be administered in combination with MTX or as a monotherapy (in case of intolerance or when treatment with MTX is not adequate). The recommended dose is 5 mg twice a day.

Tofacitinib was approved by the US Food and Drug

Administration (FDA) in November 2012 and by the EMA in March 2017 for the treatment of RA (*9,10*). The efficacy and safety of tofacitinib for the treatment of active RA in adults has been studied through numerous phase II, III, IIIb/IV and IV randomized clinical trials (RCTs) (*9*). Additionally, two long-term open-label trials have been completed (*11-23*). The results show sustained efficacy and consistent safety beyond 9.5 years (*24,25*). In a large (n = 4,362) randomized post authorization safety study (ORAL Surveillance [A3921133; NCT02092467]) in patients with RA who were 50 years of age or older with at least one additional cardiovascular risk factor, an increased incidence of major adverse cardiovascular events (MACE) and malignancies was observed with tofacitinib compared to TNF inhibitors (*9*).

RCTs have been considered the *gold standard* for generating data on efficacy and safety, occupying a high position in the hierarchy of evidence that supports the registration of the product and its commercialization. However, these studies include patients with very selective profiles, and this strong internal bias may limit their external validity and, therefore, the transferability and generalizability of the results (*26,27*). Observational studies based on real-world data cannot replace RCTs to generate safety and efficacy data. However, they can help produce evidence of therapeutic effectiveness and support the RCT data, allowing comparisons in a real clinical setting (*28*). Therefore, supplementing data from clinical trials with real-world studies provides valuable information for payers, clinicians, and patients on how an intervention performs outside the narrow confines of the research environment. The importance of real-world evidence, to support marketed products and its potential role in product development/lifecycle monitoring and decision-making for regulation and evaluation, has been recognized by FDA (*29*) and EMA (*30*). Real-world data sources are administrative claims databases, clinical databases, RA patient registries, and national pharmacovigilance programs. Real-world evidence on tofacitinib has been published in different countries, including United States (*31,32*), Canada (*33*), Switzerland (*34*), and Australia (*35*) in cohort registries, as well as data sourced from other registries and hospital cohorts (*36,37*).

The purpose of this narrative review is to provide an overview of the evidence on tofacitinib use and the administration patterns in patients with active RA in the Spanish clinical practice.

2. Methods

A review of the literature was performed on diverse databases (PubMed, GoogleSchoolar) using the following keywords: "tofacitinib", "Janus kinase inhibitors", "rheumatoid arthritis", "Spain", "real-life". The search included all studies, case-series, and abstracts published between January 2019 and October 2021, written in English or Spanish. The date of search was October 22nd, 2021. Studies not involving patients with RA, those not from Spanish hospitals, those whose data is derived from clinical trials, or those without a minimal description of patient characteristics were not incorporated into the review. Given the scarce available literature, abstracts from the XLV, XLVI and XLVII National Congress of the Spanish Society of Rheumatology (held in 2019, 2020 and 2021) and from the Annual European Congress of Rheumatology (EULAR, held in 2020 and 2021) were also examined. From the identified studies, an analysis was made of patient characteristics, treatment patterns, effectiveness, and safety. To avoid duplicates, any abstracts from the same center were carefully analyzed and only the most recent data were presented.

3. Available real-world studies with tofacitinib for rheumatoid arthritis in Spain

Between 2019 and 2021, 16 retrospective studies reported the tofacitinib experience in patients with RA on routine clinical practice in Spain (*38-53*). Data from case-series were obtained from medical records and databases of a hospital's Rheumatology Service (Supplementary Table S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=94*). Data from the Spanish registry of adverse events (AEs) of biological therapies in rheumatic diseases (BIOBADASER 3.0), including information on the administration of JAK inhibitors, was not included in the analysis (*54*). The most relevant data obtained are summarized in Table 1 (demographics and baseline disease characteristics, Online Table, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=95*) and Table 2 (effectiveness and safety data, Online Table, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=95*).

3.1. Demographic characteristics

The total number of RA patients assessed in these studies were 1,108. 511 were treated with tofacitinib. The number of RA patients included in each series that were treated with tofacitinib varied, ranging from 4/81 (4.9%) (*44*) and reaching 81/81 patients (100%) in series that only included RA patients with tofacitinib (*45*). The mean age of patients analyzed ranged between 43.7 years (standard deviation, SD: 12.2 years) (*53*) and 61.2 years (SD: 13.2 years) (*43*), while the median age varied from 61.0 years (range: 40.0-74.0) (*41*) to 62.9 years (range: 49.9-74.4 years) (*46*). According to RA prevalence, a higher percentage of women received tofacitinib (between 58.0% [16/28 of patients] (*38*) and 94.4% [17/18]) (*41*). Only two studies described the comorbidities in 40 RA patients. In one study, 30.0% (12/40) had arterial hypertension, 32.5% (13/40)

dyslipidemia, 15% (6/40) diabetes mellitus, 10% (4/40) hypothyroidism, 32.5 % (13/40) smokers, and 20.0% (8/40) osteoporosis (*39*). In the other study, arterial hypertension (30.3% of patients, 20/66), diabetes mellitus (6/66, 9.1%), and dyslipidemia (26/66, 39.4%) were reported (*49*). The body mass index has been reported only in one study (*38*), where the mean value was 30.1 kg/m2 in 28 patients (19 of them had received tofacitinib). Also, only one study reported extra-articular manifestations, in 28.6% of patients (8/28) (*43*), 14 of whom had received tofacitinib.

3.2. Baseline disease characteristics

Disease duration of RA ranged from 8.7 years (SD: 6.5 years) (*49*) to 18.0 years (interquartile range: 9.0-22.0) (*50*). The percentage of patients who had failed with a previous bDMARD and received JAK inhibitors was reported to range from 36.0% (*48*) to 85.7% (24/28) (*43*). The mean treatment time with a bDMARD was between 2.6 years (SD: 3.0 years, in one study) (*44*) and 3.9 years (range: 1.2-10.9 years, in another study) (*40*). Including case-series, patients who did not achieve a therapeutic response to least one bDMARD varied from 15.4% (6/39) (*47*) to 41.4% (24/58) (*47*) of patients receiving JAK inhibitors. Those who had failed at least two previous bDMARDs ranged between 13.6% (9/66) (*49*) and 46.1% (18/39) (*46*), and those who had failed three or more previous bDMARDs ranged from 10.6% (7/66) (*49*) to 43.3% (39/90) (*45*). The use of JAK inhibitors in patients who had not received biologics previously was reported between 10.0% (9/90) (*45*) and 35.7% (10/28) (*38*). Two studies provide data about the reasons for switching to biologics. In the first study, the switching group included 35 patients (18 received JAK inhibitors) (*40*). The reasons for changing therapy were ineffectiveness (28/35, 80.0%), AEs (6/28; 17.1%), lack of follow-up (1/28, 2.9%). The mean duration on biologic therapy before switching was 3.9 years (range: 1.2-10.9 years). In the second study, the mean time in treatment with a bDMARD was 2.6 years (SD: 3.0 years) (*44*). The reasons to switch therapy in 81/252 patients were: loss of efficacy (25/81, 30.9%); AEs (31/81, 38.3%); change of address/loss of follow-up (20/81, 24.7%); and voluntary abandonment of treatment by the patient (5/81, 6.2%). One case-series provided data on sequential and switch treatment with JAK inhibitors, in any order, and evaluated the efficacy and safety of the second therapy when the first one had failed (*43*). This study included 28 patients, half of them received either tofacitinib or baricitinib.

Regarding autoantibodies status, the presence of rheumatoid factor (RF) and/or anti-cyclic citrullinated peptide (CCP) was reported in 69% (40/58) (*47*) and 72% (13/18) (*41*) of the cases. Only this latter study reported the frequencies of RF+/CCP- (1/18, 5.6%), RF-/CCP- (3/18, 16.7%), and RF-/CCP+ (1/18, 5.6%). One study reported CCP+ (19/28, 67.9%) (*43*), and another study reported RF+ (55/81, 68.0%) and CCP+ (61/81, 74.0%) (*44*) for any JAK inhibitors. Three studies reported these values separately: RF+ (32/39, 82.1%)/CCP+ (28/39, 71.2%) (*46*), and RF+ (9/9, 100.0%) / CCP+ (7/9, 77.8%) (*42*), and RF+ (87.5%, 35/40) / CCP+ (30/40, 75.0%) (*39*), in patients treated with tofacitinib. Structural damage data was included in six studies, describing erosive disease in 46.4% (13/28) (*43*), 54.5% (*51*), 62.5% (25/40) (*39*), 66.7% (6/35) (*42*), 67.8% (38/56) (*53*), and 87.2% (34/39) (*46*) of the patients treated with JAK inhibitors.

Disease activity at baseline was evaluated in nine studies with the Disease Activity Score 28 (DAS28), showing that patients who received JAK inhibitors had initially a moderate disease activity (defined as DAS28 score range: 3.2-5.1; DAS28: 4.3 (*48*), DAS28: 4.5, SD: 1.5 (*40*), DAS28/ C-reactive protein [CPR]: 4.5, range: 1.6-6.4, DAS28: 4.8, SD: 0.9 (*47*), DAS28: 4.8 (*38*), DAS28: 4.9, SD: 0.9 (*39*), and DAS28: 4.9, SD: 1.1 (*51*)); or high disease activity (DAS28: 5.2, range: 4.3-6.3; DAS28 score > 5.1, DAS28-CPR: 5.4, SD: 0.91 (*43*), DAS28-erythrocyte sedimentation rate [ESR]: 6.1, range: 3.8-5.3 (*42*)).

3.3. Tofacitinib administration patterns

The JAK inhibitors were administered either as monotherapy or in combination with csDMARDs or corticosteroids. The percentage of patients in each study where JAK inhibitors, specifically tofacitinib, were administered as monotherapy was 20.0% (8/40) (*39*), 24.8% (23/66) (*49*), 39.2% (*48*), 51.1% (46/90) (*45*), 59% (33/56) (*50*), 66.7% (12/18) (*41*), and 67.9% (19/28) (*38*). Related to combination therapy, the csDMARD which was most frequently used in combination with JAK inhibitors was MTX, ranging between 33.3% (6/18) (*41*) and 87.5% (35/40) (*39*), followed by leflunomide by up to 19% of patients (11/58), hydroxychloroquine by up to 13.8% (8/58) and sulfasalazine by up to 12.1% (7/58) (*47*). Only four case-series reported data regarding the combination of JAK inhibitors with corticosteroids, where 42.2% (46/109) (*40*), 72.5% (*52*), 73.9 (51/69) (*51*), and 84.5% (49/58) (*47*) of patients received corticosteroids. Mean administrated doses were reported in two studies, ranging from 1.8 mg (SD: 3.2 mg) (*46*) to 7.2 mg (SD: 4.2 mg) (*43*).

3.4. Effectiveness

The most relevant data regarding effectiveness and safety of tofacitinib are presented in Table 2 (Online Table, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=95*). Clinical response to JAK inhibitor therapy was assessed by determining an improvement on the DAS28 score, tender and swollen

joint counts, C-reactive protein, and erythrocyte sedimentation rate (ESR, from baseline), as well as the reporting rate of patients who achieved DAS28 remission and low disease activity (LDA). In a study with 40 patients treated with tofacitinib, the mean baseline DAS28 was 4.9 (SD: 0.94). This was reduced at 3 months to 3.1 (SD: 1.0) and remained at the same value at 6 months, *i.e*. 3.1 (SD: 1.1) (*39*). Similarly, another study reported a baseline median DAS28 of 6.1 (range: 3.8-5.3) and a final median DAS28 of 5.5 (range: 2.6-3.6) in a sample of 9 patients (*42*). Only one study reported a higher reduction in the DAS28, with a baseline median of 4.8 (range: 3.3-6.2) and a final median DAS28 of 2.61 (range: 2.5-3.7) (*46*).

The rate of RA patients in remission and the percentage of LDA were reported in three studies. In the first study (n = 40 patients) remission at month 3 was achieved in 27.5% of subjects (11/40), and LDA in up to 22.5% (9/40). At 6 months, 47.4% of patients (9/19) achieved remission and LDA in up to 42.1% (8/19) (*39*). The second study with 18 patients when it began, showed remission at 3 months as 76.9% (10/13), and LDA of up to 15.4% (2/13). At 6 months, there was a remission in all patients who continued treatment 100% (3/3) (*41*). The third study, with an initial number of 9 patients, reported a remission of 33.3% (3/9) and a LDA of up to 11.1% (1/9) (*42*).

Efficacy data for switching between JAK inhibitors (tofacitinib and baricitinib) were reported in a case series involving 28 patients (*43*). At the beginning of the study, 14 patients received tofacitinib and another 14 patients received baricitinib. After switching (both groups of patients: from tofacitinib to baricitinib, and from baricitinib to tofacitinib), the overall mean DAS28-CPR decreased at each visit: at 3 months 3.3 (SD: 1.0), at 6 months 3.2 (SD: 1.2) and finally, with 21 patients (75.0%) followed up to 12 months (mean: 2.2, SD: 0.6).

Persistence of treatment was reported in three studies, with a treatment time of 7.6 months (mean) (*43*), 8.9 months (SD: 5.1 months) (*47*), 13.2 months (median) (*46*), respectively. One study also compared the persistence between patients receiving tofacitinib in monotherapy (9/23, 39.1%) versus in combination with csDMARD (26/43, 60.5%) (*49*). One of the studies reported the survival rate for biologically experienced patients (81.7% and 78.7% at 6 and 12 months), and the pooled survival rate for JAK inhibitors (85.0% and 82.5% at 6 and 12 months). None of the JAK inhibitor treatments in patients with no biological experience were interrupted during the follow-up (18.4%, n = 18) (*38*). Another study reported survival rates of 85.0% and 70.0% at 6 and 12 months, respectively, when tofacitinib was used as first- or second-line treatment (*45*). Another study revealed a median survival of 35 months for patients receiving tofacitinib (*50*). Regarding the percentages of follow-up, one study showed 66.6% (6/9) (*46*) of patients remaining on treatment with tofacitinib, and another study showed 64.4% (29/45) (*47*).

In the study where treatment switching was done between JAK inhibitors, the mean survival for the first JAK inhibitor was 7.6 months (SD: 6.1 months). The mean follow-up after starting the second JAK inhibitor was 9.6 months (SD: 5.6 months). Survival in the second JAK inhibitor was 82% at 3 months, 76% at 6 months, and 62% at 12 months (*43*).

3.5. Safety

The objective of this review was not to evaluate safety because the number of patients included in these studies was too low for this purpose. Nonetheless, a brief review on the safety data related to JAK inhibitors were presented. Related to the occurrence of AEs, studies reported the percentage of AEs as between 15.0% (6/40) (*39*) and 39.0% (11/28) (*43*). The most frequent AEs in each study included: infections (68/122, 55.7%) (*53*), hypercholesterolemia (5/9, 55.5%) (*46*), and herpes zoster (HZ), with the latter being the most repeated AE throughout the studies, representing between 3.3% (4/122) (*53*) and 30.8% (4/13) of cases (*47*). Infections reported in several of the studies were respiratory infections 33.3% (2/6), odontogenic infections 16.7% (1/6) (*39*) and infections in general 69.7% (46/66). The rate of treatment discontinuation ranged between 12.5% (19/149) (*38*) and 33.9% (19/56) (*50*). The most frequent reasons for discontinuation were ineffectiveness (between 8.1% of patients, 5/62 (*48*) and 61.0%, 17/28 (*43*)) and AEs (from 2.6%, 4/149 (*38*), to 50.0%, 16/32 (*45*)). Infections and intolerance to treatment were only reported in one study, which occurred in 12.0% (2/16) and 22.0% (4/16) of patients respectively (*45*). Other less frequent reasons were the failure of the first treatment (between 3.0%, 2/66, and 5.3%, 1/19) (*38*), failure of the second treatment (21.1%, 4/19) (*38*), HZ (in 3/16 of patients, 16.0%) (*45*), refractory (2/3, 66.6%) (*46*), mayor embolic risk factors (10%) (*52*), and AEs (1/3, 33.3%) (*46*).

4. Integration of the real-world evidence

This review of real-world data in RA patients treated with tofacitinib is the first that has been conducted in a Spanish population. Information on clinical practice may also be influenced by geographic location as not only may the patients managed be of differing ethnic groups, but also the health systems will differ. Therefore, it is relevant to provide the results in Spain as well as contextualize them with other studies based on clinical experiences in real-world conditions. The world's most extensive data set of patients with these characteristics used for real-world studies have been primarily the Corrona registry in the United States,

with 1,544 patients (*31*), and the eXel program in Canada, with 1,226 patients (*33*). Others, such as the Australian study by Bird *et al*. (*35*) based on the OPAL dataset (Optimizing Patient outcomes in Australian RheumatoLogy), or the Swiss study by Finckh *et al*. (*34*) based on the registry SCQM-RA (Swiss Clinical Quality Management in Rheumatoid Arthritis), have reported 650 and 806 patients, respectively. In our present review, 13 retrospective case cohorts were collected from different Spanish hospitals, involving 386 patients treated with tofacitinib. The BIOBADASER 3.0 (*54*) study reported data from 669 patients treated with JAK inhibitors, but without individualizing the differentiating characteristics for JAK inhibitors. It reported comorbidities in patients such as current smokers (20%), diabetes (10%), ischemic heart disease (3%), hypertension (30%), heart failure (2%), interstitial lung disease (ILD, 2%), chronic obstructive pulmonary disease (COPD, 3%), chronic kidney disease (3%) and osteoporosis (16%) in patients treated with JAK inhibitors. Other studies reported data, such as that of Reed *et al*. (*32*) based on the registry of Corrona (402 patients, 238 in monotherapy and 164 in combination treatment), and Mueller *et al*. (*36*), based on the records of the hospitals of St. Gallen and Aarau in Switzerland. In general, all the studies showed a higher percentage of women, and the mean age of the patients was very similar, between 48 and 61 years (*43,44*). Nevertheless, compared to the Australian study by Bird *et al*. (*35*), with ages ranging between 55 and 74, Spanish RA patients treated with tofacitinib are younger in that series (median age ranges 61-62,9 and ages range 40-74.4), and in the BIOBADASER 3.0 the mean age of patients treated with JAK inhibitors was 59.6 (12.3 SD) (*54*).

It is rare to find collected data regarding comorbidities in patients. However, the Swiss register (*34*) considered CV diseases and osteoporosis of particular interest. In our review, the Gómez-Lechón Quirós *et al*. (*39*) study reported on comorbidities, and in de la Morena *et al*. (*38*), the body mass index was reported, with a mean value of 30.1 kg/m^2, representing a weight above normal, which represents a significant risk factor for the development of CV diseases. However, the percentages did not exceed 50% in any of the previous studies mentioned. The BIOBADASER 3.0 reported a comorbidities prevalence in RA patients treated with JAK inhibitors, but without specifying the type of JAK or other characteristics such as treatment line or reasons for prior failure (*54*). From the clinical point of view, it is of interest to consider the comorbidity of the patients, especially the cardiovascular risk factors (CVRF). In the ORAL Surveillance study, comparing the combined tofacitinib doses with a TNF inhibitor in a cardiovascular risk-enriched population, risks of MACE and cancers were higher with tofacitinib and did not meet noninferiority criteria (*55*). Several adverse events were more common with tofacitinib. In a real-world data (RWD) multidatabase in USA, a population-based study about the safety of tofacitinib in routine care patients with RA (STAR-RA study) included 102,263 patients, of whom 12,852 (12.6%) initiated tofacitinib. In this study tofacitinib was not associated with an increased risk of cardiovascular outcomes when compared with TNF inhibitor, however, tofacitinib was associated with an increased risk of cardiovascular outcomes in patients with RA with cardiovascular risk factors (*56*).

In our data, tofacitinib was mainly used in patients with active RA after failure to bDMARD treatment (*45*). This was true despite patients with worse prognoses than those included in clinical trials, with long disease duration and often with previous treatment with two or more bDMARDs (*38,39*). Previous experience with patients treated with at least one bDMARD can be found in real-world studies carried out in Canada (*33*) and Switzerland (*34,36*). On the other hand, in line with other publications (*32,57*), the findings of our study coincide with the fact that patients who start tofacitinib tend to have a longer duration of the disease and have been exposed to more DMARDs than patients who start with bDMARDs. Similar to other real-world series (US Corrona registry, Canadian registry) (*32,33*), tofacitinib is administered as monotherapy in a considerable percentage of patients (between 20% to 67.9%). Concerning combined therapy, the most frequently used csDMARD was MTX (39,41), and in the case of corticosteroid application, the doses were low (*47*). In Spain, some retrospective studies have compared the efficacy and safety of JAK inhibitors under real-world conditions, obtaining similar results (*37,38,41*).

Regarding survival, the Canadian study observed long-term survival of two years in patients receiving tofacitinib. Persistence was 62.7% and 49.6% after 1 and 2 years of treatment, respectively (*33*). In the Spanish population, the study with the most extended follow-up of survival was from Soleto *et al*. (*45*) which showed promising results with tofacitinib at 12 months. The frequency of interruption of treatment due to ineffectiveness is noteworthy, which could be related to refractory patients' clinical profile in the studies and/or the small sample used (*47*). The most commonly reported AE in retrospective studies among the Spanish population was HZ infection (*45,58*), which is in line with results by Kremer *et al*. (*31*) in the US, where the 5-year incidence rate of AEs was evaluated. A pulmonary embolism was also detected in a 70-year-old hypertensive patient (*47,49*). Finally, it is necessary to highlight that the variability found among Spanish studies (regarding evaluated variables) represents that observed in current routine clinical practice in Spain. Its causality might derive from differential features of involved patients. The main objective of this review is to show the tofacitinib usage patterns in Spanish

real-world studies. For that reason, safety trials in special populations and larger real-life series already published provide more conclusive safety results than those reflected in the present analysis. After evidence obtained from the ORAL Surveillance study, the SmPC has been updated to include recommendations in patients over 65 years of age, patients who are current or past smokers, patients with other cardiovascular risk factors, and patients with other malignancy risk factors (*9*). Prospective registers of RA patients who receive treatment with biological therapies in Spain might provide more data, from a methodological point of view (including a comparator control group), for the clinical practice in Spain; however, to date there are no published data.

5. Conclusion

This analysis describes the pattern of tofacitinib use in Spain and complements the data obtained from clinical trials. Despite being a review of real-world studies and inherently limited by the retrospective nature of the observational study (*i.e*., providing only available data), and the heterogenicity due to the different and independent cohorts, the results observed reflect patterns of treatment use in real-world settings. RA patients treated in Spain are slightly younger than in other registries, have previously used biologics and often receive tofacitinib monotherapy. The small series of patients included and the lack of data regarding ethnicity or race are some limitations of the study. Also, the mean follow-up of patients treated with tofacitinib is shorter compared to other real-world studies and clinical trials' follow-up. Long-term real-world data and pharmacovigilance information will increase knowledge about safety. Nonetheless, the study aimed to describe the tofacitinib pattern of administration in Spain. Data published from medical records and databases in Spain were consistent with the known benefit/risk profile of the drug, and with the main reason for discontinuing the drug being ineffectiveness. The most common AE was infection. A disease activity response was obtained in patients previously treated with bDMARDs. Further real-world evidence, collecting data more homogeneously, and providing novel variables (patient and clinician satisfaction, for instance) are required to strengthen the body of evidence for tofacitinib use.

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References

1. Smolen JS, Aletaha D, McInnes IB. Rheumatoid arthritis. Lancet. 2016; 388:2023-2038.
2. Gabriel SE. The epidemiology of rheumatoid arthritis. Rheum Dis Clin North Am. 2001; 27:269-281.
3. Seoane-Mato D, Sánchez-Piedra C, Silva-Fernández L, *et al*. Prevalence of rheumatic diseases in adult population in Spain (EPISER 2016 study): Aims and methodology. Reumatol Clin. 2019; 15:90-96.
4. Burmester GR, Pope JE. Novel treatment strategies in rheumatoid arthritis. Lancet. 2017; 389:2338-2348.
5. Chatzidionysiou K, Emamikia S, Nam J, Ramiro S, Smolen J, van der Heijde D, Dougados M, Bijlsma J, Burmester G, Scholte M, van Vollenhoven R, Landewé R. Efficacy of glucocorticoids, conventional and targeted synthetic disease-modifying antirheumatic drugs: A systematic literature review informing the 2016 update of the EULAR recommendations for the management of rheumatoid arthritis. Ann Rheum Dis. 2017; 76:1102-1107.
6. Smolen JS, Landewé RBM, Bijlsma JWJ, *et al*. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2019 update. Ann Rheum Dis. 2020; 79:685-699.
7. Singh JA, Saag KG, Bridges SL, *et al*. 2015 American college of rheumatology guideline for the treatment of rheumatoid arthritis. Arthritis Rheumatol. 2016; 68:1-26.
8. Meyer DM, Jesson MI, Li X, Elrick MM, Funckes-Shippy CL, Warner JD, Gross CJ, Dowty ME, Ramaiah SK, Hirsch JL, Saabye MJ, Barks JL, Kishore N, Morris DL. Anti-inflammatory activity and neutrophil reductions mediated by the JAK1/JAK3 inhibitor, CP-690,550, in rat adjuvant-induced arthritis. J Inflamm (Lond). 2010; 7:41.
9. EMA XELJANZ® (tofacitinib citrate). Summary of Product Characteristics. *https://www.ema.europa.eu/en/documents/product-information/xeljanz-epar-product-information_en.pdf* (accessed November 10, 2021).
10. US FDA (Food and Drug Administration). XELJANZ(R) (tofacitinib) tablets/XELJANZ(R) XR (tofacitinib) extended-release tablets: Prescribing information. *https://www.accessdata.fda.gov/* (Accessed March 24, 2021).
11. Kremer JM, Bloom BJ, Breedveld FC, Coombs JH, Fletcher MP, Gruben D, Krishnaswami S, Burgos-Vargas R, Wilkinson B, Zerbini CA, Zwillich SH. The safety and efficacy of a JAK inhibitor in patients with active rheumatoid arthritis: Results of a double-blind, placebo-controlled phase IIa trial of three dosage levels of CP-690,550 versus placebo. Arthritis Rheum. 2009; 60:1895-1905.
12. Tanaka Y, Suzuki M, Nakamura H, Toyoizumi S, Zwillich SH. Phase II study of tofacitinib (CP-690,550) combined with methotrexate in patients with rheumatoid arthritis and an inadequate response to methotrexate. Arthritis Care Res. 2011; 63:1150-1158.
13. Fleischmann R, Cutolo M, Genovese MC, Lee EB, Kanik KS, Sadis S, Connell CA, Gruben D, Krishnaswami S, Wallenstein G, Wilkinson BE, Zwillich SH. Phase IIb

dose-ranging study of the oral JAK inhibitor tofacitinib (CP-690,550) or adalimumab monotherapy versus placebo in patients with active rheumatoid arthritis with an inadequate response to disease-modifying antirheumatic drugs. Arthritis Rheum. 2012; 64:617-629.
14. Fleischmann R, Kremer J, Cush J, Schulze-Koops H, Connell CA, Bradley JD, Gruben D, Wallenstein GV, Zwillich SH, Kanik KS; ORAL Solo Investigators. Placebo-controlled trial of tofacitinib monotherapy in rheumatoid arthritis. N Engl J Med. 2012; 367:495-507.
15. Kremer JM, Cohen S, Wilkinson BE, Connell CA, French JL, Gomez-Reino J, Gruben D, Kanik KS, Krishnaswami S, Pascual-Ramos V, Wallenstein G, Zwillich SH. A phase IIb dose-ranging study of the oral JAK inhibitor tofacitinib (CP-690,550) versus placebo in combination with background methotrexate in patients with active rheumatoid arthritis and an inadequate response to methotrexate alone. Arthritis Rheum. 2012; 64:970-981.
16. van Vollenhoven RF, Fleischmann R, Cohen S, Lee EB, García Meijide JA, Wagner S, Forejtova S, Zwillich SH, Gruben D, Koncz T, Wallenstein GV, Krishnaswami S, Bradley JD, Wilkinson B; ORAL Standard Investigators. Tofacitinib or adalimumab versus placebo in rheumatoid arthritis. N Engl J Med. 2012; 367:508-519.
17. Burmester GR, Blanco R, Charles-Schoeman C, Wollenhaupt J, Zerbini C, Benda B, Gruben D, Wallenstein G, Krishnaswami S, Zwillich SH, Koncz T, Soma K, Bradley J, Mebus C; ORAL Step investigators. Tofacitinib (CP-690,550) in combination with methotrexate in patients with active rheumatoid arthritis with an inadequate response to tumour necrosis factor inhibitors: A randomised phase 3 trial. Lancet. 2013; 381:451-460.
18. Kremer J, Li ZG, Hall S, Fleischmann R, Genovese M, Martin-Mola E, Isaacs JD, Gruben D, Wallenstein G, Krishnaswami S, Zwillich SH, Koncz T, Riese R, Bradley J. Tofacitinib in combination with nonbiologic disease-modifying antirheumatic drugs in patients with active rheumatoid arthritis: A randomized trial. Ann Intern Med. 2013; 159:253-261.
19. Van Der Heijde D, Tanaka Y, Fleischmann R, *et al*. Tofacitinib (CP-690,550) in patients with rheumatoid arthritis receiving methotrexate: Twelve-month data from a twenty-four-month phase III randomized radiographic study. Arthritis Rheum. 2013; 65:559-570.
20. Lee EB, Fleischmann R, Hall S, Wilkinson B, Bradley JD, Gruben D, Koncz T, Krishnaswami S, Wallenstein GV, Zang C, Zwillich SH, van Vollenhoven RF; ORAL Start Investigators. Tofacitinib versus methotrexate in rheumatoid arthritis. N Engl J Med. 2014; 370:2377-2386.
21. Wallenstein GV, Kanik KS, Wilkinson B, Cohen S, Cutolo M, Fleischmann RM, Genovese MC, Gomez Reino J, Gruben D, Kremer J, Krishnaswami S, Lee EB, Pascual-Ramos V, Strand V, Zwillich SH. Effects of the oral Janus kinase inhibitor tofacitinib on patient-reported outcomes in patients with active rheumatoid arthritis: Results of two Phase 2 randomised controlled trials. Clin Exp Rheumatol. 2016; 34:430-442.
22. Cohen SB, Tanaka Y, Mariette X, Curtis JR, Lee EB, Nash P, Winthrop KL, Charles-Schoeman C, Thirunavukkarasu K, DeMasi R, Geier J, Kwok K, Wang L, Riese R, Wollenhaupt J. Long-term safety of tofacitinib for the treatment of rheumatoid arthritis up to 8.5 years: Integrated analysis of data from the global clinical trials. Ann Rheum Dis. 2017; 76:1253-1262.
23. Fleischmann R, Mysler E, Hall S, *et al*. Efficacy and safety of tofacitinib monotherapy, tofacitinib with methotrexate, and adalimumab with methotrexate in patients with rheumatoid arthritis (ORAL Strategy): a phase 3b/4, double-blind, head-to-head, randomised controlled trial. Lancet. 2017; 390:457-468.
24. Cohen SB, Tanaka Y, Mariette X, Curtis JR, Lee EB, Nash P, Winthrop KL, Charles-Schoeman C, Wang L, Chen C, Kwok K, Biswas P, Shapiro A, Madsen A, Wollenhaupt J. Long-term safety of tofacitinib up to 9.5 years: A comprehensive integrated analysis of the RA clinical development program. RMD Open. 2020; 6:e001395.
25. Wollenhaupt J, Lee EB, Curtis JR, Silverfield J, Terry K, Soma K, Mojcik C, DeMasi R, Strengholt S, Kwok K, Lazariciu I, Wang L, Cohen S. Safety and efficacy of tofacitinib for up to 9.5 years in the treatment of rheumatoid arthritis: final results of a global, open-label, long-term extension study. Arthritis Res Ther. 2019; 21:89.
26. Barnish M, Turner S. The value of pragmatic and observational studies in health care and public health. Pragmatic Obs Res. 2017; 8:49-55.
27. Favalli EG, Bugatti S, Biggioggero M, Caporali R. Treatment comparison in rheumatoid arthritis: Head-to-head trials and innovative study designs. Biomed Res Int. 2014; 2014:831603.
28. Blonde L, Khunti K, Harris SB, Meizinger C, Skolnik NS. Interpretation and impact of real-world clinical data for the practicing clinician. Adv Ther. 2018; 35:1763-1774.
29. FDA. Developing a framework for regulatory use of real-world evidence. Public Work. *https://www.gpo.gov/fdsys/pkg/FR-2017-07-31/pdf/2017-16021.pdf* (Accessed March 24, 2021).
30. EMA. Update on real world evidence data collection. Published online 2016. *https://ec.europa.eu/health/sites/health/files/files/committee/stamp/2016-03_stamp4/4_real_world_evidence_ema_presentation.pdf* (accessed March 24, 2021).
31. Kremer J, Bingham C, Cappelli L, Greenberg J, Geier J, Madsen A, Chen C, Onofrei A, Barr C, Pappas D, Litman H, Dandreo K, Shapiro A, Connell C, Kavanaugh A. Postapproval Comparative safety study of tofacitinib and biological disease-modifying antirheumatic drugs: 5-year results from a United States-based rheumatoid arthritis registry. ACR Open Rheumatol. 202; 3:173-184.
32. Reed GW, Gerber RA, Shan Y, Takiya L, Dandreo KJ, Gruben D, Kremer J, Wallenstein G. Real-World comparative effectiveness of tofacitinib and tumor necrosis factor inhibitors as monotherapy and combination therapy for treatment of rheumatoid arthritis. Rheumatol Ther. 2019; 6:573-586.
33. Pope J, Bessette L, Jones N, Fallon L, Woolcott J, Gruben D, Crooks M, Gold D, Haraoui B. Experience with tofacitinib in Canada: Patient characteristics and treatment patterns in rheumatoid arthritis over 3 years. Rheumatology (Oxford). 2020; 59:568-574.
34. Finckh A, Tellenbach C, Herzog L, Scherer A, Moeller B, Ciurea A, von Muehlenen I, Gabay C, Kyburz D, Brulhart L, Müller R, Hasler P, Zufferey P; physicians and patients of the SCQM. Comparative effectiveness of antitumour necrosis factor agents, biologics with

an alternative mode of action and tofacitinib in an observational cohort of patients with rheumatoid arthritis in Switzerland. RMD Open. 2020; 6:e001174.
35. Bird P, Littlejohn G, Butcher B, Smith T, da Fonseca Pereira C, Witcombe D, Griffiths H. Real-world evaluation of effectiveness, persistence, and usage patterns of tofacitinib in treatment of rheumatoid arthritis in Australia. Clin Rheumatol. 2020; 39:2545-2551.
36. Mueller RB, Hasler C, Popp F, Mattow F, Durmisi M, Souza A, Hasler P, Rubbert-Roth A, Schulze-Koops H, Kempis JV. Effectiveness, tolerability, and safety of tofacitinib in rheumatoid arthritis: A retrospective analysis of real-world data from the St. Gallen and Aarau cohorts. J Clin Med. 2019; 8:1548.
37. Cohen S, Curtis JR, DeMasi R, Chen Y, Fan H, Soonasra A, Fleischmann R. Worldwide, 3-year, post-marketing surveillance experience with tofacitinib in rheumatoid arthritis. Rheumatol Ther. 2018; 5:283-291.
38. de la Morena I, Paz Solarte JA, Bedoya D, Larraz PT. AB0435 real world data of a patient cohort with rheumatoid artritis treated with jak/stat inhibitors. Ann Rheum Dis. 2019; 78:1680.
39. Gómez-Lechón Quirós L, Hidalgo Calleja C, López González R, *et al*. RC047. Tofacitinib en el tratamiento de artritis reumatoide: un estudio multicéntrico con experiencia en vida real. Reumatol Clin. 2019; 15:231-232. (in Spanish)
40. Pérez García C, Carballo Martínez N, Martínez Casanova Quirante J, Fernández Sala X. RC057. Impacto de los inhibidores jak en el perfil de tratamiento de la artritis reumatoide. Reumatol Clin. 2019; 15:238-239. (in Spanish)
41. Rusinovich O, Campos Esteban J, de la Torre Rubio N, Espinosa Malpartida M, Barbadillo Mateos C, Godoy Tundidor H, Villa Alcázar LF, Sanz Sanz J, Fernández Castro M, Isasi Zaragoza CM, Jiménez Palop M, Andreu Sánchez JL. RC053. Eficacia y seguridad de los inhibidores de quinasas janus en pacientes con artritis reumatoide en un hospital terciario. Reumatol Clin. 2019; 15:236-237. (in Spanish)
42. Calvo Garcia A, García Castañeda N, Valero C, Llorente I, Varas B, García-Vadillo A, González-Álvaro I, Morell A, Ramírez E, Garcia de Vicuna R, on behalf of HUP IMID Therapy Unit. AB0334 Cost-effectiveness of jak inhibitors in rheumatoid arthritis in the real world practice. Ann Rheum Dis. 2020; 79:1465-1466.
43. Retuerto-Guerrero M, Trujillo E, Valero C, Fernández-Espartero C, Soleto CY, García-Valle A, Aurrecoechea E, Garijo Bufort M, López Robles A, Loricera J, Pablos JL. FRI0132 Efficacy and safety of switching jakinibs in rheumatoid arthritis. Ann Rheum Dis. 2020; 79:648-649.
44. Rosas J, Pons A, Senabre Gallego JM, Santos Soler G, Bernal JA, García-Gómez JA, Cano C, Ivars E, Lorente Betoret M, Barber X, on behalf of AIRE-MB Group. AB0318 Characteristics of patients with rheumatoid arthritis who have withdrawn the last biological drug: Real-life results from a local registry. Ann Rheum Dis. 2020; 79:1458.
45. Soleto CY, Serrano Benavente B, Torrens Cid LA, Martínez-Barrio J, Molina Collada J, Rivera J, González T, Monteagudo I, González C, Castrejón I, Álvaro-Gracia JM. AB0357 Use of tofacitinib and reasons for discontinuation in clinical practice. Ann Rheum Dis. 2020; 79:1478-1479.
46. Valero C, Calvo García A, García Castañeda N, Ortiz A, Llorente I, Varas B, Castañeda S, García de Vicuna R, Ramirez E. AB1339-HPR safety and adherence of the jak inhibitors in clinical practice in rheumatoid arthritis. Ann Rheum Dis. 2020; 79:1957-1958.
47. Velloso Feijoo ML, Cid Boza N, Pérez MJ. AB0363 safety of jak inhibitors in patients with arthritis rheumatoid under real-life conditions. Ann Rheum Dis. 2020; 79:1482.
48. Campos Fernández C, Almela Molina C, Rueda Cid A, Balaguer Trull I, Lerma Garrido JJ, Pastor Cubillo MD, Calvo Catalá J. P045. Evaluación clínica y ecográfica de pacientes con artritis reumatoide que inician tratamiento con FAME sintético dirigido. Reumatol Clin. 2021; 17:43-307. XLVII National Congress of the Spanish Society of Rheumatology. Available from: *https://static.elsevier.es/reuma/reumacongreso2021.pdf* (in Spanish)
49. Compán Fernández O, Acosta ME, Pastor S, Ibáñez M, Fernández de la Fuente MA, Montilla C, Martínez O, Turrión A, Pino Montes J, da Casa C, Hidalgo C. P063. Evaluación de la efectividad, persistencia y seguridad de tofacitinib en artritis reumatoide en distintos escenarios. Estudio en práctica clínica habitual. Reumatol Clin. 2021; 17:43-307. XLVII National Congress of the Spanish Society of Rheumatology. Available from: *https://static.elsevier.es/reuma/reumacongreso2021.pdf* (in Spanish)
50. Espartal E, López-Lasanta M, Michelena X, Borrell H, Pluma A, de Agustín JJ, Caparrós R, Erra A, Almirall M, Marsal S. RC08. 7 años de experiencia clínica con inhibidores de JAK: Análisis retrospectivo de la eficacia y la seguridad de tofacitinib en una cohorte de pacientes con artritis reumatoide en condiciones de práctica clínica. Reumatol Clin. 2021; 17:308-355. XLVII National Congress of the Spanish Society of Rheumatology. Available from: *https://static.elsevier.es/reuma/reumacongreso2021.pdf* (in Spanish)
51. Fragío Gil JJ, González Mazarío R, Pávez Perales C, *et al*. P062. Efectividad y seguridad de los JAK inhibidores en la artritis reumatoide: Datos de vida real. Reumatol Clin. 2021; 17:43-307. XLVII National Congress of the Spanish Society of Rheumatology. Available from: *https://static.elsevier.es/reuma/reumacongreso2021.pdf* (in Spanish)
52. Haro Martínez AC, Moreno MJ, Peñas E, Mayor M, Palma D, Castillo P, Moreno MJ. P020. Eficacia de los inhibidores JAK en práctica clínica real. Reumatol Clin. 2021; 17:43-307. XLVII National Congress of the Spanish Society of Rheumatology. Available from: *https://static.elsevier.es/reuma/reumacongreso2021.pdf* (in Spanish)
53. Úbeda-Portugués Palomino S, Márquez Pete N, Pérez Ramírez C, Maldonado Montoro MM, Cáliz Cáliz R. P050. Influencia de polimorfismos en el gen EGFR en la respuesta al tratamiento con fármacos inhibidores de la JAK en pacientes diagnosticados de artritis reumatoide. Reumatol Clin. 2021; 17:43-307. XLVII National Congress of the Spanish Society of Rheumatology. Available from: *https://static.elsevier.es/reuma/reumacongreso2021.pdf* (in Spanish)
54. Rodriguez-García SC, Sánchez-Piedra C, Castellanos-Moreira R, Ruiz Montesinos D, Pombo M, Sánchez-Alonso F, Gómez-Reino JJ on behalf of the BIOBADASER Working group. POS0610. Epidemiologic profile and channeling to treatment in rheumatoid arthritis patients treated with abatacept over the last 5 years: data from the Spanish register

BIOBADASER 3.0. Ann Rheum Dis. 2021; 80:542.
55. Ytterberg SR, Bhatt DL, Mikuls TR, Koch GG, Fleischmann R, Rivas JL, Germino R, Menon S, Sun Y, Wang C, Shapiro AB, Kanik KS, Connell CA; ORAL Surveillance Investigators. Cardiovascular and cancer risk with tofacitinib in rheumatoid arthritis. N Engl J Med. 2022; 386:316-326.
56. Khosrow-Khavar F, Kim SC, Lee H, Lee SB, Desai RJ. Tofacitinib and risk of cardiovascular outcomes: results from the safety of tofacitinib in routine care patients with rheumatoid arthritis (STAR-RA) study. Ann Rheum Dis. 2022; annrheumdis-2021-221915.
57. Caporali R ZD. Real-world experience with tofacitinib for the treatment of rheumatoid arthritis. Clin Exp Rheumatol. 2019; 37:485-495.
58. Freites Nuñez D, Abasolo L, Peñuelas M, Candel FJ, Font J, Lois P, Martínez Prada C, Fernández B, Madrid García A, Rodríguez Rodríguez L, Colomer JI, León L. AB0290 Herpes zoster infections in rheumatoid arthritis patients exposed to biological agents and jak inhibitors. Real-world evidence. Ann Rheum Dis. 2020; 79:1444.

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Original Article

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Physicochemical properties and detergency of special electrolytic-reduction ion water

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SUMMARY The detergency of special electrolytic-reduction ion water (S-100) was evaluated in comparison with typical synthetic surfactants. Furthermore, to examine the cleaning mechanism of S-100, various physicochemical characteristics of S-100 were measured and a comprehensive evaluation of cleaning was performed. S-100 (10%) had a detergency comparable to that of various surfactants, such as sodium dodecyl sulfate and sodium dodecyl benzene sulfonate, which are generally blended or mixed in residential detergents. In addition, concentrated aqueous solutions of 10% or more of S-100 showed stronger detergency. The cleaning mechanism of S-100 is mainly attributed to the effect of surface tension reduction due to dissolved hydrogen or hydrogen nanobubbles in S-100, and the alkalinity generated by electrolysis charged the surface of the dirt or adherend, resulting in a peeling effect.

Keywords Detergency, electrolyzed deoxidized and ionized water, soiled fabric, hydrogen nano-bubble, CMC

1. Introduction

Surfactants have a high detergency against oil stains and are essential for daily use as the main component of detergents for homes, tableware and laundry. However, since there are many opportunities for contact with living bodies, many skin and mucosal disorders caused by surfactants have been reported (*1*). In the health hazard hospital monitor report related to household goods from the Ministry of Health, Labour and Welfare, many skin or mucous membrane disorders due to detergents have been published. Furthermore, environmental pollution of rivers and seawater by detergents, as well as toxicity to fish (*2*), are well-known issues.

Electro-reduced water is a strong alkaline and has extremely strong reducing power that can be obtained in the cathode chamber when electrolytically diluting a solution. Electrolyzed water has a high concentration of dissolved hydrogen (*3*), and is used for cleaning by exfoliation (*4*), deodorization, sterilization and dust prevention, as well as rust and antiseptic effects by antioxidant action. Utilizing these characteristics, it is now industrially used as a surface cleaning solution for cutting and precision equipment.

The effectiveness of washing using electrolytically reduced water has been investigated by comparing the detergency with other aqueous solutions (*5*). It has also been reported that special electrolytic reduced water has an emulsifying effect (*6*), and it is possible to prepare a medical emulsion that can be emulsified without using a surfactant. The reported effects of applying electrolytically reduced water to living bodies include antimicrobial properties (*7*), improvement effect on skin injury (*8,9*), protecting DNA from oxidative damage (*10*), anti-diabetic effects (*11*) and antitumor effects (*12*). In contrast, few adverse events have been reported to date.

Specially reduced electrolytic water (S-100) is manufactured using a method that differs from the conventional electrolytic reduced water method. The general production method of electrolyzed reduced water is purified by the flow of tap water → soft water → electrolyte → electrolysis, but the production method of S-100 is purified by the flow of tap water → pure water → deaeration → electrolyte → "electrolysis at high voltage" → "stabilization tank". In general, electrolyzed reduced water uses sodium chloride, fatty acid sodium, potassium chloride or fatty acid potassium as the electrolyte, whereas S-100 utilizes multiple electrolytes originating from natural seawater and minerals.

As mentioned earlier, S-100 as a cleaning agent generally contains properties that are safe for the living body and has little impact on the environment. However, there have been no published studies comparing the

cleaning action of S-100 with synthetic surfactants commonly used as residential detergents. In this study, the detergency of S-100 was evaluated in comparison with typical synthetic surfactants. Furthermore, to investigate the cleaning mechanism of S-100, various physicochemical characteristics of S-100 were measured and a comprehensive evaluation of cleaning was performed.

2. Materials and Methods

2.1. Materials

Special electrolytic-reduction ion water (product name S-100) was provided by A. I. System Products Corp. (Aichi, Japan). Sodium dodecyl sulfate (SDoS) was purchased from FUJIFILM Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Sodium dodecyl benzene sulfonate (DBS) and Sodium 1-decane sulfonate (SDeS) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). All other reagents were special grade.

Contaminated cloth for measuring detergency was standard artificially contaminated cloth EMPA 101 (Fabric type: cotton, dirt component: carbon black/olive oil) purchased from Nippon Shizai Co., Ltd. (Osaka, Japan).

2.2. Methods

2.2.1. Preparation of sample solution

S-100 stock solution was used as 100% sample solution, and S-100 stock solution was diluted with purified water to prepare aqueous solutions of various concentrations (10, 30, 50 and 80%). Three different anionic surfactants of SDoS, DBS and SDeS were used as comparative controls. Each surfactant was prepared as an aqueous solution with various concentrations (0.1, 0.5, 1.0 and 1.5%).

2.2.2. Detergency test

Each sample solution (25 mL) was placed in a sample bottle, immersed in a contaminated cloth (EMPA 101) cut to 1.5 × 1.5 cm, attached to a shaker and shaken at 185 rpm for 30 minutes. The contaminated cloth was taken out and rinsed with purified water for 5 minutes × 2 times. After which, the reflectance of the contaminated fabric was measured before and after shaking using a compact color and whiteness meter (Color Mater NW-11, Nippon Denshoku Industries Co., Ltd., Tokyo, Japan). The cleaning efficiency was calculated using the following formula. First, the amount of dirt was calculated from the reflectance using the Kubelka-Munk formula (I):

$$K/S = (1 - R)^2/2R \quad (I)$$

(K = Absorbance coefficient, S = Light scattering coefficient, R = Surface reflectance)

Furthermore, the soil removal rate (%) (II) was calculated using the following equation:

$$\text{Dirt removal rate (\%)} = \frac{\text{contaminated cloth K/S - cleaning cloth K/S}}{\text{contaminated cloth K/S - Clean cloth K/S}} \times 100 \quad (II)$$

2.2.3. Evaluation of various physicochemical properties

The surface tension of each sample solution was measured by the ring method using a Du Noüy surface tension meter. The oxidation-reduction potential (ORP) of various sample solutions was measured using HORIBA ORP electrodes. The pH of each sample solution was measured using a HORIBA pH electrode. Both measurements were performed at room temperature (25 ± 1°C).

3. Results

3.1. Cleaning efficiency of S-100 and various synthetic surfactants

The concentration of each surfactant was set to 0.1, 0.5, 1.0 and 1.5% with reference to the critical micelle concentration (CMC) of each component. In addition, S-100 was tested in aqueous solutions with concentrations of 50% and 100% for confirming concentration dependence, as well as 10%, which is a concentration used industrially as a cleaning agent. The cleaning efficiency of 10% S-100 aqueous solution was about 90% of the cleaning efficiency of 0.1% SDoS or 0.1% DBS aqueous solution (Figure 1); it also showed higher cleaning efficiency than 0.1-0.5% SDeS aqueous solution. Furthermore, in order to investigate the concentration dependence of S-100 cleaning efficiency in more detail, a test to determine the cleaning efficiency for 30% and 80% concentrations was also conducted in addition to the 10, 50 and 100% aqueous solutions (Figure 2). The cleaning efficiency of S-100 increased in a concentration-dependent manner.

3.2. pH of S-100 and various synthetic surfactants at various concentrations

The pH of the S-100 stock solution (100%) was 12.3 and the pH of the 50% and 10% aqueous solutions were 12.0 and 11.3, respectively (Figure 3). In contrast, various synthetic surfactants were almost neutral at pH 5.5-7.0, irrespective of concentration.

3.3. Surface tension of S-100 and various synthetic surfactants

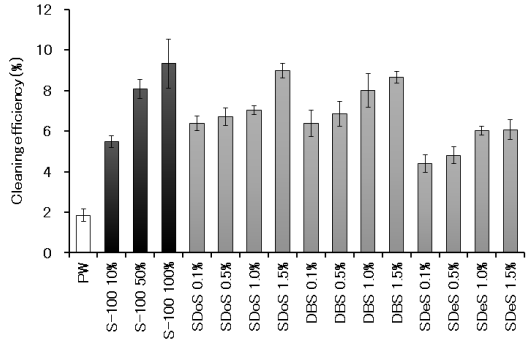

Figure 1. Cleaning efficiency of S-100 and various synthetic surfactants. (*n* = 5, mean ± S.D.). PW: purified water.

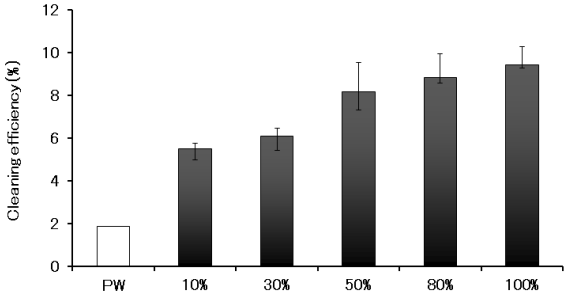

Figure 2. Cleaning efficiency of S-100 at various concentrations. (*n* = 5, mean ± S.D.). PW: purified water.

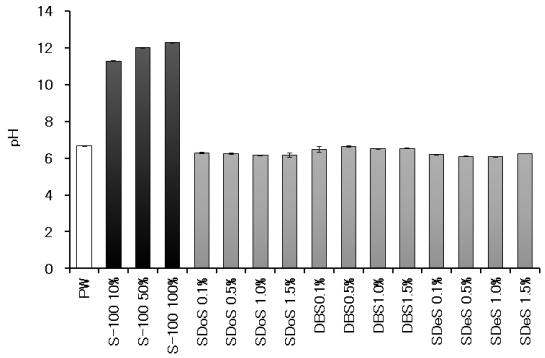

Figure 3. pH of S-100 and various synthetic surfactants at various concentrations. (*n* = 5, mean ± S.D.). PW: purified water.

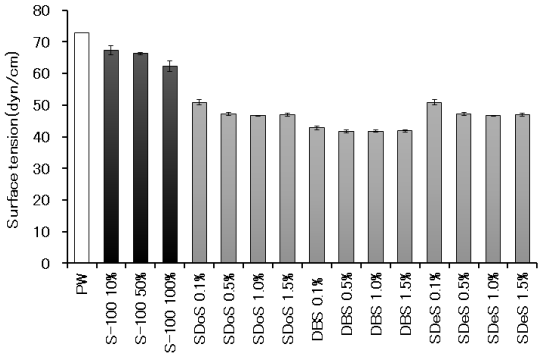

Figure 4. Surface tension of S-100 and various synthetic surfactants. (*n* = 5, mean ± S.D.). PW: purified water.

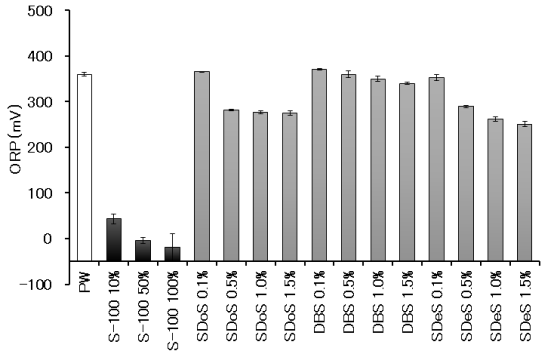

Figure 5. ORP of S-100 and various synthetic surfactants. (*n* = 5, mean ± S.D.). PW: purified water, ORP: oxidation-reduction potential.

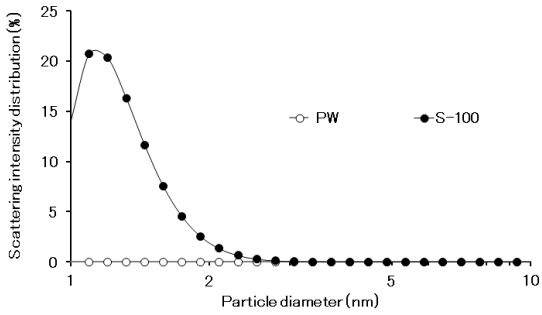

Figure 6. Scattering intensity distribution of hydrogen nanobubbles in S-100 solution and purified water (%). PW: purified water.

The surface tension of the S-100 stock (100%), 50% aqueous and 10% aqueous solutions were 62.3 dyn/cm, 66.3 dyn/cm and 67.4 dyn/cm, respectively. These values were all lower than that of purified water (72.8 dyn/cm) (Figure 4). In contrast, the surface tensions of various synthetic surfactants were in the range of 41.8-50.9 dyn/cm, and all the surfactants showed values lower than that for S-100.

3.4. ORP of S-100 and various synthetic surfactants

The ORP (oxidation-reduction potential) of the S-100 stock (100%), 50% and 10% solutions were -18.9 mV, -4.3 mV and 43.4 mV, respectively, which were significantly lower than that of purified water (360.0 mV) (Figure 5). In contrast, the ORPs of various surfactants were between 250-370 mV, which were similar to or lower than the ORP of purified water.

3.5. Scattering intensity distribution of hydrogen nanobubbles in S-100 solution

The particle size and particle size distribution were measured using the zeta-potential and particle size analyzer system ELSZ-1000ZS (Otsuka Electronics Co., Ltd., Osaka Japan). S-100 was confirmed to contain particles with an average particle size of 1-3 nm (average particle size 1.3 nm) (Figure 6).

Table 1. Names and structural formulas of surfactants used in this study

Surfactant name	Chemical structural formula	MW	CMC (mM)
Sodium dodecyl sulfate (SDoS)	$C_{12}H_{25}OSO_3Na$	288.38	8.2
Sodium dodecyl benzenesulfonate (DBS)	$C_8H_{17}C_6H_4SO_3Na$	348.50	3.5~8.9
Sodium 1-decanesulfonic acid (SDeS)	$C_{10}H_{21}SO_3Na$	244.33	40

CMC (Critical micelle concentration in pure water at 25°C), MW: Molecular weight, CMC: critical micelle concentration, SDoS: Sodium dodecyl sulfate ($C_{12}H_{25}OSO_3Na$), DBS: Sodium dodecyl (octyl) benzenesulfonate ($C_8H_{17}C_6H_4SO_3Na$), SDeS: Sodium 1-decanesulfonic acid ($C_{10}H_{21}SO_3Na$)

4. Discussion

In order to evaluate the detergency of S-100 special electrolyzed reduced water in comparison with typical synthetic surfactants, the detergency of contaminated cloth immersed in different aqueous solutions was determined. Evaluated synthetic surfactants included SDoS, DBS and SDeS, which are typical anionic surfactants commonly used as house or skin cleansers (Table 1).

The 10% S-100 aqueous solution, which is currently used as a cleaning agent for precision machinery, has a comparable cleaning power to that of various surfactants generally used in residential detergents (Figure 1). Furthermore, in order to investigate the concentration dependence of S-100 cleaning efficiency in more detail, the cleaning efficiencies for 10, 30, 50, 80 and 100% concentrations were also determined. The cleaning efficiency of S-100 increased in a concentration-dependent manner (Figure 2). These results clarified that S-100 has a cleaning power comparable to that of various synthetic surfactants. In order to further investigate the mechanism of this cleaning, pH, surface tension and ORP were measured as physicochemical properties of the S-100 aqueous solution, and compared with that of various synthetic surfactant solutions (Figures 3, 4 and 5).

In general, as a mechanism by which pH contributes to cleaning, alkaline agents negatively charge the surface charge of the dirt and the adhesion surface, thereby increasing the electrostatic repulsion force, and making it easier for the dirt to detach from the adhesion surface. This mechanism is known to contribute to cleaning by preventing reattachment (*13*). Therefore, the pH values of S-100 and various synthetic surfactants were measured (Figure 3). S-100 shows alkalinity because, in the water electrolysis process, water molecules are reduced at the cathode to generate hydrogen (H_2) and subsequently hydroxide ions (OH^-) (reaction formula 1). Various aqueous surfactant solutions are almost neutral possibly because the degree of dissociation of the sulfone and sulfate groups in each surfactant is large and does not affect pH.

$$2H_2O + 2e^- \rightarrow H_2\uparrow + 2OH^- \quad \text{(reaction formula 1)}$$

As a mechanism by which the surface tension contributes to cleaning, for example, a surfactant with a surface tension-reducing action is adsorbed on the cleaning liquid and adhesion surface, and this contributes to cleaning by removing the dirt and dispersing the dirt in the cleaning liquid. Therefore, the surface tensions of S-100 special electrolytic reduced water and various synthetic surfactants at various concentrations were measured (Figure 4). Higher S-100 concentration corresponded to lower surface tension. In contrast, the surface tensions of various synthetic surfactants showed values lower than that for S-100. S-100 showed a lower surface tension than purified water because water molecules are reduced at the cathode to generate hydrogen (H_2) in the water electrolysis process (reaction formula 1).

If dissolved hydrogen is present, the ORP may be low. Therefore, ORP was measured to estimate the dissolved hydrogen concentration in S-100 electrolyzed reduced water and various synthetic surfactants (at 25°C and at each concentration) (Figure 5). The low ORP in S-100 may be due to the dissolved hydrogen generated at the cathode in the water electrolysis process to produce S-100. The ORP of dissolved hydrogen approaches that of purified water with dilution, but the hydrogen concentration does not decrease with dilution. In other words, even in a 10% solution in which S-100 is diluted 10-fold, the reducing power estimated from the reduction potential of S-100 is not simply reduced to 1/10; this can be explained by assuming that there is hydrogen dispersed in addition to the dissolved hydrogen.

Kikuchi *et al*. (*14*) found that dissolved hydrogen in electroreduction water obtained by electrolyzing an electrolyte solution contains dissolved hydrogen and colloidal nanobubbles (micro hydrogen bubbles) that are dissolved in molecular form above the saturation concentration. Therefore, if nanobubbles of hydrogen gas are also present in S-100, even if the dissolved hydrogen is diluted when the S-100 stock solution is diluted, then the hydrogen in the nanobubbles dissolves in the water. The hydrogen concentration may be higher than the concentration obtained by simply diluting S-100. Subsequently, according to the method of Takenouchi *et al*. (*15*), the particle size and particle size distribution were measured using a zeta-potential and particle size analyzer system. S-100 was confirmed to contain particles with an average particle size of 1 -

3 nm (average particle size 1.3 nm) (Figure 6). These particles are likely to be hydrogen nanobubbles. Based on these results, the cleaning mechanism of S-100 compared with each surfactant is discussed below. The two main cleaning mechanisms for the three types of surfactants (SDoS, DBS and SDeS) used in this experiment are as follows. One is that the surfactant is dissolved in the water, and the interfacial tension between the dirt or its attached surface and water is lowered, thereby removing the dirt from the attached surface. The other is that the surfactant is adsorbed on the dirt surface and the adhesion surface, such that the sulfonate ions or sulfate ions in the structure negatively charge both surfaces and creates an electrostatic repulsive force between the dirt and the adhesion surface.

On the other hand, the cleaning mechanism of S-100 electrolytically reduced water on stains may cause separation of the dirt from the adhesion surface, as well as repelling between the dirt and the adhesion surface, due to a mechanism different from that of the surfactant. The reason for this is that hydrogen dissolves in water, or the presence of hydrogen nanobubbles reduces the surface tension of the solution, which reduces the interfacial tension between the dirt or its attached surface and water, and removes the dirt from the attached surface. The pH of S-100 solution becomes extremely high due to electrolysis, the dirt surface and adhesion surface are negatively charged, and electrostatic repulsion is generated between the dirt and the adhesion surface.

The cleaning efficiency of S-100 is concentration-dependent and linear at concentrations from 10 to 100%. However, compared to purified water (the control), the relationship is not completely proportional, and cleaning efficiency is not proportional to the dilution rate (Figure 2). In a 10% aqueous solution, in which S-100 is diluted 10 times, the cleaning efficiency of S-100 is not simply reduced to 1/10. The effect of alkalinity may have a stronger influence on the cleaning power than the effect of surface tension reduction. Therefore, cleaning efficiency was measured for NaOH aqueous solution with the same pH 12 as S-100 in the same experimental system, and found to be 6.4 ± 0.58%, thus confirming the cleaning effect due to the alkaline cleaning solution.

In conclusion, S-100 special electrolyzed reduced water has the same detergency as other electrolytically reduced waters already on the market, and the 10% aqueous solution of S-100 was confirmed to have a cleaning power comparable to that of various surfactants such as SDoS and DOS that are mixed in general detergents. In addition, the cleaning mechanism considered from the various physicochemical properties of S-100 are mainly due to the effect of surface tension reduction by dissolved hydrogen or hydrogen nanobubbles in S-100 and the alkalinity generated by electrolysis. Furthermore, the peeling effect may have been produced by charging the surface of the oil stains.

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Conflict of Interest: Special electrolytic-reduction ion water (S-100) used in this study was manufactured by A. I. System products, Corp. Masahiro Okajima and Yoshinao Okajima are employees of A. I. System products, Corp.

References

1. Marks R, Dykes PJ. The effects of the detergent action of cleansing agents on the skin. Wien Med Wochenschr Suppl. 1990; 108:16-18.
2. Misra V, Lal H, Chawla G, Kumar V, Lal H, Viswanathan PN. Effect of linear alkyl benzene sulfonate in skin of fish fingerlings (Cirrhina mrigala): Observations with scanning electron microscope. Ecotoxicol Environ Saf. 1987; 13:164-168.
3. Sato U, Takeuchi M, Kawakubo H. The basic characteristics and the performance of precision washing of Na2SO4-electrolyzed reducing water. Journal of the Japan Society for Precision Engineering. 2012; 78:231-235. (in Japanese)
4. Kuzuhara A. Study of new washing system using electrolytic reduction ion water. Tokyo Kasei University, Teacher training education promotion office annual report. 2017; 4:217-226. (in Japanese)
5. Takahashi T, Aso Y, Yamamoto T. Detergency of electrolysis water in laundering. Sen'i Gakkaishi. 2007; 63:109-116. (in Japanese)
6. Konomatsu A, Sugibayashi K, Okajima M, Ishii F. Preparation and stability of surfactant free emulsions using electrolyzed deoxidized and ionized water. Mater Tech. 2003; 21:273-285. (in Japanese)
7. Okajima M, Kanzaki M, Ishibashi Y, Wada Y, Hino F, KitaharaY, Shimokawa K, Ishii F. *In vitro* bactericidal activity against periodontopathic bacteria by electrolyzed ion-reduced water. Drug Discov Ther. 2011; 5: 306-310.
8. Yoon KS, Huang XZ, Yoon YS, Kim S-K, Song SB, Chang BS, Kim DH, Lee KJ. Histological study on the effect of electrolyzed reduced water-bathing on UVB radiation-induced skin injury in hairless mice. Biol Pharm Bull. 2011; 34:1671-1677.
9. Shu T, Okajima M, Shimokawa K, Ishii F. The treatment effect of the burn wound healing by electrolytic reduction ion water lotion. Biosci Trends. 2010; 4:1-3.
10. Shirahata S, Kabayama S, Nakano M, Miura T, Kusumoto K, Gotoh M, Hayashi H, Otsubo K, Morisawa S, Katakura Y. Electrolyzed-reduced water scavenges active oxygen species and protects DNA from oxidative damage. Biochem Biophys Res Commun. 1997; 234:269-274.
11. Kim MJ, Kim HK. Anti-diabetic effects of electrolyzed

reduced water in streptozotocin-induced and genetic diabetic mice. Life Sci. 2006; 79:2288-2292.
12. Nishikawa R, Teruya K, Katakura Y, Osada K, Hamasaki T, Kashiwagi T, Komatsu T, Li Y, Ye J, Ichikawa A, Otsubo K, Morisawa S, Xu Q, Shirahata S. Electrolyzed reduced water supplemented with platinum nanoparticles suppresses promotion of two-stage cell transformation. Cytotechnology. 2005; 47:97-105.
13. Oya M. Cleaning mecanizsm. Surface Tech. 2009; 60: 85-89. (in Japanese)
14. Kikuchi K, Takeda H, Rabolta B, Okaya T, Ogumi Z, Saihara Y, Noguchi H. Hydrogen particles and supersaturation in alkaline water from an Alkali-Ion-Water electrolyzer. J Electroanal Chem. 2001; 506: 22-27.
15. Takenouchi T, Sato U, Nishio Y. Behavior of hydrogen nanobubbles generated in alkaline electrolyzed water. Electrochemistry. 2009; 77: 521-523. (in Japanese)

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Original Article

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Antibody response of smokers to the COVID-19 vaccination: Evaluation based on cigarette dependence

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SUMMARY Smokers may have lower antibody titers after vaccination with a coronavirus disease 2019 (COVID-19) mRNA vaccine. However, to the best of our knowledge, no study has evaluated antibody titers after COVID-19 vaccination based on the level of smokers' cigarette dependence. In this study, we measured the level of serum anti-severe acute respiratory syndrome coronavirus 2 (anti-SARS-CoV-2) spike protein receptor-binding domain (S-RBD) immunoglobulin-G (IgG) by enzyme linked immunosorbent assay of 55 actively smoking Japanese social workers (firefighters, paramedics, and rescue workers) who had received two doses of the BNT162b2 vaccine. Further, we assessed their cigarette dependence using the Fagerstrom Test for Nicotine Dependence (FTND), measured their serum cotinine levels, and tested for their correlation with anti-RBD IgG levels. Serum anti-SARS-CoV-2 S-RBD protein IgG levels after BNT162b2 vaccination showed a significant negative correlation with FTND ($\rho = -0.426$, $p = 0.001$). In addition, serum cotinine level showed a significant positive correlation with FTND ($\rho = 0.470$, $p = 0.000$). However, no significant negative correlation was noted between serum cotinine and serum anti-SARS-CoV-2 S-RBD protein IgG levels ($\rho = -0.156$, $p = 0.256$). Our results suggest that smokers with strong cigarette dependence have inadequate anti-SARS-CoV-2 S-RBD protein IgG levels after COVID-19 mRNA vaccination.

Keywords SARS-CoV-2, COVID-19, mRNA vaccine, anti-RBD IgG level, cigarette dependence

1. Introduction

Since the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in 2019, the situation has evolved into an unprecedented global pandemic (*1*). In response to this challenging situation, the emergency use of vaccines BNT162b2/Pfizer and mRNA-1273/Moderna was approved by the US Food and Drug Administration in December 2020 as a prophylactic strategy against novel coronavirus disease 2019 (COVID-19) (*2,3*).

Both BNT162b2 and mRNA-1273 are mRNA vaccines are developed with the spike (S) protein as the target antigen. The S protein of SARS-CoV-2 is a major surface protein that binds with high affinity to angiotensin-converting enzyme 2 (ACE2 receptor) on human cells (*4*). Therefore, S proteins, especially the S1 receptor-binding domain (RBD), has been considered as a major target for neutralizing antibodies (*5*). Previous studies have highlighted the efficacy of these two mRNA vaccines in preventing COVID-19 and reducing the risk of severe disease (*6*).

In Japan, vaccination on priority with the COVID-19 mRNA vaccine began in early February 2021 for approximately 4.8 million healthcare workers, and in early April 2021, for approximately 36 million elderly people. Factors affecting low antibody titers after COVID-19 mRNA vaccination have been studied, including age (*7*), sex (male) (*8*), central obesity, and hypertension (*9*). One of the serious concerns reported is that smokers have lower antibody titers than non-smokers (*9,10*). Previous studies have shown that

smoking shortens life expectancy, increases overall health care costs, and contributes to reduced productivity (*11*). These studies clearly emphasize the detrimental effects of smoking on human health. Furthermore, suppressed immune function due to prolonged exposure to nicotine from smoking has also been reported (*12-14*). However, in addition to nicotine, cigarette smoke contains a very large number of toxic components, such as tar and carbon monoxide. Therefore, the mechanisms responsible for smoking-induced immune modulation after COVID-19 mRNA vaccination is necessary to be studied. Furthermore, the effect of cigarette dependence on antibody titers remains unclear.

Therefore, we measured the level of serum anti-SARS-CoV-2 spike protein receptor-binding domain (S-RBD) immunoglobulin-G (IgG) by enzyme linked immunosorbent assay (ELISA) of 55 actively smoking Japanese social workers (firefighters, paramedics, and rescue workers) who had received two doses of the BNT162b2 vaccine. It is important to evaluate the serum anti-SARS-CoV-2 response of first responders who may be in contact with people infected with COVID-19 or with suspected individuals during a pandemic.

The Fagerstrom Test for Nicotine Dependence (FTND) (*15*) is one of the most widely used scales for assessing cigarette dependence. It is a self-reported test and has been used in the scientific validation of nicotine dependence and its relation to genetic predisposition (*16*) in patients with cancer (*17*). Therefore, we adopted the FTND as a measure to assess dependence on cigarettes.

Many previous studies have explained that cigarette dependence is closely related to nicotine, one of the components of cigarettes (*18,19*). In addition, the effects of nicotine on immune mechanisms have been demonstrated in previous studies (*20,21*). However, nicotine has a short half-life, making it difficult to be used as a marker of exposure to cigarette smoke. On the other hand, cotinine, a metabolite of nicotine, has a long half-life of approximately 10-20 hours (*22,23*) and has been used in several studies to assess smoking status (*24,25*). Therefore, serum cotinine levels were used as an objective index in the present study.

The objective of this study was to assess the correlation between cigarette dependence and anti-RBD IgG levels after COVID-19 mRNA vaccination in smokers. To the best of our knowledge, this is the first study to evaluate the effect of smokers' cigarette dependence on their antibody response to the COVID-19 vaccination. We believe that this study will add new insights into the association between smoking and antibody titers after COVID-19 mRNA vaccination.

2. Materials and Methods

The participants of this study were explained in detail about the purpose of the study, methods involved, sample collection processes, and the management of personal information in the study in advance, and written informed consent was obtained. The study was performed in accordance with the principles of the Declaration of Helsinki. This study was approved by the Chubu University Ethics Review Board (Approval No.: 20200042).

2.1. Volunteer donor sample

We used a cross-sectional study design. The participants were social workers (firefighters, paramedics, and rescue workers) working at five fire stations located in a single city in Japan. We recruited participants by distributing a request form for research cooperation to the managers of each station. Subsequently, 55 active smokers were enrolled in the study. The participants had completed their second dose of BNT162b2 vaccine in mid-May 2021. All participants had a three-week interval between the first and second vaccination. We conducted blood collection in mid-June, *i.e.*, about four weeks after most participants received their second dose. This period of blood collection was between the fourth and fifth wave of COVID-19 epidemic in Japan. We collected blood from the fingertips of the participants using safety lancets, paying close attention to hygiene and infection control. The blood samples were centrifuged at 3,000 rpm for 5 min after standing for 30 min after collection, and the serum was frozen and stored at -20°C.

2.2. Survey

Participants were requested to fill a questionnaire with questions on age, gender, smoking classification, medical history, and drug history.

2.3. FTND

FTND was used to assess cigarette dependence. The FTND is a scale with a minimum score of 0 and a maximum score of 10, with scores assigned based on six factors. Our analysis showed that the FTND had a Cronbach's coefficient alpha of 0.636, showing a high reliability when compared to findings of previous studies (*26,27*).

2.4. Measurement of anti-SARS-CoV-2 S-RBD protein IgG levels

Serum anti-RBD IgG levels were measured using ELISA. The Anti-SARS-CoV-2 S-RBD protein Human IgG Kit (Proteintech Group, Rosemont, IL, USA) was used to measure serum anti-RBD IgG levels according to the manufacturer's instructions. This ELISA kit utilizes indirect ELISA as the measurement principle.

2.5. Cotinine concentration

Serum cotinine concentrations were measured using the Cotinine ELISA Kit (Abnova Corporation, Neihu District, Taipei, Taiwan) according to the manufacturer's instructions. The Cotinine ELISA Kit produces a calibration curve that relates cotinine concentrations by solid phase competition. The microplate reader used to measure absorbance was a POWERSCAN HT (Bio Tec Instruments, Winooski, VT, USA).

2.6. Statistical analysis

Descriptive statistical data are presented as the median (interquartile range: IQR) depending on the distribution. The Kruskal-Wallis test was used to evaluate statistical differences between groups for FTND, serum anti-RBD IgG and serum cotinine concentrations. Spearman's rank correlation coefficients were calculated to assess bivariate correlations. Two-sided *p*-values < 0.05 were considered statistically significant. IBM SPSS Statistics version 27 (IBM Corp., Armonk, NY, USA) was used for statistical analysis.

3. Results

3.1. Study population

A breakdown of age, sex and smoking equipment used is given in Table 1. All participants were male. None of the participants reported the use of e-cigarettes. With respect to medical history, two (3.6%) participants had history of COVID-19, two (3.6%) had hypertension, one (1.8%) had cardiac disease, and one (1.8%) had hyperlipidemia. As for oral medication use, six (10.9%) participants took antiallergic drugs, one (1.8%) took hyperlipidemia medication, and one (1.8%) participant took antihypertensive drugs. Neither medical history nor oral medication use was employed as an explanatory variable during statistical analysis, because none of these factors were found to affect serum anti-RBD IgG levels, serum cotinine, and FTND, and because there was a large difference among the samples compared (Data not shown).

Table 1. Volunteer donor profile

	n	%
Age group		
20-29	13	23.6
30-39	29	52.8
40-49	9	16.4
50-59	2	3.6
60-69	2	3.6
Sex		
Male	55	100
Female	0	0
Smoking device		
Cigarette	14	25.5
Heat-not-burn tobacco	29	52.7
Combination of cigarette and Heat-not-burn tobacco	12	21.8

3.2. Anti-RBD protein IgG levels

The median level of anti-RBD IgG was 15.5 μg/mL (11.3-36.5 μg/mL). Anti-RBD IgG levels neither showed any significant difference between the age groups (p = 0.286) (Figure 1A) nor between the smoking devices (p = 0.278) (Figure 2A). Therefore, age and smoking device were not used as explanatory variables affecting serum antibody titers in this study.

3.3. FTND and serum cotinine concentration

The median FTND was 3.0 (2.0-5.0). Comparison of FTND by age group showed no significant differences (p = 0.144) (Figure 1B). Comparison of FTND by smoking device showed no significant difference (p = 0.078) (Figure 2B).

The median serum cotinine concentration was 60.4 ng/mL (44.1-150.0). There was a large variance in serum cotinine levels in this population, and individual differences were observed. Serum cotinine concentration did not show significant difference between the age groups (p = 0.284) (Figure 1C) as well as smoking devices (p = 0.868) (Figure 2C). Therefore, age and smoking device were not used as explanatory variables affecting FTND and serum cotinine concentration in this study.

3.4. Correlations between FTND, anti-RBD IgG, and serum cotinine levels

FTND showed a significant negative correlation with anti-RBD IgG levels (ρ = -0.426, p = 0.001) (Figure 3A). By contrast, FTND showed a significant positive correlation with serum cotinine concentration (ρ = 0.470, p = 0.000) (Figure 3B). Being consistent with these findings, there was a weak negative correlation between serum cotinine concentration and anti-RBD IgG levels, but the correlation was not statistically significant (ρ = -0.156, p = 0.256) (Figure 3C).

4. Discussion

We examined smokers in their 20s to 60s who were vaccinated with BNT162b2 vaccine in Japan. First, we found that serum anti-RBD IgG levels after BNT162b2 vaccination was negatively correlated with FTND. Smoking is not only a factor that increases the risk of severity of COVID-19 (*28*), but is also clearly detrimental to antibody production after COVID-19 mRNA vaccination (*9,10*). Additionally, in the present study we observed a decrease in antibody production after COVID-19 mRNA vaccination among smokers, especially in case of strong cigarette dependence.

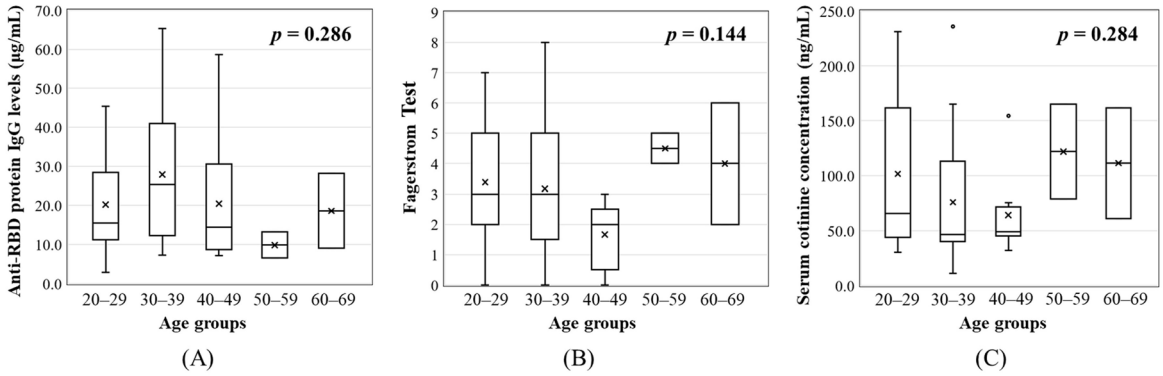

Figure 1. (A) Comparison of anti-severe acute respiratory syndrome coronavirus 2 (anti-SARS-CoV-2) spike protein receptor-binding domain (S-RBD) immunoglobulin-G (IgG) levels by age groups. (B) Comparison of the Fagerstrom Test by age groups. (C) Comparison of serum cotinine concentration by age groups. We compared anti-SARS-CoV-2 S-RBD protein IgG levels by age group showed no significant differences ($p = 0.286$) (Figure 1A). Comparison of the Fagerstrom Test by age group showed no significant differences ($p = 0.144$) (Figure 1B). Comparison of serum cotinine concentration by age group showed no significant difference ($p = 0.284$) (Figure 1C). All p values were derived from the Kruskal-Wallis test, $p < 0.05$ was considered statistically significant.

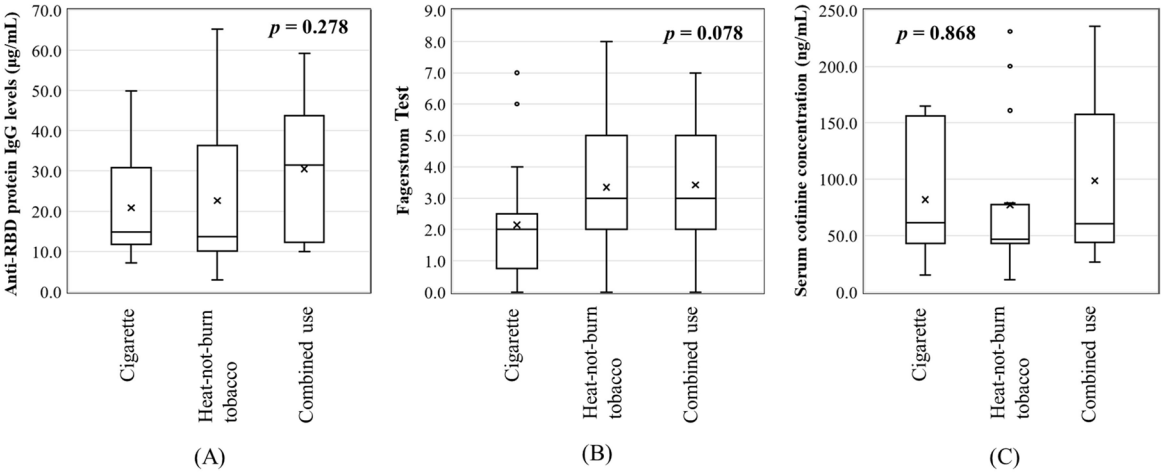

Figure 2. (A) Comparison of anti-severe acute respiratory syndrome coronavirus 2 (anti-SARS-CoV-2) spike protein receptor-binding domain (S-RBD) immunoglobulin-G (IgG) levels by smoking device. (B) Comparison of the Fagerstrom Test by smoking device. (C) Comparison of serum cotinine concentration by smoking device. We compared anti-SARS-CoV-2 S-RBD protein IgG levels by smoking device and found no significant difference ($p = 0.278$) (Figure 2A). Comparison of FTND by smoking device showed no significant difference ($p = 0.078$) (Figure 2B). Comparison of serum cotinine concentrations by smoking device category showed no significant difference ($p = 0.868$) (Figure 2C). All p values were derived from the Kruskal-Wallis test, $p < 0.05$ was considered statistically significant. FTND; Fagerstrom Test for Nicotine Dependence.

There was a significant positive correlation between FTND and serum cotinine levels, but there was no clear negative correlation between serum cotinine levels and serum anti-RBD IgG levels. These results indicate that the low antibody titers of the smokers after COVID-19 mRNA vaccination may be attributable not only to nicotine but also to other toxic substances which are contained in tobacco smoke.

To the best of our knowledge, no study has evaluated antibody titers after COVID-19 mRNA vaccination among smokers based on their level of cigarette dependence. We believe that this is the first study to demonstrate that there is a negative correlation between serum anti-RBD IgG levels and FTND after COVID-19 mRNA vaccination. This observation suggests that strong cigarette dependence may lead to reinforcement of smoking behavior, leading to a low antibody response after COVID-19 mRNA vaccination. However, the detailed mechanism is yet to be studied. COVID-19 mRNA vaccine has been reported to play an important role in reducing the risk and severity of the infection (*6*). Therefore, low antibody titers after COVID-19 mRNA vaccination in smokers with high cigarette dependence is an important concern during the pandemic. Another consideration is that smokers have unique characteristics that are influenced by their genetics, metabolism, physical and mental health status, as well as habits, personality, and lifestyle (*29*). Therefore, these characteristics may influence serum anti-RBD levels after COVID-19 mRNA vaccination. In addition, occupation of the participants and the type of mRNA vaccine administered were limited in

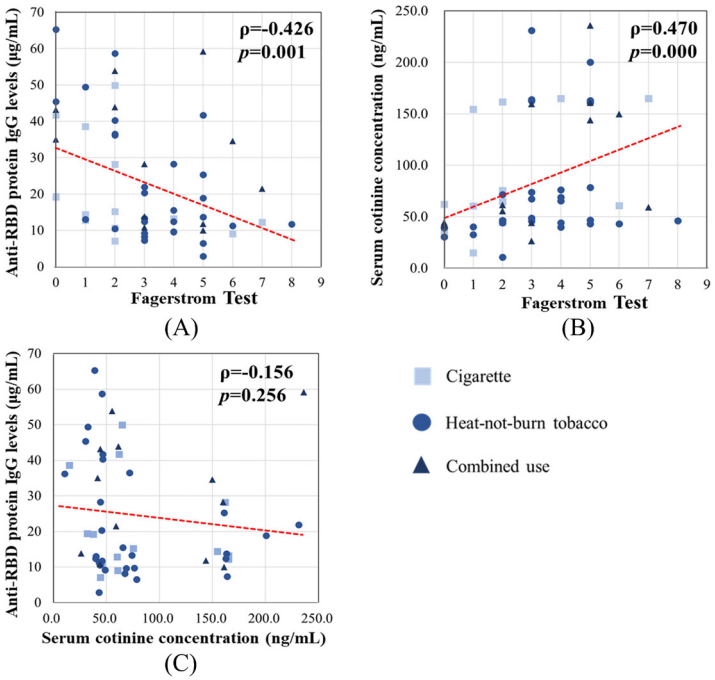

Figure 3. (A) Correlation between the Fagerstrom Test and anti-severe acute respiratory syndrome coronavirus 2 (anti-SARS-CoV-2) spike protein receptor-binding domain (S-RBD) immunoglobulin-G (IgG) level. (B) Correlation between Fagerstrom Test and serum cotinine concentration. (C) Correlation between serum cotinine concentration and anti-severe acute respiratory syndrome coronavirus 2 (anti-SARS-CoV-2) spike protein receptor-binding domain (S-RBD) immunoglobulin-G (IgG) level. FTND showed a significant negative correlation with anti-SARS-CoV-2 S-RBD protein IgG levels (ρ = -0.426, *p* = 0.001) (Figure 3A). Serum cotinine concentration showed a significant positive correlation with FTND (ρ = 0.470, *p* = 0.000) (Figure 3B). There was a negative correlation between serum cotinine concentration and anti-SARS-CoV-2 S-RBD protein IgG levels, but the correlation was not statistically significant (ρ = -0.156, *p* = 0.256) (Figure 3C). Values of ρ and *p* were derived from Spearman's test, $p<0.05$ was considered statistically significant. FTND: Fagerstrom Test for Nicotine Dependence.

this study. Furthermore, due to the nature of the study objectives, the establishment of a control group has challenging aspects. It is also necessary to take into account that the participants in this study were members of the fire department (firefighters, paramedics, and rescuers), who work hard around the clock and may have different circadian rhythms compared to other professionals. The results of the current study showed no significant differences in serum anti-RBD IgG levels and FTND between cigarette, heat-not-burn tobacco, and combined use groups. Therefore, we believe that concerns regarding low antibody titers after COVID-19 mRNA vaccination due to high cigarette dependence should be noted not only for cigarette smokers but also for heat-not-burn tobacco smokers and combination smokers. It is also possible that a history of COVID-19 could enhance antibody titers after vaccination (*30,31*). However, the two patients with a history of COVID-19 in this study were not treated as variables to be adjusted for, as there was no such trend.

Nicotine has effects on anxiety and stress reduction, pleasure, stimulation, and mood modulation (*32*), and may lead to cigarette dependence and reinforce smoking behavior. In the present study, there was a significant positive correlation between FTND and serum cotinine concentration, a metabolite of nicotine, which supports the results of previous studies, though the characteristics of the samples differed (*33*). We tested whether serum cotinine concentration also correlated negatively with serum anti-RBD IgG levels, as in the case of FTND. However, serum anti-RBD IgG levels after vaccination did not show a reliable negative correlation with serum cotinine levels.

The effects of nicotine on immune mechanisms have been shown in previous studies (*20,21*). In particular, it has been reported that prolonged exposure to nicotine through smoking can induce B cells that decrease antibody secretion, inhibit cell proliferation and development, and ultimately suppress normal immune function (*12,14*). However, cigarette smoke contains more than 4,500 components in its gaseous and particulate phases (*34*). Previous studies have shown that tobacco smoke affects a variety of host defense mechanisms (*20*), but due to the large number of toxic substances in tobacco, these mechanisms are not clearly understood (*35*). Our results suggest that when discussing the role of toxins in tobacco smoke in causing low antibody titers after COVID-19 mRNA vaccination, the risk factor may not be limited to nicotine. In addition, in our study, we did not find any significant difference in serum cotinine levels between groups according to the smoking device, suggesting that the type of smoking device may not have contributed to the present results. Japan has become an important market for companies

producing heat-not-burn tobacco (*36*), and 74.5% of the population we surveyed were either heat-not-burn tobacco smokers or combination smokers. However, none of the participants reported e-cigarette use; it was thus not included in the current study. Consistently, it is reported that nicotine concentrations in the smoke from cigarette paper and that from heat-not-burn tobacco (iQOS, distributed in Japan) were almost the same (*37*). In addition, there may be diurnal variation in blood cotinine concentration, and large individual differences were observed in this study. This may have been a factor that prevented a significant correlation between antibody titer and serum cotinine concentration.

In summary, our study represents the possibility that smokers who are heavily dependent on cigarettes may have particularly low antibody titers after COVID-19 mRNA vaccination. It also suggests that the factors influencing low antibody titers may not be limited to nicotine, but probably involve several other toxic substances. However, in the midst of the current COVID-19 pandemic, we do not necessarily argue that investigating those harmful substances is a top priority. This is because, although it can be assumed that there is some diurnal variation in blood cotinine levels, cigarette dependence itself is a persistent and constant factor for many individual smokers. Therefore, the key results we present highlight the possibility that the repeated smoking behavior, reinforced by a strong dependence on tobacco, may work against antibody titers after COVID-19 mRNA vaccination more adversely. This provides evidence that smokers with strong tobacco dependence may have insufficient protection against infection or severity of COVID-19 when vaccinated with COVID-19 mRNA vaccine.

There are several limitations to our study. First, this was a cross-sectional study and we were not able to identify any variation over time. Second, the assessment of SARS-CoV-2 antibody titers requires caution, as the assay used may yield multiple results (*38*). Third, because there are multiple measures to assess nicotine dependence other than the FTND used in this study, results may differ depending on the instrument used. Fourth, though previous studies have accounted for racial/ethnic differences in cotinine metabolism rates (*39,40*), the participants in our study were entirely of Japanese origin. Finally, though previous studies have shown that COVID-19 mRNA vaccines induce neutralizing antibody responses against three SARS-CoV-2 variants (*41*), the findings of the present study do not include the examination of neutralizing antibodies against SARS-CoV-2 variants.

5. Conclusions

In this study, we found that serum anti-RBD IgG levels were negatively correlated with FTND after BNT162b2 vaccination, while it showed no clear correlation with serum cotinine levels. These results suggest that repeated smoking behavior due to strong cigarette dependence may lead to low antibody titers after COVID-19 mRNA vaccination, and that the factors affecting low antibody titers after COVID-19 mRNA vaccination in cigarettes may not be limited to nicotine.

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References

1. Poon LLM, Peiris M. Emergence of a novel human coronavirus threatening human health. Nat Med. 2020; 26:317-319.
2. Polack FP, Thomas SJ, Kitchin N, *et al.* Safety and efficacy of the BNT162b2 mRNA Covid-19 vaccine. N Engl J Med. 2020; 383:2603-2615.
3. Walsh EE, Frenck RW, Falsey AR, *et al.* Safety and immunogenicity of two RNA-based Covid-19 vaccine candidates. N Engl J Med. 2020; 383:2439-2450.
4. Yan R, Zhang Y, Li Y, Xia L, Guo Y, Zhou Q. Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. Science. 2020; 367:1444-1448.
5. Brouwer PJM, Caniels TG, van der Straten K, *et al.* Potent neutralizing antibodies from COVID-19 patients define multiple targets of vulnerability. Science. 2020; 369:643-650.
6. Thompson MG, Burgess JL, Naleway AL, *et al.* Prevention and attenuation of Covid-19 with the BNT162b2 and mRNA-1273 vaccines. N Engl J Med. 2021; 385:320-329.
7. Müller L, Andrée M, Moskorz W, *et al.* Age-dependent immune response to the Biontech/Pfizer BNT162b2 coronavirus disease 2019 vaccination. Clin Infect Dis. 2021; 73:2065-2072.
8. Demonbreun AR, Sancilio A, Velez ME, Ryan DT, Pesce L, Saber R, Vaught LA, Reiser NL, Hsieh RR, D'Aquila RT, Mustanski B, McDade TW, McNally EM. COVID-19 mRNA vaccination generates greater immunoglobulin G levels in women compared to men. J Infect Dis. 2021; 224:793-797.
9. Watanabe M, Balena A, Tuccinardi D, *et al.* Central obesity, smoking habit, and hypertension are associated with lower antibody titres in response to COVID-19 mRNA vaccine. Diabetes Metab Res Rev. 2022; 38:e3465.
10. Nomura Y, Sawahata M, Nakamura Y, Kurihara M, Koike R, Katsube O, Hagiwara K, Niho S, Masuda N, Tanaka T, Sugiyama K. Age and smoking predict antibody titres at 3 months after the second dose of the BNT162b2 COVID-19 vaccine. Vaccines (Basel). 2021; 9:1042.
11. Das SK. Harmful health effects of cigarette smoking.

Mol Cell Biochem. 2003; 253:159-165.
12. Skok MV, Kalashnik EN, Koval LN, Tsetlin VI, Utkin YN, Changeux JP, Grailhe R. Functional nicotinic acetylcholine receptors are expressed in B lymphocyte-derived cell lines. Mol Pharmacol. 2003; 64:885-889.
13. Skok MV, Grailhe R, Agenes F, Changeux JP. The role of nicotinic receptors in B-lymphocyte development and activation. Life Sci. 2007; 80:2334-2336.
14. Skok M, Grailhe R, Changeux JP. Nicotinic receptors regulate B lymphocyte activation and immune response. Eur J Pharmacol. 2005; 517:246-251.
15. Heatherton TF, Kozlowski LT, Frecker RC, Fagerström KO. The Fagerström Test for Nicotine Dependence: a revision of the Fagerström Tolerance Questionnaire. Br J Addict. 1991; 86:1119-1127.
16. Nishizawa D, Kasai S, Hasegawa J, *et al.* Associations between the orexin (hypocretin) receptor 2 gene polymorphism Val308Ile and nicotine dependence in genome-wide and subsequent association studies. Mol Brain. 2015; 8:50.
17. Mikami I, Akechi T, Kugaya A, Okuyama T, Nakano T, Okamura H, Yamawaki S, Uchitomi Y. Screening for nicotine dependence among smoking-related cancer patients. Jpn J Cancer Res. 1999; 90:1071-1075.
18. Henningfield JE, Fant RV. Tobacco use as drug addiction: the scientific foundation. Nicotine Tob Res. 1999; 1 (Suppl 2):S31-35.
19. Breteler MH, Hilberink SR, Zeeman G, Lammers SM. Compulsive smoking: the development of a Rasch homogeneous scale of nicotine dependence. Addict Behav. 2004; 29:199-205.
20. Sopori M. Effects of cigarette smoke on the immune system. Nat Rev Immunol. 2002; 2:372-377.
21. Cloëz-Tayarani I, Changeux JP. Nicotine and serotonin in immune regulation and inflammatory processes: a perspective. J Leukoc Biol. 2007; 81:599-606.
22. Benowitz NL, Jacob P. Metabolism of nicotine to cotinine studied by a dual stable isotope method. Clin Pharmacol Ther. 1994; 56:483-493.
23. Benowitz NL. Cotinine as a biomarker of environmental tobacco smoke exposure. Epidemiol Rev. 1996; 18:188-204.
24. Duque A, Martínez PJ, Giraldo A, Gualtero DF, Ardila CM, Contreras A, Duarte S, Lafaurie GI. Accuracy of cotinine serum test to detect the smoking habit and its association with periodontal disease in a multicenter study. Med Oral Patol Oral Cir Bucal. 2017; 22:e425-e431.
25. Pontes CC, Chikte U, Kimmie-Dhansay F, Erasmus RT, Kengne AP, Matsha TE. Prevalence of oral mucosal lesions and relation to serum cotinine levels-findings from a cross-sectional study in South Africa. Int J Environ Res Public Health. 2020; 17:1065.
26. Huang CL, Lin HH, Wang HH. Psychometric evaluation of the Chinese version of the Fagerstrom Tolerance Questionnaire as a measure of cigarette dependence. J Adv Nurs. 2006; 55:596-603.
27. Etter JF. A comparison of the content-, construct- and predictive validity of the cigarette dependence scale and the Fagerström test for nicotine dependence. Drug Alcohol Depend. 2005; 77:259-268.
28. Liu W, Tao ZW, Wang L, Yuan ML, Liu K, Zhou L, Wei S, Deng Y, Liu J, Liu HG, Yang M, Hu Y. Analysis of factors associated with disease outcomes in hospitalized patients with 2019 novel coronavirus disease. Chin Med J (Engl). 2020; 133:1032-1038.
29. Prochaska JJ, Benowitz NL. Current advances in research in treatment and recovery: Nicotine addiction. Sci Adv. 2019; 5:eaay9763.
30. Krammer F, Srivastava K, Alshammary H, *et al.* Antibody responses in seropositive persons after a single dose of SARS-CoV-2 mRNA vaccine. N Engl J Med. 2021; 384:1372-1374.
31. Salvagno GL, Henry BM, di Piazza G, Pighi L, De Nitto S, Bragantini D, Gianfilippi GL, Lippi G. Anti-SARS-CoV-2 receptor-binding domain total antibodies response in seropositive and seronegative healthcare workers undergoing COVID-19 mRNA BNT162b2 vaccination. Diagnostics (Basel). 2021; 11:832.
32. Benowitz NL. Nicotine addiction. N Engl J Med. 2010; 362:2295-2303.
33. Ma E, Brown N, Alshaikh B, Slater D, Yusuf K. Comparison of the Fagerström test for cigarette dependence and the heaviness of smoking index in the second and third trimester of pregnancy. Nicotine Tob Res. 2017; 20:124-129.
34. Smith CJ, Hansch C. The relative toxicity of compounds in mainstream cigarette smoke condensate. Food Chem Toxicol. 2000; 38:637-646.
35. Stämpfli MR, Anderson GP. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. Nature Reviews Immunology. 2009; 9:377-384.
36. Tabuchi T, Kiyohara K, Hoshino T, Bekki K, Inaba Y, Kunugita N. Awareness and use of electronic cigarettes and heat-not-burn tobacco products in Japan. Addiction. 2016; 111:706-713.
37. Bekki K, Inaba Y, Uchiyama S, Kunugita N. Comparison of chemicals in mainstream smoke in heat-not-burn tobacco and combustion cigarettes. J UOEH. 2017; 39:201-207.
38. Merrill AE, Jackson JB, Ehlers A, Voss D, Krasowski MD. Head-to-head comparison of two SARS-CoV-2 serology assays. J Appl Lab Med. 2020; 5:1351-1357.
39. Benowitz NL, Bernert JT, Caraballo RS, Holiday DB, Wang J. Optimal serum cotinine levels for distinguishing cigarette smokers and nonsmokers within different racial/ethnic groups in the United States between 1999 and 2004. Am J Epidemiol. 2009; 169:236-248.
40. Signorello LB, Cai Q, Tarone RE, McLaughlin JK, Blot WJ. Racial differences in serum cotinine levels of smokers. Dis Markers. 2009; 27:187-192.
41. Jalkanen P, Kolehmainen P, Häkkinen HK, *et al.* COVID-19 mRNA vaccine induced antibody responses against three SARS-CoV-2 variants. Nat Commun. 2021; 12:3991.

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Original Article

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Dating the origin and dispersal of global hepatitis B virus genotype C in humans

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SUMMARY Hepatitis B virus genotype C (HBV/C) is one of the most prevalent HBV strains worldwide, especially in the Western Pacific and the South-East Asia. However, the origin and evolutionary timescale of HBV/C remains largely unresolved. We analyzed the evolutionary rate and molecular clock phylogeny of 101 full-genome HBV/C sequences sampled globally using a Bayesian Markov Chain Monte Carlo (MCMC) approach. We inferred the spatiotemporal dynamics of the HBV/C worldwide by the Bayesian Stochastic Search Variable Selection (BSSVS). We found that the estimated mean evolution rate of the HBV/C genotype full-genome was 4.32×10^{-5} subs/site/year (95% highest posterior density 3.02×10^{-6} - 8.97×10^{-5}). Phylogeographic reconstruction was able to identify a single location for the origin of the global HBV/C in Australia around A.D. 715. The subgenotype C4 diverged earliest and mainly circulated in Australia, C1 mainly in Southeast Asia, C2 mainly in East Asia and C3 in Remote Oceania. The effective number of HBV infection presented a rapid exponential increase between the 1760s and 1860s followed by a maintained high level until now. Our study, for the first time, provides an estimated timescale for the HBV/C epidemic, and brings new insight to the dispersal of HBV/C in humans globally. Based on the continuous presence of a highly effective viral population, this study provides further evidence of the challenge from a population-based molecular level to eliminate HBV by 2030, and calls for a concerted effort from policy makers, health providers, and society in the globalized world.

Keywords Hepatitis B virus, HBV genotype C, evolution, phylogenetic, phylogeographic

1. Introduction

Hepatitis B remains a major worldwide public health problem with approximately 257 million individuals infected with hepatitis B virus (HBV) and more than 94 million chronic hepatitis B (*1*). Current estimates place 29% of cirrhosis and 40% of hepatocellular carcinoma (HCC) can potentially be attributed to HBV infections (*2*). The HBV genome is a 3.2 kb partially double-stranded circular DNA composed of four open reading frames encoding for seven proteins. The DNA backbone is characterized by high variability and diversity due to the self-replication strategy of HBV, selection pressure imposed by the host immune system and other exogenous factors. So far, eight genotypes of HBV (A-H) have been identified defined by a diversity of greater than 8% genetic differences in the complete genome sequence (*3*).

Different genotypes have a clear geographical distribution and many risks associated with disease progression resulting in cirrhosis and HCC. HBV genotype C (HBV/C) is the major genotype circulating in Asia and the Western coast of the USA, which account for a large number of the infections worldwide (*4*). In detail, subgenotype C1 is the most common in Southeast Asia and Southern China; C2 is dominant in East Asia (South Korea and Japan) and the northern part of China; C3 persists in Oceania; and C4 is abundant in the Aborigines in Australia. Compared with genotype A and B, chronic infections with genotype C more commonly result in advanced liver diseases with an increased rate of progression to HCC (*5,6*). Additionally, several studies have suggested that disease outcomes are related to some genetic variants. A recent community-based study in

mainland China confirmed that the frequency of HCC-related mutations in HBV/C was significantly higher than that reported in genotype B cases (*7*).

A myriad of evidence has identified HBV as an ancient virus (*8,9*). Investigating the origin of HBV is crucial because it provides a framework for studying the disease burden, and subsequently an understanding of the evolution of HBV pathogenicity with respect to changes in human population size and life expectancy. Especially when considering the risk of disease progression associated with HBV/C strains, it is vital to understand the molecular evolution and epidemiological history of HBV/C genotype. However, to the best of our knowledge, only a few studies explored the origin and dissemination of HBV/C genotype in some countries (Japan) (*10*) or region (east Asia) (*11*). Based on the hypothesis of coincidence between HBV and human migrations, representative sequences from the whole world will be included in the model for analysis of the origin and dissemination of the HBV/C genotype.

Therefore, based on high-resolution phylogenetic and phylodynamic approaches, the study presented here is aimed at investigating the origin and evolution rates of HBV/C genotype, and to reconstruct its spatial and temporal global dynamics, particular attention focused on the study of subgenotypes C1-C4.

2. Materials and Methods

2.1. Sequence querying strategy and selection

HBV/C whole-genome sequences were retrieved from all uploads to the GenBank database (*https://www.ncbi.nlm.nih.gov*) up to the date May 24, 2019. Full-genome sequences were downloaded in "gb" format including the information GenBank accession number, nucleotide sequence, sequence release year, sampling time and area, and sequence length. Full-genome sequences were included with known sampling time and country. The following sequences were excluded: (1) non-human HBV sequences; (2) expression vector sequences; (3) sequences of patients co-infected with HBV/human immunodeficiency virus; (4) sequences with nucleotide length less than 3215 bp; (5) sequences containing illegal characters (*i.e.* characters other than A, T, C and G); (6) recombination sequences. After excluding ineligible sequences, one was chosen to represent the remaining sequences with similarity over 97 percentage, identical isolation country and year.

2.2. Sequence alignment, genotype, genetic distance and recombination

Nucleotide sequences were aligned using BioEdit software v7.0.5.3. Phylogenetic trees were constructed with MEGA v6.0, using the neighbor-joining method after estimation of genetic distance employing the Kimura two-parameter method. A bootstrapping test was performed with 1,000 duplicates, and the transition/transversion rate was set at 2.0. The online tool jumping profile Hidden Markov Model (jpHMM) (*http://jphmm.gobics.de/submission_hbv*), which specializes in detecting recombination events in the HBV genome, was used to analyze the selected sequences and confirm the genotyping results. We also used SimPlot to validated the recombination repeatedly.

2.3. Phylogenetic analysis

Bayesian Markov Chain Monte Carlo (MCMC) method was used to analyze the evolution rate and molecular clock phylogeny of global HBV/C strains with the BEAST software package version 1.7.5. The time of the most recent common ancestor (tMRCA) with 95% highest posterior density (HPD) of global HBV/C was estimated. The calibration point was the year that each strain was isolated. We used the general time reversible nucleotide substitution model with gamma-distributed rates of variable among sites, which were identified as the best fitting model by jModelTest v2.1.7 on the basis of Akaike Information Criterion. Multiple combinations of molecular clock and coalescent models were explored to select the best fitting model. Finally, runs were performed using the constant size, under the strict clock and Bayesian Skyline Plot molecular clock model. Bayesian MCMC analyses were run with a chain of 60 million steps and sampled every 1,000 steps. Convergence of parameters was identified by TRACER v1.5 with the effective sample size exceeding 200. The Maximum Clade Credibility (MCC) tree was calculated with TreeAnnotator with a burn-in period of 6,000 and then visualized in FigTree v1.4.2.

2.4. Phylogeographic analysis

In order to infer the spatiotemporal dynamics of HBV/C worldwide, the Bayesian Stochastic Search Variable Selection (BSSVS) was used to provide evidence for statistically supported diffusion between state variables under BEAST v1.7.5. The results of BSSVS were summarized using SPREAD v1.0.6, a keyhole markup language (KML) file was generated to identify the major routes of geographic diffusion. Bayes factor (BF) test was used to select the most probable diffusion process. To visualize the geographic dispersal of HBV, the KML file was imported to Google™ Earth to produce a graphical animation of the estimated spatiotemporal pathways of global HBV/C.

3. Result

3.1. Characteristics of included sequences

A total of 101 HBV/C full-genome sequences from

23 countries were included in this analysis (Figures S1 and S2, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=96*). The span of origin time of HBV/C sequences was 54 years with the earliest isolated from India in 1963 and the latest from Bangladesh in November 12, 2017. The highest proportion of isolated sequences was observed in 2013 (12.87%), followed by 2012 (9.90%). The included sequences were isolated from Asia (74.26%), Oceania (18.81%), America (3.96%), Europe (1.98%), and Africa (0.99%). In Asia, the highest proportion of isolated sequences was observed in China (16.83%), followed by Malaysia (12.87%); while in Oceania, the highest proportion of isolated sequences was observed in Australia (7.92%), followed by Papua New Guinea (3.96%). The spatial and temporal distribution of included sequences is summarized in Table 1.

3.2. Estimated evolution rates and tMRCA

The mean evolution rate of HBV/C full-genome sequences was evaluated using 101 isolates. After comparing the strict and relaxed clock models by BF test, the strict clock model was the best fit to the data (2lnBF = 466.45). Under the strict clock model, the estimated mean evolution rate of the HBV/C genotype full-genome was 4.32×10^{-5} subs/site/year (95% HPD 3.02×10^{-6} - 8.97×10^{-5}). In addition, we estimated the tMRCA of all the internal nodes of the MCC tree. In general, the estimated mean tMRCA of the tree root was 1,302 years ago (95% HPD 328-5139 years), which corresponds to the origin date of HBV/C genotype back to A.D. 715 (credibility interval between B.C. 3122-A.D. 1689). In particular, HBV-C4 was the first subgenotype diverging from the root in A.D. 1084 (B.C. 1646-A.D. 1794), followed by C3 with an origin date of A.D. 1211 (B.C. 1079, A.D. 1804). While the origin dates of C1 and C2 were more recent: A.D. 1268 (B.C. 913, A.D. 1820) for C1 and A.D. 1243 (B.C. 996, A.D. 1821) for C2, respectively (Table 2).

3.3. Time-scaled phylogeny and phylogeographic analysis

Phylogenetic analysis suggested that the origin of the global HBV/C genotype may likely have been in Australia. Results of the MCC tree (Figure 1) constructed using Tree Annotator revealed that the global HBV/C genotype segregated into two groups in the early stage of divergence. Lineage 1 consisted of HBV subgenotype C4 and circulated in Australia. Lineage 2 continued to diverge and finally segregated into four groups. Group 1 consisted of HBV subgenotype C1, which was mainly endemic in Southeast Asia, such as Malaysia and Thailand. Group 2 consisted of HBV subgenotype C2, which was mainly endemic in East Asia, such as China, Japan, and Korean. Group 3 consisted of HBV subgenotype C3, which mainly circulated in Oceania including Australia, New Zealand, and Tonga. Group 4 consisted of HBV subgenotype C10 circulating in Indonesia and Papua New Guinea.

The geographic dispersal of the global HBV/C genotype was conducted with the full-genome sequences and is shown in Figure 2, Figure S3 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=96*) and Figure S4 (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=96*). Phylogeographic reconstruction was able to identify a single location for the origin of the global HBV/C in Australia around 715. We discovered three main dissemination routes. Route 1 the virus crossed the Torres Strait and arrived in Papua New Guina in 992, and then spread to China in 1159, proceeding to Japan in 1620 and to Korean in 1733. Route 2 the virus crossed the Pacific and arrived in Fiji in 1550, and then spread from Fiji to New Zealand in 1749, and to Tonga in 1931. For route 3 the virus arrived in Malaysia in 1421 from China, and developed a new spreading epicenter in Malaysia, which spread to Thailand in 1615, on to Vietnam in 1641, continuing to Bangladesh in 1829, and finally to Taiwan, China in 1904.

3.4. Population dynamic analysis

The strict clock model and Bayesian skyline plot analysis was performed to reconstruct the evolutionary epidemiology of HBV/C based on the full-genome (Figure 3). Between 1642 and the 1730s, the effective number of new HBV infections remained consistent, then increased slowly until the 1760s, a rapid and exponential increase continued until the 1860s, followed by slow growth until the 1960s and maintained a plateau into the 1980s, finally decreasing gradually since 1992.

4. Discussion

The transmission of hepatitis B has a very long history and remains a major public health concern worldwide. In this study, we performed a complete and comprehensive analysis into the possible origin, temporal and spatial dynamics of global HBV/C based on full-genome sequences obtained from the GenBank database. The findings revealed that (*1*) the mean evolution rate of HBV/C full-genome was 4.32×10^{-5} subs/site/year (95% HPD 3.02×10^{-6} - 8.97×10^{-5}); (*2*) The global HBV/C may have derived from Australia around A.D. 715 and finally segregated into five lineages corresponding to the five subgenotypes; (*3*) The effective number of HBV infections presented a rapid and exponential increase during 1760s and 1860s, and maintained a high level of transmission up to now.

Due to the lack of proof-reading activity of the reverse transcriptase of HBV, nucleotide misincorporation occurs during genome replication,

Table 1. The spatial and temporal distribution of included global full HBV/C sequences

Year	ARG	AUS	BD	BEL	CHN	CHN-HK	CHN-TW	FJ	INA	IND	JPN	KOR	MAS	MYA	NZL	PAN	PHI	PNG	RSA	TG	THA	USA	VN	Total
1963	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1
1984	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1
1991	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1
1992	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	1
1993	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1
1994	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1
1996	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	1
1997	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	2
1998	-	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	4
1999	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	1
2000	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
2001	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	2
2002	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	2
2003	1	-	-	-	-	-	-	-	-	-	-	1	-	-	-	2	-	-	-	-	1	-	-	5
2004	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	-	-	1	-	3
2005	-	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-	-	2	-	-	-	-	-	4
2006	-	-	-	1	1	-	-	-	1	-	1	1	2	-	-	-	-	-	-	-	-	-	-	7
2007	-	-	-	-	-	-	-	-	2	-	1	1	1	-	-	-	-	-	1	-	-	-	-	6
2008	-	-	-	-	3	-	-	-	-	-	-	1	1	-	-	-	-	-	-	-	-	-	-	5
2009	-	-	-	-	1	-	-	-	-	-	1	-	3	-	-	-	-	-	-	-	2	-	-	7
2010	-	1	-	-	1	-	-	-	-	-	1	1	1	-	-	-	-	-	-	-	-	-	-	5
2011	-	1	-	-	1	-	1	-	-	-	1	-	-	-	-	-	-	-	-	-	1	-	-	5
2012	-	1	-	-	4	1	-	-	-	-	1	-	2	-	-	-	-	-	-	-	-	-	1	10
2013	-	1	-	-	1	1	1	-	1	1	1	1	3	-	-	-	1	-	-	-	1	-	-	13
2014	-	1	-	-	1	-	-	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-	1	5
2015	-	-	1	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	2
2016	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	2
2017	-	-	1	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	3
Total	1	8	2	2	17	2	4	2	4	2	10	6	13	1	3	2	1	4	1	2	10	1	3	101

Number in the table means the counts of sequences sampled in corresponding country and year. - means there are no sequences sampled in corresponding country and year. *Abbreviations*: ARG, Argentina; AUS, Australia; BD, Bangladesh; BEL, Belgium; CHN, China; CHN-HK, Hongkong, China; CHN-TW, Taiwan, China; FJ, Fiji; INA, Indonesia; IND, India; JPN, Japan; KOR, Korea; MAS, Malaysia; MYA, Myanmar; NZL, New Zealand; PAN, Panama; PHI, Philippines; PNG, Papua New Guinea; RSA, South Africa; TG, Tonga; THA, Thailand; USA, United States; VN, Viet Nam.

which leads to an inconsistent substitution rate. This study found that the mean evolution rate of HBV/C full-genome was 4.32×10^{-5} subs/site/year (95% HPD 3.02×10^{-6} - 8.97×10^{-5}), which was consistent with the results reported in an analysis based on the global HBV genotype A sequences (3.0×10^{-5} subs/site/year) (*12*), and higher than the result in a study based on the HBV/C sequences from East Asia (5.6×10^{-4} subs/site/year) (*11*). It is well accepted that the estimated time course of the evolution of HBV mainly depends on the nucleotide substitution rate. Furthermore, the substitution rate is dependent on the calibration approach. In particular, the estimated evolution rate may be faster when recent calibration points are used over a period of a few years (*13*), and in contrast, slower when based on more remote points (such as fossil or primate data) (*8,14,15*). This could also account for the difference in the estimated evolution rates between our own study and the results from the East Asia study. Due to calibration errors, model mis-specifications or mutational saturation, especially the fact that not all of the current circulating mutants will maintain fixed in the population, the evolutionary rates were presumed to change over time (*16*). Theoretically, it is appropriate to use a short-term evolutionary rate to calculate the time-scale of recent events (such as the intra-genotype evolution) and similarly appropriate to use a long-term substitution rate to study events that are distant in time (such as the origin, co-divergence, and cross-species transmission between human and primates). Based on this principle, we focused our studies more on the interaction of HBV/C evolution and population dynamics using sequences from humans, and their public health consequences especially in the modern history of the world.

Table 2. Estimates of the origin and evolutionary rate of global full HBV/C sequences

Genotype	Mean value	95% HPD
global C	A.D. 715	B.C. 3122, A.D. 1689
C1	A.D. 1268	B.C. 913, A.D. 1820
C2	A.D. 1243	B.C. 996, A.D. 1821
C3	A.D. 1211	B.C. 1079, A.D. 1804
C4	A.D. 1084	B.C. 1646, A.D. 1794

Although there have been many disputes on the origin of HBV infections in humans, several phylogenetic analyses provided the probable evidence that HBV is an ancient virus based on sequences from human and primates (originated 33,600-34,100 years ago) (*8,17*). Based on the representative sample of currently available sequences from human infections, we inferred that the timing of global HBV/C in humans was estimated to originate 1,302 years ago. The estimated origin time of HBV/C in our study was much earlier than the results in the study based on isolates from East Asia (*11*), which was consistent with the differences in estimated evolutionary rate. In the study reported here we found that the global HBV/C originated in Australia, the C4 subgenotype branched from the rest of genotypes first and circulated in Australia, and the C3 subgenotype spread to Remote Oceania. The phylogeographic scenarios of C3 and C4 were highly consistent with the results from Paraskevis D, *et al.* (*8*). We also found that the virus expanded from Australia

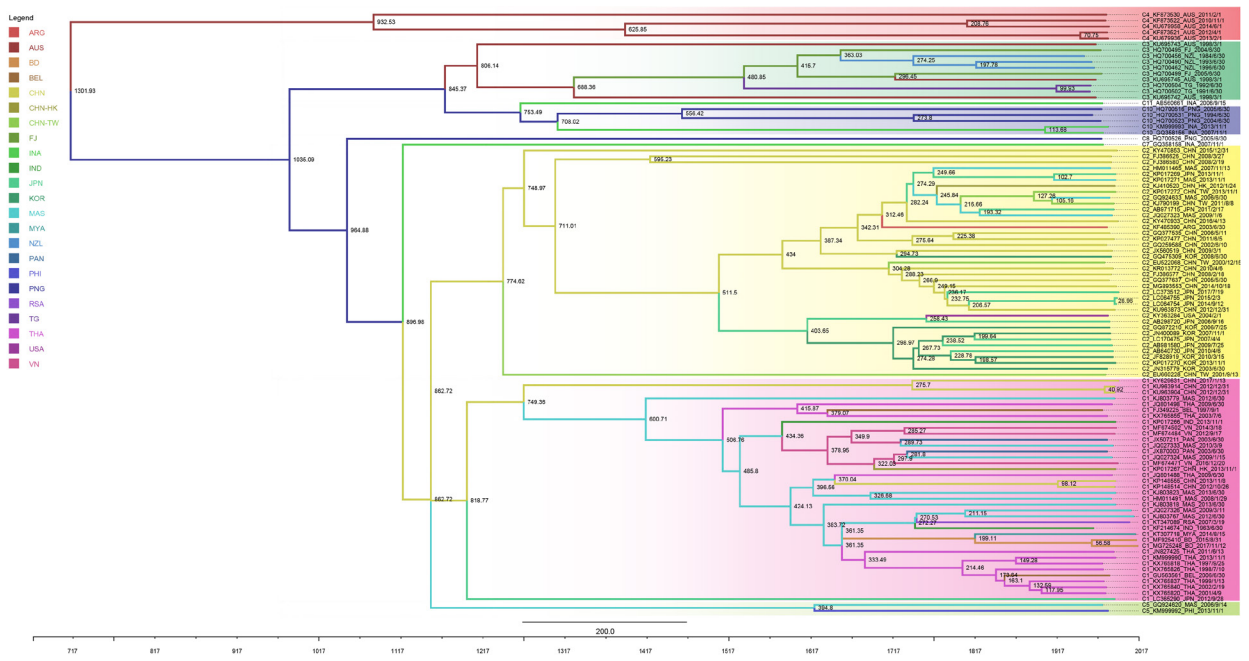

Figure 1. MCC tree of the complete HBV genotype C sequences throughout the world visualized in FigTree. Dated virus phylogeny displaying subgenotypes within genotype C. The colors of the branches corresponded to their probable geographic location. The intervals of branch reflect the 95% HPD intervals for the branch height. Numbers on the horizontal axis correspond to years before present. Abbreviations of geographic location are shown as described in the note of Table 1.

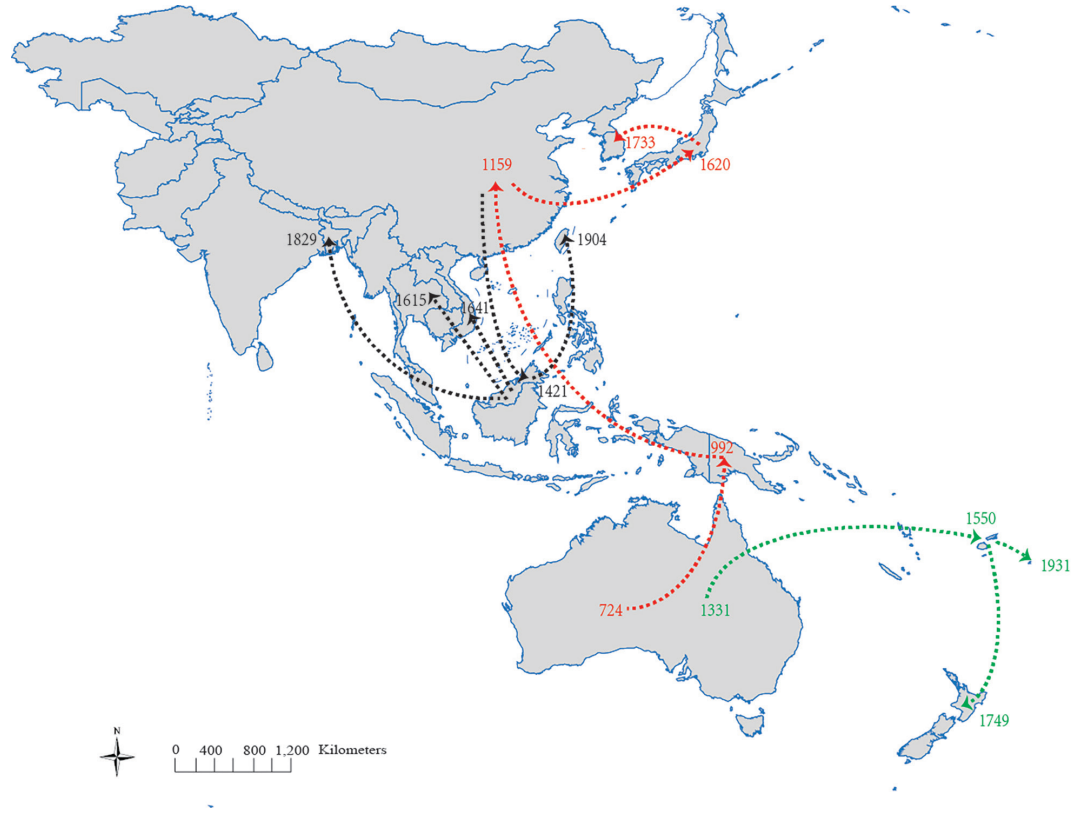

Figure 2. Geographic distribution and inferred dynamics of global HBV genotype C. The map is reconstructed using the ArcGIS (*http://www.esri.com/*), and is identical to the original image created by the SPREAD and Google™ Earth. Arrows indicate the inferred routes of spread of HBV genotype C. The number next to the arrows represent the time when the virus arrived. Main dissemination routes are colored according to their geographic location and time-scale.

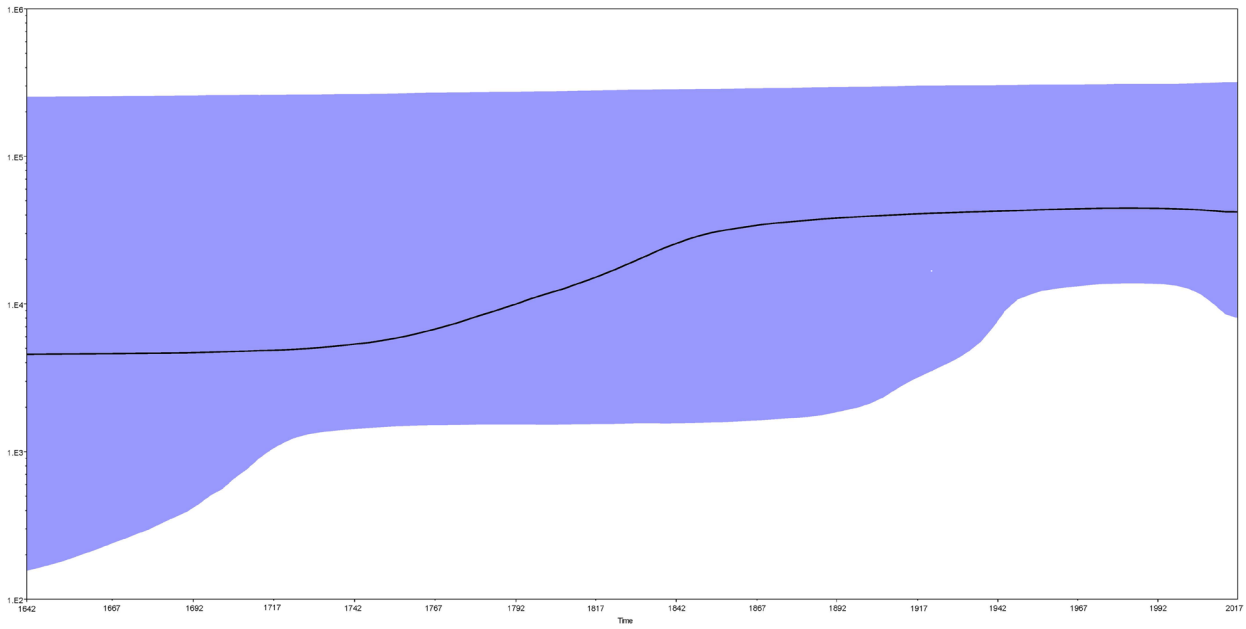

Figure 3. Bayesian skyline plot obtained by analyzing the 101 global HBV/C genotype sequences through time. The solid line stands for the median and shaded areas correspond to the 95% HPD intervals. The effective number of infections is reported on the Y-axis. Time (in years before 2017) is reported on the X-axis.

around A.D. 724, *via* Papua New Guinea, and arrived in China around A.D. 1159, and then spread to Japan in A.D. 1620 and to Korea in A.D. 1733. The detection and molecular characterization of HBV DNA isolated from a Korean Child naturally mummified in the 16th Century A.D. (*9*), together with frequent trade contacts, diplomatic activities, and wars since Tang Dynasty (7th Century A.D) (*18,19*) supplied possible evidence

to support the credibility of our results. In Southeast Asia, Malaysia became a new disseminating center from which the virus spread to Thailand, Vietnam, and Bangladesh respectively between 17th and 19th century, which could be explained by trade contacts, wars, large scale of population migration, and colonization during the Melaka Sultanate (A.D. 1402-1511) (*20,21*), and the colonial era (A.D. 1511-1914) (*22,23*).

The demographic history of the effective viral population is another important factor that influenced the rate of virus evolution, which comprehensively reflects the scale and dynamics of the host population and the epidemiology/ecological characteristics of infection. In small, stable and isolated populations, the virus may transmit widely and become hyper-endemic. In this setting, the evolutionary rate is usually low, and the dominant route of transmission is vertical. Infected populations frequently presented a high proportion of immunotolerance. On the contrary, HBV when transmitted into a large, highly mobile and susceptible population, will lead to higher evolutionary rates and patients present with a high proportion of immunocompetence. In this study, the Bayesian Skygrid plot indicated that the effective number of HBV infections present a rapid and exponential increase between the 1760s and 1860s, which corresponded to the comprehensive dissemination of HBV in Remote Oceania, Southeast Asia and East Asia as shown by the phylogeographic analysis. Interestingly, the effective viral population began to slowly decline since 1992, possibly due to increasing immunization coverage, the scale-up of antiviral treatment and the prevention of mother to child transmission. However, the continuous high level of the effective viral population remains a large obstacle to end the HBV epidemic.

There are several implications based on this study: (*1*) To the best of our knowledge, this is the first comprehensive analysis of the phylogeographic and phylodynamic spread of the global HBV/C genotype in humans based on a representative sample of current available HBV/C genotype complete sequences. (*2*) The results of this study supplied further evidence that the HBV/C genotype is an ancient virus, chronologically diverged and disseminated companion of the population dynamics, mainly in Oceania, Southeast Asia and East Asia. Especially, the results exhibited an ongoing and concerning condition of higher effective viral population present in the Asian-Pacific region based on molecular evolution. (*3*) In 2017, the World Health Organization (WHO) published a report defining the criteria for hepatitis elimination and outlining a strategy to achieve this goal by 2030 (*24*). However, the latest global hepatitis report revealed that the Western Pacific Region had the highest prevalence of HBV infection and the largest infected population, and the South-East Asia Region has the third highest prevalence of HBV infection. HBV/C is the dominant genotype in the two regions. Thus, the Western Pacific and the South-East Asia are key regions in achieving the goal of HBV elimination by 2030 due to the heavy burden of HBV infection, which was especially caused by HBV/C genotype. This study also provided molecular evidence of the high effective viral population in these areas. Together with the epidemiologic and molecular data, it calls for a concerted effort from policy makers, health providers, and society in these regions to assist (*25*) with the HBV crisis.

There are some limitations in this study. First, HBV evolution rate, time-scaled phylogeny and phylogeographic analysis are all influenced by the number of included sequences and their isolation location and time. However, not all the countries uploaded HBV/C sequences and we also could not obtain earlier sequences restrained by the development of molecular biological technique. Based on 101 sequences from 23 countries and a timespan of 54 years, the study may underestimate the origin time of HBV/C and provide a probable scenario of molecular evolution. Second, similar to all other studies, we also presumed the evolutionary rate did not change over time, which could influence the precision of our estimation. Thus, further studies are needed to confirm the time-dependency characteristics of the evolutionary rate. Third, recombination revealed the interaction of different virus genotypes and could at times bias evolutionary relationships when constructing phylogenetic trees. Thus, recombination sequences were commonly excluded. However, recombination of different genotypes could also influence the molecular evolution of HBV. Thus, additional innovative analysis methods are also needed to address this issue.

Our study, for the first time, provides an estimated timescale for the HBV/C epidemic, and brings new insight to the dispersal of HBV/C in humans globally. Our study also added additional evidence for the hypothesis of HBV/C divergence and co-expanding with human populations. Based on the continuous condition of high effective viral population, this study further demonstrated a challenge from a population-based molecular level to eliminate HBV by 2030, and calls for a concerted effort from policy makers, health providers, and societies in the globalized world.

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References

1. Polaris Observatory Collaborators. Global prevalence, treatment, and prevention of hepatitis B virus infection in

2016: A modelling study. Lancet Gastroenterol Hepatol. 2018; 3:383-403.
2. GBD 2017 Causes of Death Collaborators. Global, regional, and national age-sex-specific mortality for 282 causes of death in 195 countries and territories, 1980-2017: a systematic analysis for the Global Burden of Disease Study 2017. Lancet (London, England). 2018; 392:1736-1788.
3. Pourkarim MR, Amini-Bavil-Olyaee S, Kurbanov F, Van Ranst M, Tacke F. Molecular identification of hepatitis B virus genotypes/subgenotypes: revised classification hurdles and updated resolutions. World J Gastroenterol. 2014; 20:7152-7168.
4. Kramvis A, Kew M, François G. Hepatitis B virus genotypes. Vaccine. 2005; 23:2409-2423.
5. Toan NL, Song le H, Kremsner PG, Duy DN, Binh VQ, Koeberlein B, Kaiser S, Kandolf R, Torresi J, Bock CT. Impact of the hepatitis B virus genotype and genotype mixtures on the course of liver disease in Vietnam. Hepatology. 2006; 43:1375-1384.
6. Lin CL, Kao JH. The clinical implications of hepatitis B virus genotype: Recent advances. J Gastroenterol Hepatol. 2011; 26 Suppl 1:123-130.
7. Yin J, Zhang H, He Y, Xie J, Liu S, Chang W, Tan X, Gu C, Lu W, Wang H, Bi S, Cui F, Liang X, Schaefer S, Cao G. Distribution and hepatocellular carcinoma-related viral properties of hepatitis B virus genotypes in Mainland China: a community-based study. Cancer Epidemiol Biomarkers Prev. 2010; 19:777-786.
8. Paraskevis D, Magiorkinis G, Magiorkinis E, Ho SY, Belshaw R, Allain JP, Hatzakis A. Dating the origin and dispersal of hepatitis B virus infection in humans and primates. Hepatology. 2013; 57:908-916.
9. Kahila Bar-Gal G, Kim MJ, Klein A, Shin DH, Oh CS, Kim JW, Kim TH, Kim SB, Grant PR. Tracing hepatitis B virus to the 16th century in a Korean mummy. Hepatology. 2012; 56:1671-1680.
10. Lin SY, Toyoda H, Kumada T, Liu HF. Molecular evolution and phylodynamics of acute hepatitis B virus in Japan. PloS One. 2016; 11:e0157103.
11. Lin SYC, Magalis BR, Salemi M, Liu HF. Origin and dissemination of hepatitis B virus genotype C in East Asia revealed by phylodynamic analysis and historical correlates. J Viral Hepat. 2019; 26:145-154.
12. Zehender G, Svicher V, Gabanelli E, *et al*. Reliable timescale inference of HBV genotype A origin and phylodynamics. Infect Genet Evol. 2015; 32:361-369.
13. Zehender G, De Maddalena C, Giambelli C, Milazzo L, Schiavini M, Bruno R, Tanzi E, Galli M. Different evolutionary rates and epidemic growth of hepatitis B virus genotypes A and D. Virology. 2008; 380:84-90.
14. Godoy BA, Alvarado-Mora MV, Gomes-Gouvêa MS, Pinho JR, Fagundes N, Jr. Origin of HBV and its arrival in the Americas--the importance of natural selection on time estimates. Antivir Ther. 2013; 18:505-512.
15. Ho SY, Lanfear R, Bromham L, Phillips MJ, Soubrier J, Rodrigo AG, Cooper A. Time-dependent rates of molecular evolution. Mol Ecol. 2011; 20:3087-3101.
16. Ho SY, Shapiro B, Phillips MJ, Cooper A, Drummond AJ. Evidence for time dependency of molecular rate estimates. Syst Biol. 2007; 56:515-522.
17. Paraskevis D, Angelis K, Magiorkinis G, Kostaki E, Ho SY, Hatzakis A. Dating the origin of hepatitis B virus reveals higher substitution rate and adaptation on the branch leading to F/H genotypes. Mol Phylogenet Evol. 2015; 93:44-54.
18. Wei CC. China's cultural routes to korean peninsular and japan and its cultural exchange in the han dynasty and tang dynasty. Jilin Da Xue She Hui Ke Xue Xue Bao. 2008; 1:89-95,159-160. (in Chinese)
19. Atwell WS. Some observations on the "seventeenth-century crisis" in china and japan. J Asian Stud. 1986; 45:223-244.
20. Wilkinson RJ. The Malacca Sultanate. Journal of the Malayan Branch of the Royal Asiatic Society. 1935; 13:22-67.
21. Borschberg P. The Melaka Empire. c.1400-1528. Brill, Leiden, Netherlands, 2019.
22. Andaya, Leonard Y. A history of Malaysia. St Martin's Press. 1982.
23. Hack K, Rettig T. Colonial armies in Southeast Asia. Routledge, London, UK, 2006.
24. World Health Organization. Combating hepatitis B and C to reach elimination by 2030, Advocacy brief. Geneva. *http://apps.who.int/iris/bitstream/10665/206453/1/WHO_HIV_2016.04_eng.pdf; ua=1* (Accessed 23 Oct 2019).
25. World Health Organization. Global hepatitis report, Geneva. 2017.

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Communication

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Suppressing leukocyte Kv1.3-channels by commonly used drugs: A novel therapeutic target for schizophrenia?

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SUMMARY Recent studies revealed the involvement of "chronic inflammation" in the pathogenesis of schizophrenia. In schizophrenia and some neurodegenerative disorders that are caused by inflammation, T-lymphocytes and macrophages were hyperactivated or proliferated in the central nervous system, being accompanied by the overexpression of delayed rectifier K^+-channels (Kv1.3) within the cells. In our previous basic studies, in addition to nonsteroidal anti-inflammatory drugs (NSAIDs) and statins, antibiotics (clarithromycin, chloroquine), anti-hypertensive drugs (nifedipine, benidipine, diltiazem, verapamil) and anti-allergic drugs (cetirizine, fexofenadine, azelastine, terfenadine) strongly suppressed the Kv1.3-channel activity and pro-inflammatory cytokine production from lymphocytes. Given such pharmacological properties of these commonly used drugs, they may be useful in the treatment of schizophrenia, in which the enhanced cellular immunity and the subsequent release of excessive cytokines are responsible for the pathogenesis.

Keywords Schizophrenia, chronic inflammation, lymphocyte, Kv1.3-channels, nonsteroidal anti-inflammatory drugs (NSAIDs), statins

Schizophrenia is a chronic brain disorder which affects approximately 0.7 to 1.1% of world population (*1*). It is characterized by continuous or relapsing episodes of psychosis, presenting with symptoms such as hallucinations, delusions, paranoia and disorganized thinking. Besides the contribution of genetic or environmental factors, studies revealed that abnormalities of neurotransmitters, such as dopamine and glutamate, play major roles in the pathogenesis of schizophrenia (*1*). Therefore, targeting hyperactivated dopamine system, antipsychotics have commonly been used in the treatment of schizophrenia, since they persistently block postsynaptic dopamine 2 (D2) receptors (*1*). However, both typical and atypical antipsychotics can cause serious side effects, including movement disorders, metabolic syndrome, cardiac arrhythmia and sexual dysfunction (*2*). Such side effects frequently cause the drug discontinuation in the schizophrenia patients and the subsequent relapse of psychotic symptoms.

Recent advances in molecular pathology have additionally revealed the involvement of "chronic inflammation" in the pathogenesis of schizophrenia (*3,4*). In patients with schizophrenia, besides the inflammatory markers, such as serum C-reactive protein (CRP) levels and the neutrophil-lymphocyte ratio (*5,6*), pro-inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α), were actually increased in both peripheral blood and the cerebral spinal fluid (*7*). These cytokines directly or indirectly contribute to the psychopathology of schizophrenia by disturbing the brain connectivity, neurodevelopment, neurogenesis and the neurotransmitter function. Microglia are the brain-resident macrophages that produce pro-inflammatory cytokines within the central nervous system (*3,4*). In patients with schizophrenia, in addition to microglia, T-lymphocytes, which also produce pro-inflammatory cytokines (*8*), were activated or proliferated in both peripheral blood and the central nervous system (*3,9,10*). These findings strongly suggest the involvement of enhanced cellular immunity in the pathogenesis of schizophrenia.

T-lymphocytes and macrophages predominantly express delayed rectifier K^+-channels (Kv1.3) in their plasma membranes (*8*). These channels play crucial roles in the activation and proliferation of these leukocytes, which consequently stimulates the cellular immunity (*8,11*). Using animal models with advanced-stage chronic kidney disease (CKD), we previously revealed that both T-lymphocytes and macrophages were markedly increased and the cytokine levels, such as IL-2 and TNF-α, were significantly elevated within the fibrotic kidneys (*8,12*). In these leukocytes, Kv1.3-channels were

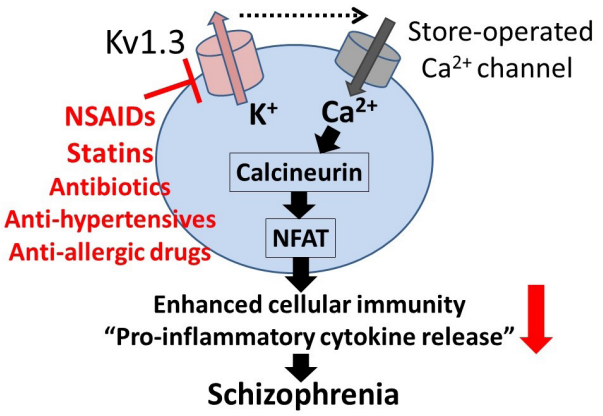

Figure 1. Roles of Kv1.3-channels in the activation pathway of T-lymphocytes or brain macrophages (microglia) and as the targets of commonly used drugs for schizophrenia. Kv1.3-channels promote calcium influx and trigger the proliferation and activation of T-lymphocytes or brain macrophages (microglia). The increased cytosolic calcium concentration stimulates the phosphatase calcineurin, which de-phosphorylates the nuclear factor of activated T cells (NFAT), causing its accumulation in the nucleus and binding to the promoter region of cytokine-encoding genes. Nonsteroidal anti-inflammatory drugs (NSAIDs), statins, antibiotics, anti-hypertensives and anti-allergic drugs, which inhibit Kv1.3-channels, suppress the enhanced cellular immunity and the subsequent release of excessive cytokines.

over-expressed and the pharmacological blockade of the channels actually ameliorated the disease progression. Therefore, the Kv1.3-channels were thought to be responsible for the overactivation of cellular immunity and the subsequent progression of renal fibrosis (*8,12*). Recently, in addition to chronic diseases, including CKD, chronic obstructive pulmonary disease and inflammatory bowel disease (*8*), some neurodegenerative disorders, such as multiple sclerosis, Alzheimer's disease and Parkinson' s disease, are also considered to be caused by inflammation (*13*). In such diseases, T-lymphocytes and macrophages were hyperactivated or proliferated in the central nervous system, being accompanied by the overexpression of Kv1.3-channels within the cells (*13*).

In the treatment of schizophrenia, recent clinical studies have additionally revealed the therapeutic efficacy of anti-inflammatory agents, including nonsteroidal anti-inflammatory drugs (NSAIDs), antibiotics and anti-cholesterol drugs (statins) (*14,15*). Despite the lack of pharmacological evidence, these agents actually lowered the serum CRP levels in schizophrenia patients and suppressed the release of pro-inflammatory cytokines in the brain (*15*). According to our previous patch-clamp studies using murine thymocytes, both NSAIDs, such as indomethacin, diclofenac and salicylate, and statins, such as pravastatin, lovastatin and simvastatin, strongly suppressed the activity of lymphocyte Kv1.3-channels and thus reduced the production of pro-inflammatory cytokines (*16,17*). These findings provide an additional pharmacological mechanism by which NSAIDs and statins were effective for schizophrenia, where the enhanced cellular immunity and the subsequent release of excessive cytokines were responsible for the pathogenesis (Figure 1).

In our series of patch-clamp studies thus far, we further demonstrated the inhibitory properties of antibiotics (clarithromycin, chloroquine), anti-hypertensive drugs (nifedipine, benidipine, diltiazem, verapamil) and anti-allergic drugs (cetirizine, fexofenadine, azelastine, terfenadine) on lymphocytes Kv1.3-channels (*8,18,19*). Considering such pharmacological properties of these commonly used drugs, they would also be useful in the treatment of schizophrenia, since the channel inhibition suppresses the activity of brain lymphocytes or macrophages and thus represses their cytokine production (Figure 1). Compared to the highly selective Kv1.3-channel inhibitors that were originally derived from scorpion venom or sea anemone peptide toxins (*20*), the drugs, such as NSAIDs, statins, antibiotics, anti-hypertensive drugs and anti-allergic drugs, could be used more harmlessly, because they have commonly been prescribed in a general clinical practice for longer periods of time.

Conclusion

In addition to NSAIDs and statins, some of the antibiotics, anti-hypertensive drugs and anti-allergic drugs strongly suppressed the Kv1.3-channel activity and pro-inflammatory cytokine production from lymphocytes. Given such pharmacological properties of these commonly used drugs, they may be useful in the treatment of schizophrenia, in which the enhanced cellular immunity and the subsequent release of excessive cytokines are responsible for the pathogenesis

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References

1. Jauhar S, Johnstone M, McKenna PJ. Schizophrenia. Lancet. 2022; 399:473-486.
2. Stroup TS, Gray N. Management of common adverse effects of antipsychotic medications. World Psychiatry. 2018; 17:341-356.
3. Muller N. Inflammation in schizophrenia: Pathogenetic aspects and therapeutic considerations. Schizophr Bull. 2018; 44:973-982.
4. Fond G, Lancon C, Korchia T, Auquier P, Boyer L. The role of inflammation in the treatment of schizophrenia. Front Psychiatry. 2020; 11:160.
5. Faugere M, Micoulaud-Franchi JA, Alessandrini M, Richieri R, Faget-Agius C, Auquier P, Lancon C, Boyer L. Quality of life is associated with chronic inflammation in schizophrenia: a cross-sectional study. Sci Rep. 2015; 5:10793.
6. Semiz M, Yildirim O, Canan F, Demir S, Hasbek E,

Tuman TC, Kayka N, Tosun M. Elevated neutrophil/lymphocyte ratio in patients with schizophrenia. Psychiatr Danub. 2014; 26:220-225.
7. Miller BJ, Goldsmith DR. Evaluating the hypothesis that schizophrenia is an inflammatory disorder. Focus (Am Psychiatr Publ). 2020; 18:391-401.
8. Kazama I. Physiological significance of delayed rectifier K^+ channels (Kv1.3) expressed in T lymphocytes and their pathological significance in chronic kidney disease. J Physiol Sci. 2015; 65:25-35.
9. Muller N, Hofschuster E, Ackenheil M, Eckstein R. T-cells and psychopathology in schizophrenia: relationship to the outcome of neuroleptic therapy. Acta Psychiatr Scand. 1993; 87:66-71.
10. Schlaaff K, Dobrowolny H, Frodl T, Mawrin C, Gos T, Steiner J, Bogerts B. Increased densities of T and B lymphocytes indicate neuroinflammation in subgroups of schizophrenia and mood disorder patients. Brain Behav Immun. 2020; 88:497-506.
11. Kazama I, Senzaki M. Does immunosuppressive property of non-steroidal anti-inflammatory drugs (NSAIDs) reduce COVID-19 vaccine-induced systemic side effects? Drug Discov Ther. 2021; 15:278-280.
12. Kazama I, Baba A, Matsubara M, Endo Y, Toyama H, Ejima Y. Benidipine suppresses *in situ* proliferation of leukocytes and slows the progression of renal fibrosis in rat kidneys with advanced chronic renal failure. Nephron Experimental nephrology. 2014; 128:67-79.
13. Wang X, Li G, Guo J, Zhang Z, Zhang S, Zhu Y, Cheng J, Yu L, Ji Y, Tao J. Kv1.3 Channel as a key therapeutic target for neuroinflammatory diseases: State of the art and beyond. Front Neurosci. 2019; 13:1393.
14. Hong J, Bang M. Anti-inflammatory strategies for schizophrenia: A review of evidence for therapeutic applications and drug repurposing. Clin Psychopharmacol Neurosci. 2020; 18:10-24.
15. Cakici N, van Beveren NJM, Judge-Hundal G, Koola MM, Sommer IEC. An update on the efficacy of anti-inflammatory agents for patients with schizophrenia: a meta-analysis. Psychol Med. 2019; 49:2307-2319.
16. Kazama I, Maruyama Y, Murata Y. Suppressive effects of nonsteroidal anti-inflammatory drugs diclofenac sodium, salicylate and indomethacin on delayed rectifier K^+-channel currents in murine thymocytes. Immunopharmacol Immunotoxicol. 2012; 34:874-878.
17. Kazama I, Baba A, Maruyama Y. HMG-CoA reductase inhibitors pravastatin, lovastatin and simvastatin suppress delayed rectifier K^+-channel currents in murine thymocytes. Pharmacol Rep. 2014; 66:712-717.
18. Kazama I, Tamada T, Tachi M. Usefulness of targeting lymphocyte Kv1.3-channels in the treatment of respiratory diseases. Inflamm Res. 2015; 64:753-765.
19. Baba A, Tachi M, Maruyama Y, Kazama I. Suppressive effects of diltiazem and verapamil on delayed rectifier K^+-channel currents in murine thymocytes. Pharmacol Rep. 2015; 67:959-964.
20. Han S, Yi H, Yin SJ, Chen ZY, Liu H, Cao ZJ, Wu YL, Li WX. Structural basis of a potent peptide inhibitor designed for Kv1.3 channel, a therapeutic target of autoimmune disease. J Biol Chem. 2008; 283:19058-19065.

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Commentary

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Linezolid for patients with multidrug-resistant tuberculosis/ extensively drug-resistant tuberculosis in China

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SUMMARY Linezolid has been one of the key anti-tuberculosis agents for the treatment of multidrug-resistant tuberculosis (MDR-TB)/extensively drug-resistant tuberculosis (XDR-TB). It used to be very expensive and was not covered by social insurance from local governments. Nevertheless, a growing number of patients in China received linezolid in their anti- MDR/XDR TB regimens over the past decade. Many scholars in China have reported their experience using linezolid to treat patients with MDR/XDR-TB. In view of this, existing evidence of the efficacy and safety of linezolid and problems faced by Chinese patients with MDR/XDR-TB are summarized here.

Keywords Linezolid, MDR/XDR-TB, China, drug resistance

1. Introduction

Linezolid has been endorsed by the WHO as one of the Group A anti-tuberculosis agents for the treatment of multidrug-resistant tuberculosis (MDR-TB)/extensively drug-resistant tuberculosis (XDR-TB) since 2018 (*1*). China is one of the world's countries with the highest TB burden (*2*). Concerns have led to an emphasis on the care and control of MDR-TB and XDR-TB in China (*3*). Over the past decade, a growing number of patients in China received linezolid in their anti-MDR/XDR TB regimens. Many scholars in China have reported their experience using linezolid to treat patients with MDR/XDR-TB (*4-7*). In view of this, existing evidence of the efficacy and safety of linezolid and problems faced by patients with MDR/XDR-TB in China have been summarized here.

2. Use of linezolid to treat Chinese patients with MDR/XDR TB

Linezolid is an artificial synthetic oxazolidinone antibiotic. It binds to the 50S subunit of the ribosome and prevents protein synthesis. Linezolid has proven to be a potent anti-tuberculosis drug both *in vitro* and *in vivo* (*8,9*). Before 2021, the cost of the drug rendered it unaffordable for typical patients with MDR/XDR-TB in China except for those participating in clinical trials, and this even included patients covered by government-subsidized medical insurance (*10*). Uneven economic development may have also contributed to the underutilization of linezolid in China since MDR/XDR-TB is markedly more prevalent in low-income groups. Moreover, MDR/XDR TB is extremely costly and can significantly burden individuals and the healthcare system. The exact proportion of patients receiving an anti-TB regimen that includes linezolid is unknown. As such, linezolid was presumably only included in the treatment regimen received by a small number of patients with MDR/XDR-TB in China over the past decade. In recent years, the drug has become more accessible cost-wise as the central government has revised the subsidy for medical insurance and more local pharmaceutical manufacturers have started production. Linezolid will presumably be given to more patients to treat MDR/XDR-TB in China in the coming days.

3. The efficacy and safety of linezolid in MDR/XDR-TB

The current authors searched for use of linezolid to treat MDR/XDR-TB in articles and reports by Chinese authors published in CNKI, PubMed, Embase, and the Cochrane Central Library from the start of the given database until December 31, 2021. There is growing evidence of the efficacy of linezolid against MDR-TB, from studies of varying scales carried out across different cities in China. From the earliest study of 8 patients with XDR-TB in Shanghai in 2009 to an RCT initiated by Tang *et al*. in 2015 in Beijing, these sources have all found linezolid

to be efficacious against MDR/XRD-TB (*4,11*). A recent multi-center study on administration of bedaquiline in a regimen to treat MDR/XDR-TB in China found that more than 90% of patients administered linezolid in that regimen were also administered bedaquiline (*12*). The high rate of culture conversion further demonstrated the efficacy of linezolid combined with bedaquiline. The potential efficacy of linezolid against MDR/XDR-TB was evident even in a small group of pediatric patients (*7*). A prospective non-randomized controlled single center trial conducted by the current authors' team found no difference in treatment outcomes between patients with MDR/XDR-TB receiving or not receiving bedaquiline in a regimen that included linezolid (*6*). The most commonly used dose of linezolid in these regimens was 600 mg/d, though this was sometimes reduced to 300 mg/d due to linezolid -related toxicity (*6,12*). All of these studies noted an average two- to three-month sputum culture conversion rate of more than 80% within six months from the initiation of treatment (*6,12,13*). Patients receiving linezolid were more likely to have favorable treatment outcomes compared to those not receiving linezolid (*13*).

Adverse events are believed to be associated with the dose and duration of treatment, which are consistent with the international literature (*14-16*). Frequently reported adverse reactions to long-term use of linezolid are peripheral neuropathy, anemia, and thrombocytopenia, which are the main risk factors for reduced patient compliance and discontinuation of treatment. Optimizing both the dose and duration of treatment to improve the antibiotic's tolerability profile is a major focus for larger scale studies in the future. At present, the prospective efficacy of linezolid in China is supported mainly by small-scale studies. Randomized controlled studies on a massive scale need to be conducted to accumulate more evidence. Numerous clinical studies in China are recruiting patients with MDR/XDR-TB to receive a treatment regimen including linezolid (NCT: ChiCTR:2000032298, ChiCTR:2100042287, and ChiCTR:210004593) (*17*). These studies are expected to yield more definitive clinical findings.

4. Resistance to linezolid

Findings from *in vitro* testing and molecular testing have indicated that only a small percentage (4.5-5.6%) of patients with MDR/XDR-TB in China exhibited linezolid resistance; this resistance was mainly caused by alterations to the specific linezolid binding site (*18,19*). A study of 399 isolates from southwest China revealed that 4 were linezolid-resistant and 2 carried mutations in the *rplC* gene (*19*). Another study of 93 isolates reported that 5 were linezolid-resistant; only 2 (40.0%) were found to contain the Cys154Arg allele in the *rplC* gene (18). In a multi-center study involving the use of bedaquiline and linezolid in 277 patients, 115 patients with prior linezolid exposure yielded 19 isolates (6.9%) exhibiting linezolid resistance. Genetic mutations were observed in 10 (52.6%, 10/19) linezolid-resistant isolates, the most prevalent of which was a Cys154Arg (36.8%, 7/19) substitution within ribosomal protein L3 (*20*). A subsequent study of nationwide drug resistance surveillance in China revealed linezolid resistance in 15 (3.8%) of 391 culture-positive specimens (*21*). Notwithstanding the low resistance rate, induced linezolid resistance caused by intermittent administration as a result of drug cost and adverse reactions remains a major concern in China in the coming future.

5. Conclusion

In the face of the limited number of new drugs to treat MDR/XDR-TB, a regimen that includes linezolid will remain an important weapon to treat MDR/XDR-TB in China over the next few decades. Considering the importance of linezolid, precision dosage and close monitoring of treatment are vital to achieving optimal drug efficacy and balancing its tolerability profile. These efforts will benefit more patients with drug-resistant TB and reduce community transmission.

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References

1. World Health Organization. Rapid communication: Key changes to treatment of multidrug-and rifampicin-resistant tuberculosis (MDR/RR-TB). Geneva: World Health Organization, 2018.
2. World Health Organization. Global tuberculosis report 2021. Geneva: World Health Organization, 2021.
3. Chen B, Bao H, Chen X, Liu K, Peng Y, Wang W, Wang F, Jiang J, Xu B. Identification and attribute analysis of key stakeholders who influence multidrug-resistant tuberculosis prevention and control in China. Infect Dis Poverty. 2021; 10:108.
4. Tang S, Yao L, Hao X, Zhang X, Liu G, Liu X, Wu M, Zen L, Sun H, Liu Y, Gu J, Lin F, Wang X, Zhang Z. Efficacy, safety and tolerability of linezolid for the treatment of XDR-TB: A study in China. Eur Respir J. 2015; 45:161-170.
5. Zhu H, Guo SC, Liu ZQ, Wang B, Fu L, Chu NH, Lu Y. Therapeutic drug monitoring of cycloserine and linezolid during anti-tuberculosis treatment in Beijing, China. Int J Tuberc Lung Dis. 2018; 22:931-936.
6. Fu L, Weng T, Sun F, Zhang P, Li H, Li Y, Yang Q, Cai Y, Zhang X, Liang H, Chen X, Wang Z, Liu L, Zhang W, Deng G. Insignificant difference in culture conversion between bedaquiline-containing and bedaquiline-free all-oral short regimens for multidrug-resistant tuberculosis. Int J Infect Dis. 2021; 111:138-147.
7. Zhang Z, Cheng Z, Liu Q, Shang T, Jiang L, Fu Z, Zhu K, Wang X, Chen J, Xu H. Safety of longer linezolid regimen

in children with drug-resistant tuberculosis and extensive tuberculosis in Southwest China. J Glob Antimicrob Resist. 2020; 21:375-379.
8. Erturan Z, Uzun M. *In vitro* activity of linezolid against multidrug-resistant *Mycobacterium tuberculosis* isolates. Int J Antimicrob Agents. 2005; 26:78-80.
9. Anger HA, Dworkin F, Sharma S, Munsiff SS, Nilsen DM, Ahuja SD. Linezolid use for treatment of multidrug-resistant and extensively drug-resistant tuberculosis, New York City, 2000-06. J Antimicrob Chemother. 2010; 65:775-783.
10. Zhang Y, Liu X, Yang L, Zhang G, Gu Z, Chen Z, Sun J. Barriers and strategies: A review of access to affordable multi-drug resistant tuberculosis medication in China. Infect Drug Resist. 2020; 13:3679-3687.
11. Yao L, Tang S, Xiao H, Zeng L, Sun H, Gu J, Hao X, Liu Y. Examination of the clinical efficacy of using linezolid to treat extensively drug-resistant tuberculosis. Zhonghua Lin Chuang Yi Shi Za Zhi. 2010; 4:6. (in Chinese).
12. Gao M, Gao J, Xie L, *et al*. Early outcome and safety of bedaquiline-containing regimens for treatment of MDR- and XDR-TB in China: A multicentre study. Clin Microbiol Infect. 2021; 27:597-602.
13. Pang Y, Lu J, Huo F, Ma Y, Zhao L, Li Y, Liang Q, Chu N, Gao M, Huang H. Prevalence and treatment outcome of extensively drug-resistant tuberculosis plus additional drug resistance from the National Clinical Center for Tuberculosis in China: A five-year review. J Infect. 2017; 75:433-440.
14. Agyeman AA, Ofori-Asenso R. Efficacy and safety profile of linezolid in the treatment of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis: A systematic review and meta-analysis. Ann Clin Microbiol Antimicrob. 2016; 15:41.
15. Conradie F, Diacon AH, Ngubane N, *et al*. Treatment of highly drug-resistant pulmonary tuberculosis. N Engl J Med. 2020; 382:893-902.
16. Jaspard M, Butel N, El Helali N, Marigot-Outtandy D, Guillot H, Peytavin G, Veziris N, Bodaghi B, Flandre P, Petitjean G, Caumes E, Pourcher V. Linezolid-associated neurologic adverse events in patients with multidrug-resistant tuberculosis, France. Emerg Infect Dis. 2020; 26:1792-1800.
17. Chinese Clinical Trial Registry. *http://www.chictr.org.cn/enIndex.aspx*
18. Pang Y, Zong Z, Huo F, Jing W, Ma Y, Dong L, Li Y, Zhao L, Fu Y, Huang H. *In vitro* drug susceptibility of bedaquiline, delamanid, linezolid, clofazimine, moxifloxacin, and gatifloxacin against extensively drug-resistant tuberculosis in Beijing, China. Antimicrob Agents Chemother. 2017; 61:e00900-17.
19. Zheng H, He W, Jiao W, Xia H, Sun L, Wang S, Xiao J, Ou X, Zhao Y, Shen A. Molecular characterization of multidrug-resistant tuberculosis against levofloxacin, moxifloxacin, bedaquiline, linezolid, clofazimine, and delamanid in southwest of China. BMC Infect Dis. 2021; 21:330.
20. Du J, Gao J, Yu Y, Li Q, Bai G, Shu W, Gao M, Liu Y, Wang L, Wang Y, Xue Z, Huo F, Li L, Pang Y. Low rate of acquired linezolid resistance in multidrug-resistant tuberculosis treated with bedaquiline-linezolid combination. Front Microbiol. 2021; 12:655653.
21. Wang G, Jiang G, Jing W, Zong Z, Yu X, Chen S, Li W, Huang H. Prevalence and molecular characterizations of seven additional drug resistance among multidrug-resistant tuberculosis in China: A subsequent study of a national survey. J Infect. 2021; 82:371-377.

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Commentary

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Prospects of contezolid (MRX-I) against multidrug-resistant tuberculosis and extensively drug-resistant tuberculosis

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SUMMARY Tuberculosis has become a great global public health threat. Compared with drug-susceptible tuberculosis (TB), the treatment regimens for multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) involve more severe adverse events and poorer treatment outcomes. Linezolid (LZD) is the first oxazolidinones used for TB. Thanks to its potent activity against *Mycobacterium tuberculosis*, LZD has become one of the key agents in the regimens against MDR/XDR-TB. However, this drug may cause intolerability and other adverse events. Contezolid, another novel oxazolidinone, can also inhibit *M. tuberculosis*, still with fewer adverse effects compared with LZD. This paper is to prospect the potentials of contezolid in the treatment of MDR/XDR-TB, with focus on its efficacy and possible adverse effects.

Keywords *Mycobacterium tuberculosis*, contezolid, MDR-TB, XDR-TB

1. Introduction

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*. A total of about 2 billion people are infected with *M. tuberculosis* worldwide, and 5-10% of them will develop TB disease during their lifetime (*1*). Tuberculosis has become a great global public health threat. Compared with drug-susceptible TB, the treatment regimens for multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) involve a longer course of treatment, heavier economic burden, and higher drug toxicity, as well as more severe adverse events and poorer treatment outcomes (*1*). Therefore, new and fast-acting anti-mycobacterial drugs with better efficacy to cure MDR/XDR-TB are in urgent demand. Oxazolidinones have been found potential inhibition against MDR Gram-positive bacteria and *Mycobacterium tuberculosis*, among which linezolid (LZD) is the first used for TB. Thanks to its potent activity against *M. tuberculosis*, LZD has become one of the key agents in the regimens against MDR/XDR-TB (*2*). However, this drug may cause intolerability and other adverse events, such as peripheral and optic neuropathy as well as myelosuppression. Contezolid, another novel oxazolidinone is preliminarily developed for Gram-positive infections. Some studies have shown that contezolid can also inhibit *M. tuberculosis* (*3*), still with fewer adverse effects compared with LZD (*4*). This paper is to prospect the potentials of contezolid in the treatment of MDR/XDR-TB, with focus on its efficacy and possible adverse effects.

2. The disadvantage of LZD for MDR/XDR-TB

Oxazolidinones are a series of antibiotics against MDR Gram-positive bacteria, including vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* (MRSA) (*5*). The mechanics of action is to bind the 50S ribosomal subunit of archaea and bacteria, inhibiting the biosynthesis of their proteins, but without influence on human cytoplasmic ribosomes (*6*). Their promising activity against MDR-TB was also found soon after their discovery. LZD, the first oxazolidinone used for TB, is now one of the key drugs in both longer and shorter MDR/XDR-TB regimens. However, myelosuppression greatly limits the use of LZD. Potential irreversible optic neuropathy and peripheral neuropathy are also the major adverse effects of LZD (*7*). These neurotoxic adverse effects are due to the inhibition of MAOs, a family of enzymes that are essential for the metabolic inactivation of the neurotransmitters, such as serotonin, dopamine, and epinephrine. To avoid such adverse effects and improve tolerability and safety, the development of better drugs is imperative.

3. The superiority of contezolid

Contezolid, a new member of oxazolidinone antibiotics,

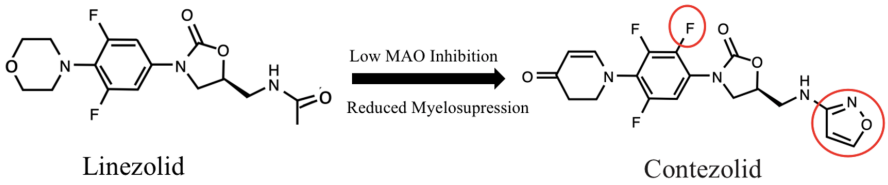

Figure 1. The superiority of contezolid.

can inhibit the formation of a functional 70S initiation complex that is necessary for bacterial reproduction (*4*). It is rationally designed to address the myelosuppression and MAO inhibition associated with linezolid. Serotonergic profiles for contezolid *in vitro* and in rodents have been reported. Compared with LZD, contezolid exhibits a reduction in reversible inhibition of MAO-A and MAO-B isoforms by 2-and 148-fold respectively (Figure 1) (*8*). The safety and tolerability studies of contezolid show that it was well tolerated and safe in healthy Chinese subjects (*9-11*). Even at doses up to 800 and 1,200 mg every 12 hours for 28 days, no severe adverse events were observed, and nobody discontinued the drug due to any adverse events. Compared with LZD, contezolid is associated with a lower incidence of myelosuppression. Noteworthily, in the high dose contezolid group, approximately half of the subjects had merely slight ALT elevations; however, with most of them < 2 ULN, and none of them persistent (*10*). Contezolid may prolong the QT interval slightly at a supratherapeutic dose (1,600 mg/d) but does not influence the QT interval at a therapeutic dose (800 mg/d) (*12,13*).

4. The anti-tuberculosis activity of contezolid

Only a few studies have been published on contezolid for its effect on tuberculosis. One study (*3*) tested the oxazolidinones (including contezolid and LZD) against both susceptible and MDR/XDR *M. tuberculosis* isolates *in vitro*. The MIC_{50} and MIC_{90} for LZD and contezolid were 1 mg/mL and 0.5 mg/mL, *vs*. 0.5 mg/mL and 0.125 mg/mL, respectively. Contezolid showed a same activity as LZD against all *M. tuberculosis* isolates. Because of the promising results *in vitro*, the evaluation of the drugs' efficacy was then performed *in vivo*. LZD and contezolid were studied in *M. tuberculosis*-infected mice further. The mice were randomly assigned into 6 groups as follows: untreated early control (EC) group for the *M. tuberculosis* baseline, late control (LC) group to determine the bacterial load at the end of therapy, one LZD group (100 mg/kg once daily), three contezolid groups (100 mg/kg once daily, 50 mg/kg twice daily, and 25 mg/kg twice daily). Treatment was started one week after infection, with administrations for 5 days per week for 4 weeks. Then the bacterial load in mouse lungs was determined, showing the bacteria in the LZD group and the contezolid 100 mg/kg group significantly reduced compared with the EC and LC groups ($p < 0.05$). The efficacy in the LZD group was equivalent to the contezolid 100 mg/kg group ($p < 0.05$). The contezolid 100 mg/kg once daily group showed significant better efficacy than the contezolid 50 mg/kg and 25 mg/kg twice daily groups ($p < 0.05$). The efficacy in the contezolid 50 mg/kg group was equivalent to the contezolid 25 mg/kg group ($p < 0.05$). The contezolid 50 mg/kg and 25 mg/kg groups showed significant favorable result than the LC group ($p < 0.05$). However, currently no clinic study on contezolid against *M. tuberculosis* is reported. Meanwhile, contezolid is active against *M. abscessus in vitro* too, with compatibility to those antibiotics most frequently used to treat such infections. It inhibits intracellular replication of *M. abscessus*, exhibiting equivalent activity in culture compared with linezolid. Therefore, contezolid is also a potential candidate to be included in novel therapeutic anti-*M. abscessus* regimens (*14*).

5. Conclusion

In summary, compared to LZD, contezolid has a similar activity against both drug-resistant and drug-susceptible *M. tuberculosis in vitro* and *in vivo*, but with fewer side effects, especially neuropathy and myelosuppression. The inclusion of contezolid possibly makes MDR-TB/XDR-TB therapy regimens more efficacious and less toxic than LZD. However, further clinical studies are required to confirm it.

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References

1. World Health Organization. Global Tuberculosis Report 2021. *https://www.who.int/publications/i/item/9789240037021* (accessed March 17, 2022).
2. Mirzayev F, Viney K, Linh NN, Gonzalez-Angulo L, Gegia M, Jaramillo E, Zignol M, Kasaeva T. World Health

Organization recommendations on the treatment of drug-resistant tuberculosis, 2020 update. Eur Respir J. 2021; 57:2003300.
3. Shoen C, DeStefano M, Hafkin B, Cynamon M. *In vitro* and *in vivo* activities of contezolid (MRX-I) against *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2018; 62:e00493-18.
4. Hoy SM. Contezolid: First approval. Drugs. 2021; 81:1587-1591.
5. Foti C, Piperno A, Scala A, Giuffrè O. Oxazolidinone asntibiotics: Chemical, biological and analytical aspects. Molecules. 2021; 26:4280.
6. Yamada M, Yasuhara H. Clinical pharmacology of MAO inhibitors: Safety and future. Neurotoxicology. 2004; 25:215-221.
7. Vinh DC, Rubinstein E. Linezolid: a review of safety and tolerability. J Infect. 2009; 59:S59-74.
8. Wang W, Voss KM, Liu J, Gordeev M. Nonclinical evaluation of antibacterial oxazolidinones contezolid and contezolid acefosamil with low serotonergic neurotoxicity. Chem Res Toxicol. 2021; 34:1348-1354.
9. Wu X, Li Y, Zhang J, *et al*. Short-term safety, tolerability, and pharmacokinetics of MRX-I, an oxazolidinone antibacterial agent, in healthy Chinese subjects. Clin Ther. 2018; 40:322-332.e5.
10. Eckburg PB, Ge Y, Hafkin B. Single- and multiple-dose study to determine the safety, tolerability, pharmacokinetics, and food effect of oral MRX-I versus linezolid in healthy adult subjects. Antimicrob Agents Chemother. 2017; 61:e02181-16.
11. Wu J, Wu H, Wang Y, Chen Y, Guo B, Cao G, Wu X, Yu J, Wu J, Zhu D, Guo Y, Yuan H, Hu F, Zhang J. Tolerability and pharmacokinetics of contezolid at therapeutic and supratherapeutic doses in healthy Chinese subjects, and assessment of contezolid dosing regimens based on pharmacokinetic/pharmacodynamic analysis. Clin Ther. 2019; 41:1164-1174.e4.
12. Wu J, Cao G, Wu H, Chen Y, Guo B, Wu X, Yu J, Ni K, Qian J, Wang L, Wu J, Wang Y, Yuan H, Zhang J, Xi Y. Evaluation of the effect of contezolid (MRX-I) on the corrected QT interval in a randomized, double-blind, placebo- and positive-controlled crossover study in healthy Chinese volunteers. Antimicrob Agents Chemother. 2020; 64:e02158-19.
13. Wu J, Wang K, Chen Y, Yuan H, Li L, Zhang J. Concentration-response modeling of ECG data from early-phase clinical studies to assess QT prolongation risk of contezolid (MRX-I), an oxazolidinone antibacterial agent. J Pharmacokinet Pharmacodyn. 2019; 46:531-541.
14. Guo Q, Xu L, Tan F, Zhang Y, Fan J, Wang X, Zhang Z, Li B, Chu H. A novel oxazolidinone, contezolid (MRX-I), expresses anti-mycobacterium abscessus activity *in vitro*. Antimicrob Agents Chemother. 2021; 65:e0088921.

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Letter to the Editor

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Pulse corticosteroids for the management of extensive CNS tuberculosis presenting with acute-onset quadriparesis

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SUMMARY Myelopathy in central nervous system tuberculosis is notorious for poor outcomes, determined by the severity of inflammation and cord level involved. Acute-onset quadriplegia or paraplegia in these cases represents a neuro-emergency. We report a young female with disseminated tuberculosis who presented with acute onset flaccid quadriparesis with loss of bladder and bowel function. Imaging helped identify the extensive involvement of the neuraxis. We propose that, in addition to anti-tubercular therapy, high-dose corticosteroids such as pulse methylprednisolone may result in a meaningful improvement and show greater rapidity of response in cases of severe central nervous system inflammation such as arachnoiditis or myelopathy.

Keywords Tuberculosis, meningitis, myelopathy, immunosuppression

To the Editor,

Central nervous system (CNS) tuberculosis is a severe form of extrapulmonary tuberculosis with mortality rates of 30-40%, and up to 60% with advanced disease at presentation (*1*). Clinical manifestations include fever, headache, vomiting, seizures, and altered sensorium. Acute-onset quadriparesis is an unusual presentation due to spinal cord involvement (*2*). Rapid onset of disease indicates an acute insult which may be reversed by aggressive treatment, reducing the severity of long term sequelae. We present a case wherein early administration of supraphysiologic doses of steroid ('pulse') therapy led to early sustained recovery.

A 14-year-old girl was symptomatic with low-grade fever, weight-loss and anorexia for one-year, abdominal distension for two months, and persistent headache for one month. She had been clinically diagnosed earlier as disseminated tuberculosis based on peritoneal thickening and moderate ascites on ultrasound of the abdomen, patchy consolidation in both lung fields, and close contact with a known case of pulmonary tuberculosis (father). She had been non-adherent to the prescribed anti-tubercular therapy (ATT), interrupting treatment twice for several weeks at a time due to frequent vomiting.

She presented to us in the emergency department with weakness of all four limbs, loss of sensation below the umbilicus, and urinary retention for one day. On examination, she had intact sensorium and normal cranial nerves. The visual acuity was found reduced to 6 ft/60 ft in both eyes, and fundus examination was normal. Motor testing revealed decreased power in both upper limbs (3/5, modified Medical Research Council (mMRC) grading) and no movements in the lower limbs (0/5) or trunk. Deep tendon reflexes in bilateral ankle, knee, and triceps were absent while bilateral biceps and supinator were weak (1+). Both plantars were mute. Sensory testing revealed a loss of vibration, touch, and pain below the level of the umbilicus, spontaneous paraesthesias in upper limbs, and normal facial sensation. Bowel and bladder function were lost.

Within 24 hours of presentation, power declined in upper limbs to 0/5 (mMRC). In the setting of disseminated tuberculosis, we suspected spinal cord involvement. The symmetric involvement, acute onset, and absence of bony deformity, girdle sensation and root pain was suggestive of non-compressive myelopathy (tuberculous transverse myelitis, anterior spinal cord infarction) instead of compressive myelopathy (spine tuberculosis, tuberculomas, epidural abscess). Blood investigations revealed normocytic anemia (hemoglobin 9 gm/dL), leukocytosis (12,000/μL, 75% neutrophils, 9% lymphocytes and 10% monocytes), and elevated erythrocyte sedimentation rate (56 mm/hour). Platelet count, renal and liver functions were normal, and antibodies against human immunodeficiency virus (HIV)

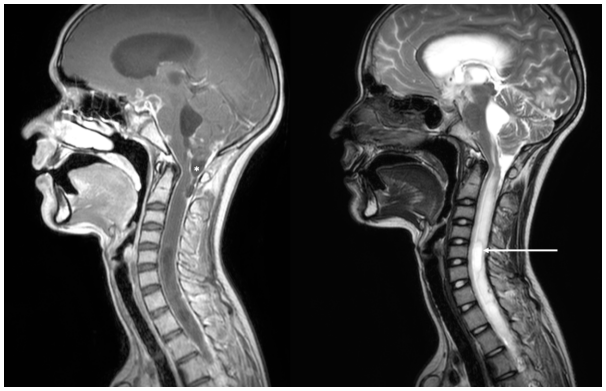

Figure 1. Magnetic resonance imaging (MRI) of the brain and spine demonstrates several manifestations of CNS tuberculosis. Post-contrast T1 weighted (T1w, left panel) and T2w(right panel) sagittal MRI shows diffuse leptomeningeal enhancement and thickening over the brainstem and spinal cord, exudates at the suprasellar cistern, base of the fourth ventricle, and the cervico-medullary junction leading to focal CSF loculation posterior to it (marked with an asterisk). Significant oedema and syrinx extending throughout the cord are evident, most prominent at C5-C6 level (white arrow).

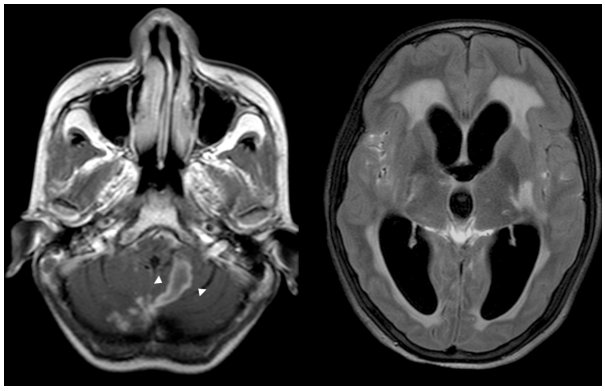

Figure 2. MRI Brain T1w post-contrast (left panel) and T2-FLAIR (right panel) shows ring and disc enhancing lesions in the cerebellar vermis measuring 2.8 cm (white arrowhead), and severe hydrocephalus with periventricular ooze.

were absent. Lumbar puncture was attempted twice by different experienced operators, but both attempts failed. The cause of quadriparesis was evident on neuroimaging: florid arachnoiditis, cord oedema, and syringomyelia were prominent (Figure 1), along with hydrocephalus (Figure 2).

She was started on ATT (rifampicin, isoniazid, pyrazinamide, and levofloxacin) and intravenous steroids (pulse methylprednisone (500 mg for five days) followed by dexamethasone). Power in both upper limbs improved gradually and symmetrically in a proximal to distal manner to 3/5 (mMRC) by day 3, and to 4+/5 (mMRC) by day 7 of pulse corticosteroids. A ventriculoperitoneal shunt was subsequently inserted. There was no incidence of dyselectrolytemia, hypertension, hyperglycemia, fluid overload, or hospital-acquired infection during her admission. She was prescribed high dose dexamethasone (0.4mg/kg) for one month after discharge. At two months of follow-up, she had regained functionality in upper limbs but remained paraplegic.

In over two-third cases, quadriparesis or paraparesis in tuberculosis results from compressive myelopathy due to tubercular spondylitis (*2*). Less often, it is attributable to the direct involvement of spinal cord or nerve roots by the arachnoiditis, resulting in tuberculous myelopathy (*3*). This can present with either an upper or lower motor neuron pattern of weakness, is frequently associated with the development of a syrinx and bears poor prognosis (*4*). It has reportedly been confused with Guillain–Barré syndrome when presenting as only flaccid weakness without bladder or sensory involvement (*5*). Binocular or monocular visual impairment frequently occurs in tuberculous meningitis. It is usually a consequence of the basal meningeal exudates encasing the optic nerve or chiasma resulting in retrobulbar optic neuritis, with possible contribution from raised intracranial pressure, vasculitic infarcts, and use of certain anti-tubercular drugs (ethambutol, fluoroquinolones, and linezolid) (*6*). The absence of papillitis or papilledema on fundoscopy in the setting of extensive basal exudates is consistent with retrobulbar neuritis as the cause of vision loss in our patient.

The treatment of CNS tuberculosis targets the mycobacterium, the inflammatory response, and prevention of complications such as hydrocephalus (*e.g.*, ventricular drainage) or ischemic stroke (*e.g.*, aspirin) (*7*). ATT usually combines four drugs of which rifampicin, isoniazid, and pyrazinamide are well accepted, while ethambutol is often the fourth agent. Fluoroquinolones (levofloxacin, moxifloxacin), aminoglycosides (streptomycin, amikacin) and linezolid have been studied as alternatives to ethambutol owing to its poor CNS penetration, and are non-inferior in terms of mortality (*7*).

Corticosteroids are remarkable adjuncts to ATT in CNS tuberculosis, particularly in meningitis where short-term mortality is reduced by 25% (*8*). Prevention of hydrocephalus, vasculitic infarction and IRIS (immune reconstitution syndrome) probably underly the mortality benefit, although without significant improvement in neurological sequelae in survivors (*9*). The standard doses of dexamethasone for meningitis described by Thwaites *et al*. have often been empirically applied to the other manifestations of CNS tuberculosis (*1*). However, the specific steroid used, its dose, route of administration, and the rate of taper have been a topic of intense discussion. In children with tubercular meningitis, small randomized studies have found no mortality difference between high and low dose steroids (4 mg/kg *vs*. 2 mg/kg prednisone) (*10*). These doses were equivalent to 0.75 mg/kg dexamethasone, thus not comparable to contemporary practice. A retrospective analysis reported an early switch from intravenous to oral route of dexamethasone in patients with tuberculomas or basal exudates and better neurological status at baseline but did not evaluate the outcomes with this approach (*11*).

The present case explored the role of pulse steroid therapy in CNS tuberculosis prompted by the abrupt clinical presentation and radiological findings of severe inflammation. Pulse therapy implies the intermittent administration of suprapharmacologic doses of steroids to enhance the therapeutic effect and reduce the side effects, defined arbitrarily as 250 mg prednisone or equivalent per day (*12*). Although methylprednisone is the typical steroid employed in pulse therapy, dexamethasone is longer acting (biological t1/2 36-72 hours *vs*. 12-36 hours), more potent, cheaper and does not cause significant fluid retention (*12*). A comparative study of intravenous dexamethasone and pulse methylprednisolone in tubercular meningitis found a similar reduction in death and visual impairment between the groups (*13*). Pulse steroids have also been tried for tuberculous arachnoiditis and cerebral vasculitis (*5,14*). However, none of these reports describe the time to clinical improvement. The improvement in upper limb power from 0 to 3/5 (mMRC) within three days of pulse steroids indicates an improvement in nervous tissue function at the cervical cord level. Of the multiple pathologies that plagued the cervical cord in our patient, cord oedema would possibly respond the earliest. At the same time, syringomyelia and arachnoiditis involve significant structural damage and may take longer to respond to therapy. Clinical recovery in the present cases was faster than we had expected based on our previous experience managing such cases at our tertiary center. We propose that pulse therapy, with either methylprednisolone or dexamethasone, may be worth exploring to induce a faster clinical response for the torment of CNS tuberculosis.

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

Consent: Informed consent has been obtained from the patient's guardian (mother).

References

1. Thwaites GE, Nguyen DB, Nguyen HD, *et al*. Dexamethasone for the treatment of tuberculous meningitis in adolescents and adults. N Engl J Med. 2004; 351:1741-1751.
2. Vaishnav B, Suthar N, Shaikh S, Tambile R. Clinical study of spinal tuberculosis presenting with neuro-deficits in Western India. Indian J Tuberc. 2019; 66:81-86.
3. Hristea A, Constantinescu RVM, Exergian F, Arama V, Besleaga M, Tanasescu R. Paraplegia due to non-osseous spinal tuberculosis: report of three cases and review of the literature. Int J Infect Dis. 2008; 12:425-429.
4. Gupta R, Garg RK, Jain A, Malhotra HS, Verma R, Sharma PK. Spinal cord and spinal nerve root involvement (myeloradiculopathy) in tuberculous meningitis: Medicine (Baltimore). 2015; 94:e404.
5. Singanamalla B, Saini AG, Sidana V, Saini L, Sankhyan N, Singh P. Progressive quadriparesis and inflammation: A common disease, a rare presentation. Indian J Tuberc. 2020; 67:336-339.
6. Garg RK, Malhotra HS, Kumar N, Uniyal R. Vision loss in tuberculous meningitis. J Neurol Sci. 2017; 375:27-34.
7. Davis A, Meintjes G, Wilkinson RJ. Treatment of tuberculous meningitis and its complications in adults. Curr Treat Options Neurol. 2018; 20:5.
8. Prasad K, Singh MB, Ryan H. Corticosteroids for managing tuberculous meningitis. Cochrane Database Syst Rev. 2016; 4:CD002244.
9. Thwaites GE, Macmullen-Price J, Chau TTH, Mai PP, Dung NT, Simmons CP, White NJ, Hien TT, Summers D, Farrar JJ. Serial MRI to determine the effect of dexamethasone on the cerebral pathology of tuberculous meningitis: an observational study. Lancet Neurol. 2007; 6:230-236.
10. Shah I, Meshram L. High dose versus low dose steroids in children with tuberculous meningitis. J Clin Neurosci. 2014; 21:761-764.
11. Paliwal VK, Das A, Anand S, Mishra P. Intravenous steroid days and predictors of early oral steroid administration in tuberculous meningitis: A retrospective study. Am J Trop Med Hyg. 2019; 101:1083-1086.
12. Sinha A, Bagga A. Pulse steroid therapy. Indian J Pediatr. 2008; 75:1057-1066.
13. Malhotra HS, Garg RK, Singh MK, Agarwal A, Verma R. Corticosteroids (dexamethasone versus intravenous methylprednisolone) in patients with tuberculous meningitis. Ann Trop Med Parasitol. 2009; 103:625-634.
14. Javaud N, Certal Rda S, Stirnemann J, Morin AS, Chamouard JM, Augier A, Bouchaud O, Carpentier A, Dhote R, Dumas JL, Fantin B, Fain O. Tuberculous cerebral vasculitis: Retrospective study of 10 cases. Eur J Intern Med. 2011; 22:e99-104.

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