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Editorial Office
ul. Marcinkowskiego 2–6
50-368 Wrocław, Poland
Tel.: +48 71 784 12 05
E-mail: redakcja@umw.edu.pl

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Contents

Research letters

Microbiota-derived psychedelics: Lessons from COVID-19

Adonis Sfera[1,2,A], Sabine Hazan[3,B], Zisis Kozlakidis[4,E], Carolina Klein[5,F]

[1] Department of Psychiatry, Patton State Hospital, San Bernardino, USA
[2] Department of Psychiatry, University of California Riverside, USA
[3] ProgenaBiome, Ventura, USA
[4] The International Agency for Research on Cancer, Lyon, France
[5] Department of Psychiatry, Napa State Hospital, USA

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Address for correspondence
Adonis Sfera
E-mail: dr.sfera@gmail.com

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Abstract

Emil Kraepelin believed that dementia praecox, the disorder we now call schizophrenia, was caused by the brain being poisoned with toxins generated in other parts of the body, especially the mouth, intestine or genitals. In this regard, Kraepelin hinted at the microbiome and conceptualized microbial molecules as drivers of severe psychiatric illness. However, it was not until the coronavirus disease (COVID-19) pandemic that Kraepelin's paradigm gained traction, particularly because this virus was associated with both gut barrier disruption and new-onset psychosis.

Likewise, despite numerous studies linking severe psychiatric illness to genomic damage and dysfunctional DNA repair, this pathogenetic mechanism was underappreciated before the COVID-19 pandemic.

The use of the psychotomimetic anesthetic, ketamine, for treatment-resistant depression has reawakened the interest in endogenous serotonergic hallucinogens, especially tryptamine and N,N-dimethyltryptamine (DMT), which are beneficial for depression but associated with psychosis.

In this editorial, we take a closer look at the role of the microbiome in psychopathology, attempting to answer 2 questions:

1. Why may psychosis-predisposing serotonergic hallucinogens alleviate depression?
2. Are microbiota-derived psychedelics part of an inbuilt antidepressant system similar to endogenous opioids?

Key words: gut microbes, serotonergic hallucinogens, severe psychiatric illness

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Introduction

Two major advances, the discovery of trace amine-associated receptors (TAARs) in 2001 and ketamine use for treatment-resistant depression, have contributed to a better understanding of the gut microbiota's role in psychopathology.[1,2]

These findings were further corroborated by the discovery, in 2014, of tryptophan decarboxylase-expressing gut commensals capable of converting dietary tryptophan into tryptamine, a serotonergic hallucinogen previously implicated in schizophrenia.[3–5] Although the physiological role of endogenous hallucinogens so far unknown, translocation of tryptophan decarboxylase-expressing microbes into the host systemic circulation may enable tryptamine to access the central nervous system (CNS) and interact with brain TAARs.[6] Indeed, the discovery of N,N-dimethyltryptamine (DMT) and N-acetyltryptamine (NAT) in rat pineal gland suggests that endogenous hallucinogens are either synthesized in the CNS or derived from gut microbes.[7,8]

We hypothesize that serotonergic hallucinogens and their CNS receptors comprise an endogenous antidepressant system mediated by TAARs, and that elevated levels of tryptamine or DMT due to a disrupted gut barrier may activate 5-hydroxytryptamine 2A receptors (5HT2ARs) and the aryl hydrocarbon receptor (AhR), and in consequence trigger psychosis. In other words, the activation of TAAR may drive the antidepressant effect, while stimulation of 5HT2ARs and AhR may engender psychosis.

Loose microbes and aberrant microglia

Studies on intestinal barrier disruption and an increased prevalence of psychiatric disorders after the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection have contributed to a better understanding of the physiological role of microbiota-generated serotonergic hallucinogens as well as the participation of other gut microorganisms in the pathogenesis of severe psychiatric illness (SPI), conceptualized as mental or behavioral conditions which substantially interfere with patients' life activities.[9–16]

Along this line, a recent retrospective study found that 19.5% of coronavirus disease (COVID-19) survivors developed depression or anxiety, and nearly 2.8% developed new-onset psychosis, linking the SARS-CoV-2 disruption of the gut barrier to neuropathology.[17] The psychological stress caused by contracting COVID-19, a potentially fatal disease, as well as the subsequent restrictive measures, including mandatory isolation and social distancing, can trigger mood and anxiety disorders. However, psychological stress has been associated with the impairment of both the gut barrier and microglial activation.[18–20] Indeed, several viral infections, including COVID-19 and human immunodeficiency virus (HIV), were associated with higher rates of posttraumatic stress disorder (PTSD) than those found in war

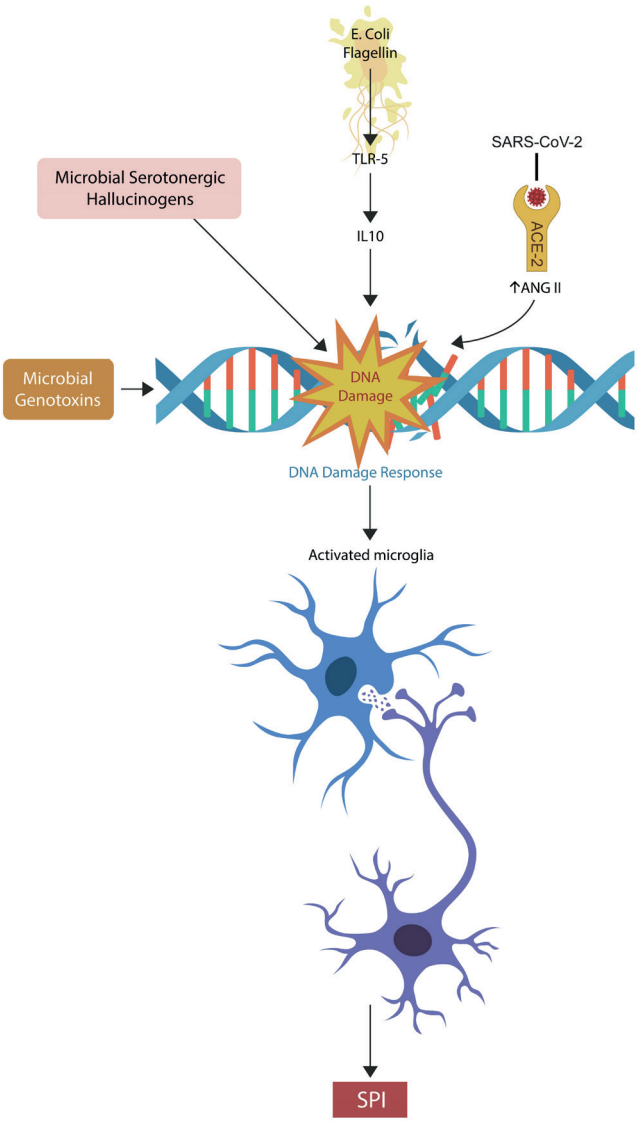

Fig. 1. Numerous pathogens and their molecules can damage the host genome. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) virus upregulates angiotensin II (ANG II), a genotoxin, by inhibiting its hydrolyzing enzyme, ACE-2. The *Escherichia coli* spp. (*E. coli*) protein, flagellin, activates toll-like receptor-5 (TLR-5), upregulating interleukin 10 (IL-10) that in turn can damage the DNA. Dysfunctional serotonergic hallucinogens and bacterial genotoxins can inflict additional genomic damage, activate microglia and cause severe psychiatric illness (SPI) by aberrant phagocytosis of viable neurons

veterans as well as military and law enforcement personnel, suggesting that aside from psychosocial stress, viruses could directly interfere with the gut and/or blood–brain barrier (BBB).[21–23] Moreover, microbial translocation markers, including zonulin, intestinal fatty acid binding protein (I-FABP) and soluble CD14 (sCD14), were documented in people with depression and suicidal behavior.[24] Along this line, a novel study linked suicidal ideation with depletion of the oral microbe *Alloprevotella rava* spp., further emphasizing the role of microbiota in neuropathology.[25] Indeed, previous studies have associated SPI with increased gastrointestinal (GI) tract permeability measured by the levels of microbial translocation markers.[26,27] In this regard, earlier data linked SPI

to the translocation of *Escherichia coli* spp. (*E. coli*) into the host systemic circulation.[28,29] In addition, neuropsychiatric symptoms, including psychosis, were documented in 2011 during an *E. coli* outbreak in northern Germany, further connecting this bacterium to neuropathology.[30,31] Moreover, *E. coli* antigens, including lipopolysaccharide (LPS) and flagellin, were shown to aberrantly activate microglia, potentially leading to pathological phagocytosis of viable neurons and/or synapses.[32,33] Flagellin, a Toll-like receptor-5 (TLR-5) agonist, was demonstrated to activate neurotoxic microglia, inducing gray matter loss in patients with SPI[34,35] (see the section on microbial genotoxins and SPI). Interestingly, serotonergic hallucinogen DMT was found capable of protecting neurons by deactivating microglia, emphasizing an important physiological function of this biomolecule.[36]

Taken together, these studies show that the systemic translocation of bacteria and/or their molecules can activate neurotoxic microglia that aberrantly eliminate healthy neurons. On the other hand, serotonergic hallucinogens may show neuroprotective properties by inhibiting neurotoxic microglia.

Endogenous hallucinogens – friends or foes?

Early studies, in the 1960s and 1970s, have connected SPI with endogenous hallucinogens derived from several sources, including the microbial metabolism of tryptophan.[3,4] These older studies were disregarded at the time due to the levels of endogenous psychedelics being considered too low to produce significant biological effects. However, the discovery of TAARs capable of sensing nanomolar ligand quantities has reawakened the interest in endogenous hallucinogens and their involvement in SPI.[37,38] As both ketamine, an N-methyl-D-aspartate (NMDA) receptor blocker, and serotonergic hallucinogens exhibit fast-acting antidepressant effects, it begs the question: how can psychosis-associated molecules alleviate depression?[39]

We surmise that microbiota-derived serotonergic hallucinogens are beneficial for severe depression as they induce limited genomic disruption that activates DNA damage repair (DDR), a neuroplasticity-mediating physiological process. Indeed, novel studies have shown that DDR comprises an adaptive mechanism that enhances neuronal plasticity and long-term potentiation.[40,41] On the other hand, irreparable genomic damage, leading to neuronal death, contributes to the pathogenesis of neurodegenerative disorders and/or psychosis.[42,43] For example, recreational use of exogenous hallucinogens may inflict extensive DNA damage, triggering psychosis, a pathology documented in users of d-lysergic acid diethylamide (LSD) or psilocybin.[44,45]

Taken together, limited DNA damage enhances neuroplasticity, inducing antidepressant effects. On the other hand, extensive genomic damage may trigger psychosis or neurodegeneration by converting microglia into a neurotoxic phenotype.

Microbial genotoxins and SPI

The SARS-CoV-2 virus was found to disrupt the human genome, probably accounting for the new-onset psychosis that sometimes accompanies COVID-19 critical illness.[46,47] Indeed, DNA damage-induced neuropathology, though a phenomenon already known before the COVID-19 pandemic, remained underappreciated as a pathogenetic factor of SPI until recently.[48–53]

Several *E. coli* species were demonstrated to produce colibactin, a genotoxic molecule associated with inflammatory bowel disease (IBD), colorectal carcinoma (CRC) and possibly schizophrenia. Moreover, *Morganella morganii* spp. (*M. morganii*) was found to damage the host DNA by releasing indolimines, genotoxic colibactin-like molecules.[54] Interestingly, both *E. coli* and *M. morganii* were previously implicated in schizophrenia, further linking DNA damage to psychopathology.[55] Interestingly, *M. morganii* was demonstrated to cause food poisoning by secreting serotonergic hallucinogens and histamine, connecting this gut commensal to SPI.[56–58]

Aside from damaging the DNA, both colibactin and indolimine can increase the permeability of the gut barrier, facilitating microbial translocation from the GI tract into host tissues.[59–63] In addition, colibactin and indolimine were demonstrated to activate bacteriophages in gut microbes, likely increasing the abundance of bacteriophage-resistant tryptophan decarboxylase-expressing commensals and the levels of endogenous hallucinogens.[64,65] Notably, patients with schizophrenia were recently reported to exhibit altered oropharyngeal bacteriophages (phageome), connecting this disorder to microbial genotoxin-induced DNA damage.[66] Several studies connected interleukin 10 (IL-10) with *E. coli* infection, a microbe associated with genomic damage and schizophrenia (Fig. 1).[67]

Taken together, some gut microbes release genotoxic molecules that can reactivate both latent viruses, such as Epstein–Barr virus (EBV), as well as bacteriophages, triggering various pathologies, including SPI.[68]

The link between the microbiome and SPI

Several studies have reported altered microbial composition in patients with SPI.[69] For example, compared to healthy controls, the fecal microbiome of patients with schizophrenia exhibits an increased abundance of phylum Proteobacteria, especially genus *Succinivibrio*.[70] On the other hand, the oropharyngeal microbiome of individuals with first-episode psychosis (FEP) was found to display increased levels of lactic acid bacteria and *Lactobacillus* phage.[66,71] As lactic acid microbes utilize glucose as a carbon source for generating pyruvate, these changes in the microbiome likely point to the preponderance of glycolysis as compared to oxidative phosphorylation

(OXPHOS) in SPI.[72] Indeed, mitochondrial dysfunction and impaired OXPHOS were previously demonstrated in schizophrenia.[73]

Conclusions

Gut microbiota, comprised of bacteria, fungi and viruses, can underlie pathological circumstances and translocate outside the GI tract, triggering immunogenicity and hyperinflammation that may disrupt both the host genome and DDR, engendering SPI.

Gut microbiota-generated endogenous hallucinogens, acting via TAARs, likely comprise an inbuilt antidepressant system akin to endogenous opioids. Dysfunctional AhR signaling can damage neuronal DNA, inducing SPI by neurotoxic microglia.

More studies are needed to elucidate the antidepressant function as well as the nonmicrobial sources of endogenous hallucinogens, as it is currently uncertain whether these compounds are also synthesized in the human brain.

ORCID iDs

Adonis Sfera https://orcid.org/0000-0002-2406-715X
Sabine Hazan https://orcid.org/0000-0002-6592-3776
Zisis Kozlakidis https://orcid.org/0000-0002-3836-7209

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Special roles of rural primary care and family medicine in improving vaccine hesitancy

Giuliano Piccoliori[1,A,C–F], Verena Barbieri[1,B,C,F], Christian J. Wiedermann[1,2,A,B,D,F], Adolf Engl[1,A,F]

[1] Institute of General Practice and Public Health, Province College for Health-Care Professions "Claudiana", Bolzano, Italy
[2] Department of Public Health, Medical Decision Making and Health Technology Assessment, University for Health Sciences, Medical Informatics and Technology, Hall, Austria

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Giuliano Piccoliori
E-mail: giuliano.piccoliori@am-mg.claudiana.bz.it

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Abstract

Family medicine plays a crucial role in overcoming vaccine hesitancy in rural areas with a limited access to healthcare services and a higher prevalence of vaccine hesitancy. Competent information on diseases and vaccinations provided in a trusting relationship is important to overcome vaccination hesitancy and reach acceptance. This article aims to provide a critical analysis of this issue using methodologically rigorous research and evidence-based recommendations from nonsystematic literature research. Studies on the coronavirus pandemic conducted in South Tyrol, Italy, confirmed that vaccination hesitancy is more common in rural areas than in urban areas, even in economically well-developed Central European regions. The reason for this increased hesitancy is that groups with at-risk sociodemographic characteristics associated with hesitancy are more prevalent in rural areas. This fact assigns a special role to rural primary care and family medicine to be the mediators of vaccinations. Healthcare systems should invest in targeted continuing medical education to promote vaccination literacy among rural healthcare workers and physicians. Therefore, it is important to provide general practitioners with information on common vaccinations. Effective and efficient communication techniques should be improved for successful communication with patients.

Key words: vaccine hesitancy, rural medicine, primary care, prevention

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Introduction

Coronavirus disease 2019 (COVID-19) pandemic presented healthcare services with completely new medical and organizational facts.[1] The infection risk of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and severity of respiratory illness due to unestablished immune protection until the COVID-19 vaccination was developed necessitated new forms of triage as well as isolation of infectious and noninfectious patients in ambulatory and hospital care. This affected acute and intensive care medicine on the one hand,[2,3] and care of the chronically ill on the other.[4] Due to the nature of things, acute medical care could not be delayed and was prioritized, whereas other medical services, such as elective specialist care, had to be postponed, and preventive medicine interrupted.

In addition to the emergency rooms and intensive care units, primary care and family medicine were particularly challenged.[5] Online medical consultations through standard video conferencing systems were initiated to care for COVID-19 patients as pilot projects, but only a limited number of patients participated.[6] In many healthcare systems, including South Tyrol, Italy, virtual consultation disappeared after the pandemic. In South Tyrol, during the COVID-19 pandemic, there was a list of telephone numbers allowing family physicians to communicate directly with hospital departments; however, this is no longer active. In Italy, the electronic generation of prescriptions for patients (in direct communication with pharmacies) was rapidly introduced in the context of pandemic emergency regulations, which the Italian National Health Service has recently decided to maintain. The attempt to communicate online with patients has contributed little to the overall management of the pandemic. Family medicine was largely unprepared, and the pandemic highlighted weaknesses in general medical care.

Even though, especially in primary care and family medicine, new ways of communicating with patients, hospitals, health administrators, and authorities – innovations that had been considered unsuccessful before the pandemic – were quickly found within the framework of organizational adjustments, the weaknesses of the respective healthcare systems and institutions became obvious.[4] In one way or the other, better digitalization in the whole healthcare system would have been desirable to allow a better communication and information flow between the several actors of the health system and patients.[7] The negative effects resulting in the overstrain of human actors have been documented in numerous ways, including among general practitioners.[7] Moreover, the inevitable inadequacy of medical services for some patients has been reported.[4]

This article aimed to raise awareness on the issue of vaccine hesitancy in family medicine and offer recommendations for healthcare providers on how to address it. The commentary should contribute to a wider discussion about vaccine hesitancy and promote evidence-based practices in family medicine.

Materials and methods

A nonsystematic review of the literature in PubMed and EMBASE for data collection and analysis was performed in order to identify the key factors contributing to vaccine hesitancy in family medicine, and provide evidence-based recommendations for healthcare providers on how to address vaccine hesitancy, such as effective communication techniques and addressing patient concerns. This highlights the importance of addressing vaccine hesitancy in family medicine for public health and community wellbeing.

Results

SARS-CoV-2 vaccines

In dealing with the pandemic, attention was initially limited to nonpharmacological measures. Vaccines against SARS-CoV-2 were then developed, tested and approved at unprecedented speed.[8] It soon became clear that because of the global spread of the disease, herd immunity could no longer be achieved even with mass vaccination.[9] However, observational studies and pharmacovigilance were conducted globally to create large datasets and confirm the safety and effectiveness of the vaccines, making it clear that vaccinations would end the pandemic.[10] Thus, global vaccine availability, vaccination rates, and overcoming vaccination hesitancy and aversion have become a focus of public health research.[11,12]

It is critical to better understand what determines vaccine acceptance, including vaccination intention and the factors that could determine hesitancy and resistance. The determining factors for hesitancy included individual and demographic characteristics such as distrust in authority, risk aversion, disgust sensitivity, age, economic situation, educational status, type of parenthood, and rural residence.[13] From a public health perspective, special attention must be paid to vaccination hesitancy among the rural population. Overcoming vaccination hesitancy is accomplished by providing information through a trusted and competent medical specialist.[14] This task falls primarily to primary care and family medicine.

Urban–rural disparities in vaccine hesitancy

There are several urban–rural disparities in vaccine hesitancy which may affect the vaccination rates of different geographic areas.[15] Rural areas can have limited access to healthcare facilities and providers, which makes it more difficult for residents to receive vaccinations. This lack of access to healthcare contributes to vaccine hesitancy, as patients may not trust healthcare providers or may not have received adequate education regarding

the importance of vaccinations. Rural areas may have political and cultural beliefs different from those in urban areas, which can influence vaccine hesitancy. For example, some rural areas may have higher rates of conservatism or religious fundamentalism, which may be associated with lower vaccination rates. Also, such areas may have lower levels of education and awareness of the importance of vaccinations, which contributes to vaccine hesitancy. Lack of access to information and education about vaccines and their benefits can lead to misconceptions and misinformation regarding their safety and efficacy. Social networks can also influence vaccination hesitancy. Rural areas have tight-knit communities, where social norms may discourage vaccination and increase vaccine hesitancy. Rural residents can have a greater mistrust of medical authorities, which also contributes to vaccine hesitancy. This may be due to historical or current experiences of discrimination, limited access to healthcare or lack of familiarity with medical practices.[15]

Overall, urban–rural disparities leading to vaccine hesitancy can have significant public health implications, contribute to lower vaccination rates in rural areas and increase the risk of disease outbreaks. It is important to address these disparities through targeted public health campaigns and educational programs that discuss the unique factors contributing to vaccine hesitancy in rural areas.

Urban–rural discrepancies in vaccination in South Tyrol, Italy

Italy's COVID-19 vaccination rate is among the highest in Europe.[16] Within Italy, however, significant regional differences have been reported. The northernmost province, close to the border with Austria, South Tyrol, had the lowest vaccination rate in the country and highest infection incidence in September 2021.[17] South Tyrol is an autonomous province in Italy with a special statute and language groups, and the majority of its 535,000 inhabitants are native German speakers.

In South Tyrol, a representative survey on the COVID-19 pandemic, including hesitant vaccination behaviors, was conducted in March 2021. The results showed vaccination hesitancy in 15.6% of participants.[18] Increased hesitancy was mostly observed in young patients with an absence of chronic disease, mistrust of institutions and conspiracy thinking. Also, worse economic situation was linked to vaccine hesitancy. Importantly, rural residence was associated with greater hesitancy, although due to the alpine landscape and the relatively small inhabitable area, the population density was high, and the country was economically well developed. Vaccine hesitancy differed significantly among language groups and was higher in German speakers than in Italian speakers (15.8% compared to 11.7%). However, a linguistic group was not an independent predictor of vaccine hesitancy.[18]

Since more members of the German-language group live in rural areas than in urban areas, and because rurality is a known risk factor for increased vaccination hesitancy, it was natural to look more closely at the question of the influence of residence type on vaccination hesitancy. A second analysis of the survey data showed that hesitant vaccination behaviors were significantly higher in rural than in urban areas (17.6% compared to 12.8%, $p = 0.013$). Sociodemographic differences between rural and urban areas were numerous: rural populations tended to have worsened economic situations as a result of the pandemic, were at an educational disadvantage and more often had young children (under 6 years of age) – all of these factors were associated with increased vaccination hesitancy. The fact that there were fewer chronic diseases and more COVID-19-related deaths in rural areas, other predictors of vaccination hesitancy according to the survey, are likely to be responsible for the urban–rural differences. Finally, national public health institutions placed less trust in pandemic management, as was their trust in local authorities, civil protection and local health services. Similarly to the effects of language group membership on vaccine hesitancy,[18] neither rural nor urban residence was an independent predictor in the regression analyses. The relatively higher vaccination hesitancy in rural areas of more than 1/3 can be adequately explained by known predictors.[19] Thus, several predictors of COVID-19 vaccine hesitancy were more prevalent in rural than in urban areas and among members of the German-speaking linguistic group, which may explain the lower vaccine uptake in rural areas.

Hesitant people are more likely to talk to their primary care physician about vaccinations than people who are vaccinated when it is generally recommended.[20] Larson et al. wrote in their review on vaccine hesitancy: "Given that physicians and other healthcare providers are still among the most trusted when it comes to healthcare advice, local information about the nature and scope of vaccine hesitancy in their communities may help them anticipate and support important conversations in the clinic."[21] Factors linked to vaccine hesitancy were found to be more prevalent in rural populations[19] that are amenable to targeted medical counseling.

Discussion

Family medicine plays a crucial role in overcoming vaccine hesitancy in rural areas where there may be limited access to healthcare services and a higher prevalence of vaccine hesitancy.[22,23] Details and practical examples of ways in which family medicine physicians can help address vaccine hesitancy in rural areas are presented in Table 1. By taking these steps, family physicians can increase vaccination rates and improve the health of their communities.[23]

Table 1. Family physician's steps to increase vaccination rates

Measure	Explanation
Building trust[24]	Family physicians are often highly respected members of the community in rural areas. They can use their existing relationships and credibility to build trust with patients who are hesitant about vaccines. By engaging in conversations and providing evidence-based information about vaccines, family physicians can address concerns and help patients make informed decisions about vaccination.
Educating patients[25]	Family physicians are in a unique position to educate patients about the importance of vaccines and the potential benefits and risks. They can also address myths and misinformation surrounding vaccines, which are often major drivers of vaccine hesitancy. By providing accurate information, family physicians can help patients understand the risks of vaccine-preventable diseases as well as the safety and effectiveness of vaccines.
Offering convenient access[26]	Rural areas may have limited healthcare resources, which can make it difficult for patients to access vaccines. Family physicians can help overcome this barrier by offering vaccines in their clinics or by coordinating with local health departments or pharmacies to provide vaccines in the community. By offering convenient access to vaccines, family physicians can increase vaccination rates in their communities.
Encouraging vaccination among vulnerable populations[27]	Rural areas may have populations that are particularly vulnerable to vaccine-preventable diseases, such as the elderly or those with chronic illnesses. Family physicians can play a key role in identifying these populations and encouraging them to get vaccinated. They can also work with local community organizations and public health agencies to promote vaccination among these populations.

Table 2. Communication attitudes when counselling people who are hesitant to vaccinate[29,30]

Recommendation	Explanation
Active listening and empathy[31]	An important first step is to actively listen to the patients and show empathy for their concerns and fears. It is important to understand the patient's perspective in order to address their concerns effectively.
Asking open-ended questions[32]	Open-ended questions can help initiate and guide the conversation. Open-ended questions encourage the patient to share their thoughts and concerns. For example: "What are your concerns regarding vaccination?"
Providing information[33]	It is important to provide patients with the facts and information they need in order to make an informed decision. The information should be clear and understandable to avoid any misunderstandings or misinformation.
Showing empathy[34,35]	Empathy can help build a relationship between the healthcare provider and the patient, and gain the patient's trust. Empathy involves putting oneself in the patient's shoes and understanding their feelings and concerns.
Motivational interviewing[36]	Motivational interviewing is a technique that aims to increase the patient's motivation to vaccinate. By using open-ended questions, positive reinforcement and collaboration, the patient can be encouraged to reconsider and change their attitude towards vaccination.
Avoiding persuasion[37]	The use of persuasion or pressure can cause the patient to become defensive and further entrench their position. Instead, the healthcare provider should take an open and supportive approach and help the patient make an informed decision.

Effective interviewing is crucial in the medical counseling of people who are hesitant to be vaccinated.[28] Evidence-based recommendations and techniques can be applied to improve the course of the conversation (Table 2).

Communicating with conspiracy thinkers about vaccinations can be challenging, as their beliefs can be deeply held and difficult to change. Concrete recommendations on how to counsel such individuals are presented in Table 3. It is important to keep in mind that changing the beliefs of conspiracy thinkers is a difficult and ongoing process. Healthcare providers should aim to establish a respectful and trusting relationship with their patients and continue to provide accurate information and support for vaccinations over time.

These techniques can be learned and practiced through specialized training and courses for healthcare providers aimed at ensuring effective and patient-centered interviews. For example, the United Kingdom National Health Service offers courses and training programs. However, further research on vaccine hesitancy in family medicine and the effectiveness of different interventions is warranted.

This study is limited by the nonsystematic nature of data collection and analysis. The weighting of the results could have been biased by the authors' study, which was conducted in South Tyrol.

Conclusions

Observations indicate that there is no need for a fundamentally strategic difference in communication aimed at improving vaccination readiness between urban and rural populations. Competent information on the diseases and vaccinations provided in a trusting relationship is important to overcome vaccination hesitancy and increase acceptance. Studies on the COVID-19 pandemic conducted in South Tyrol, Italy, confirmed that vaccination hesitancy is more common in rural than in urban areas, even in economically well-developed Central European regions. The reason for this is that risk groups with sociodemographic characteristics associated with hesitancy are more prevalent in rural than in urban areas.

Table 3. Communication attitudes when counselling people who are conspiracy thinkers

Recommendation	Explanation
Avoid confrontation[38,39]	It is important to avoid confrontation and argumentation with conspiracy thinkers. Instead, the focus should be on building rapport, finding common ground and addressing their concerns in a respectful and nonjudgmental manner.
Acknowledge their concerns[40]	Conspiracy thinkers may have legitimate concerns or fears related to vaccination. It is important to acknowledge their concerns and show empathy for their perspective.
Provide accurate information[40]	Provide accurate and credible information about vaccination to counter misinformation and conspiracy theories. Use trusted sources of information, such as the World Health Organization or the Centers for Disease Control and Prevention.
Use plain language	Use plain language and avoid medical jargon to ensure that the information is understandable to the patient.
Address mistrust of authorities[40]	Conspiracy thinkers may have a general mistrust of authorities, including healthcare providers. It is important to address their mistrust and build trust through respectful communication and by providing accurate information.
Use storytelling	Stories can be a powerful way to communicate the benefits of vaccination. Share stories of individuals who have been vaccinated and have had positive outcomes.
Provide resources[40]	Provide resources, such as websites, brochures or videos, that patients can access to learn more about vaccination.

Thus, rural family doctors play special roles as mediators of vaccination.[41] Healthcare systems should invest in targeted continuing medical education to promote vaccination literacy among rural healthcare workers and physicians.[42,43] It should be emphasized that family physicians need specific training not only on particular vaccinations but also on how to talk to vaccination skeptics and opponents, which is not easy and often leads to conflicts rather than success.

ORCID iDs

Giuliano Piccoliori https://orcid.org/0000-0003-1974-4184
Verena Barbieri https://orcid.org/0000-0002-5934-9472
Christian J. Wiedermann https://orcid.org/0000-0002-4639-8195

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The relationship between non-alcoholic fatty liver disease and incidence of chronic kidney disease for diabetic and non-diabetic subjects: A meta-analysis

Yueqiao Chen[1,A–C,E,F], Wenjie Bai[2,A–C,E,F], Dewen Mao[3,A–D,F], Fuli Long[3,A,C–F], Na Wang[3,A–E], Kejing Wang[4,A–F], Qinglan Shi[3,A–D,F]

[1] Department of Liver Diseases, The First Affiliated Hospital of Guangxi University of Chinese Medicine, China
[2] Foreign Language Department, Guangxi University of Chinese Medicine, China
[3] Department of Liver Diseases, Ruikang Hospital Affiliated to Guangxi University of Chinese Medicine, China
[4] Hepatobiliary Surgical Department, Ruikang Hospital Affiliated to Guangxi University of Chinese Medicine, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Kejing Wang
E-mail: zy98314@163.com

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Abstract

Introduction. The prevalence of chronic kidney disease (CKD) rises with age and co-morbid diseases such as liver diseases.

Objectives. The main aim of the current meta-analysis is to assess the relationship between Non-alcoholic fatty liver disease (NAFLD) and chronic kidney disease incidence in both diabetic and non-diabetic subjects compared with control.

Materials and methods. A systematic literature search of papers published from January 1, 2005, till April 30, 2022, found 19 studies including 1,111,046 subjects; 310,804 were diagnosed with NAFLD, and 800,242 were non-NAFLD. The measured outcome was the incidence of CKD among NAFLD subjects compared to non-NAFLD subjects in diabetic and non-diabetic subjects. Dichotomous analysis methods were used within the random effects model to calculate the odds ratio (OR) with 95% confidence intervals (95% CIs).

Results. The incidence of CKD is highly significant in NAFLD subjects compared with controls (OR: 1.95; 95% CI: 1.65–2.31). The diabetic non-NAFLD subjects showed a significantly increased incidence of CKD compared to the non-diabetic subjects with NAFLD (OR: 1.79; 95% CI: 1.35–2.38).. In addition, the incidence of CKD was significantly higher in the NAFLD group compared with the non-NAFLD non-diabetic subjects (OR: 2.52; 95% CI: 1.91–3.32). Diabetes acts as an independent risk factor for CKD, as proven by a significant increase in incidence of diabetic subjects compared to non-diabetic NAFLD subjects (OR: 1.82; 95% CI: 1.15–2.88).

Conclusions. Non-alcoholic fatty liver disease is significantly related to an increased incidence of CKD, which is significantly higher in diabetic subjects.

Key words: diabetes, NAFLD, kidney function, chronic kidney disease

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Introduction

The prevalence of chronic kidney disease (CKD) rises with age, affecting around 25% of those aged over 65 in the Western world.[1] Consistent with the epidemic expansion of major risk factors including aging, diabetes, obesity, metabolic syndrome, smoking, and hypertension, the prevalence of CKD is increasing.[2,3] More than 400,000 Americans are already undergoing renal replacement therapy, and this figure is expected to increase to 2,200,000 by 2030.[2] The majority of patients with CKD die from cardiovascular disease (CVD) before the renal replacement treatment can begin,[4] making CKD a key risk factor for end-stage renal disease (ESRD), as well as CVD. The health effects of CKD may be minimized by an early detection and treatment that slows the progression of renal disease and reduces CVD.[3] Early referral efforts for patients with stage 3 of CKD are the most useful. Despite these facts, CKD is frequently misdiagnosed; according to the Third National Health and Nutrition Examination Survey (NHANES III), only 8.2% of persons with stage 3 of CKD were aware of their disease.[5] Due to the disease's high morbidity and mortality rates, as well as high related healthcare expenditures, researchers are searching for novel modifiable risk factors for CKD. Thirty percent of the adult population has non-alcoholic fatty liver disease (NAFLD), the hepatic manifestation of metabolic syndrome.[6] Sixty to seventy percent of those with diabetes and obesity are affected. Under normal conditions, reactive oxygen species (ROS) are a key part of cell signaling, which is involved in cell growth, division, death, and immune defense in many cell lineages, including renal cells. However, in diabetes, the kidneys produce too much ROS. This causes inflammation which changes the structure and function of the kidneys and eventually leads to ESRD. The production of ROS caused by hyperglycemia encourages the recruitment of many inflammatory cells and increases the production of inflammatory cytokines, growth factors and transcription factors that are involved in the pathological processes of diabetic nephropathy.[7] Non-alcoholic fatty liver disease can range histologically from simple steatosis to non-alcoholic steatohepatitis (NASH), the latter of which can involve significant fibrosis. Independently of metabolic syndrome and existing risk factors, NAFLD raises the risk of cirrhosis, which is primarily limited to NASH, as well as CVD.[8] Experiments and epidemiological research are accumulating evidence indicating that NAFLD and CKD interact and share pathogenic mechanisms.[9] In the published literature, small study populations and marginal relationships between NAFLD and recognized risk factors for CKD cast doubt on the existence of a link between NAFLD and CKD.

Objectives

The main aim of the current meta-analysis is to assess the relationship between NAFLD and CKD incidence in diabetic and non-diabetic subjects compared with controls.

Materials and methods

Based on the epidemiological declaration, a methodology was developed and the eligible studies were analyzed.

Criteria for study selection and eligibility criteria of the study

The purpose of the current meta-analysis was to examine the association between NAFLD and the occurrence of CKD in diabetic and non-diabetic subjects compared with controls (non-NAFLD subjects) using statistical methods, such as frequency rate, odds ratio (OR), relative risk, or mean difference (MD) with a 95% confidence interval (95% CI).

The current meta-analysis was open to studies of any size, but research letters and review articles were not included since they did not provide sufficient evidence of causality to meet the inclusion criteria. The conceptualization of the meta-analysis is presented in Fig. 1. Diabetic and non-diabetic patients with and without NAFLD were compared for their sensitivity to CKD.

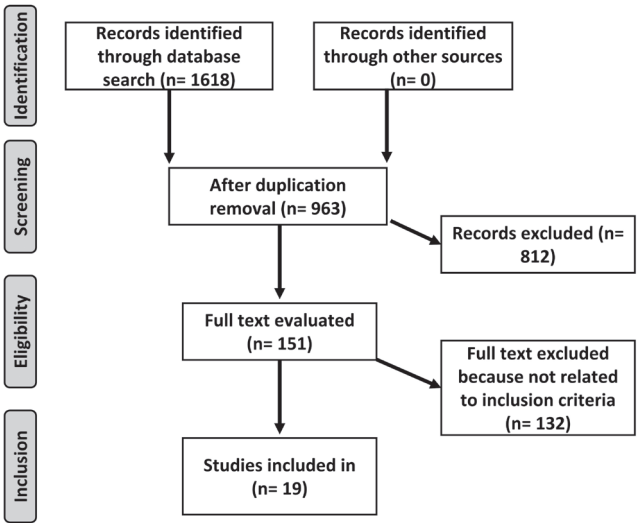

Fig. 1. Illustration diagram presenting the mode of meta-analysis

Inclusion criteria

Randomized controlled trials, prospective studies and retrospective studies were included in this study. Studies comparing NAFLD and controls conducted on human population and estimating the role of NAFLD and diabetes in the development of CKD (glomerular filtration rate

(GFR) < 60 mL/min/1.73 m^2) were taken into account. Finally, studies that examine the prevalence of CKD in people with NAFLD who are either diabetic or non-diabetic were also included.

Exclusion criteria

Studies excluded from the current analysis were those that did not investigate the impact of NAFLD or diabetes on CKD incidence or did not analyze feeding habits. In addition, studies using outcome measures that are unreliable, incomplete or deceptive were also excluded. Finally, studies that did not compare subjects with NAFLD to subjects without NAFLD, or those that did not compare subjects with diabetes to people without diabetes, were considered low quality and unsuitable for inclusion.

Search strategy, study selection and data extraction

Identification

The search strategy is shown in Table 1. "NAFLD", "CKD", "diabetic", "kidney function", and similar terms were used to conduct a comprehensive literature search in MEDLINE/PubMed, the Cochrane Library, OVID, Embase, and Google Scholar published from January 1, 2005, till April 30, 2022. The PICOS process had been used during the identification and screening of the articles: 1) population (P): NAFLD; 2) intervention/exposure (I): monitoring CKD incidence in NAFLD subjects compared with control for both diabetic and non-diabetic subjects (comparison (C)); 3) outcome (O): incidence of CKD. Study types (S) include both randomized clinical trials and retrospective studies. The EndNote software (Clarivate, London, UK) was used to classify the research publications to eliminate duplicates. To further assess the relationship between NAFLD and CKD incidence in both diabetic and non-diabetic subjects compared with controls, we reviewed all titles and abstract data. All relevant data for this topic were collected from the studies we considered.

Table 1. Strategy of searching scientific databases

Database	Search strategy
PubMed	#1 "non-alcoholic fatty liver disease" (MeSH terms) OR "chronic kidney disease" (MeSH terms) OR "diabetes" (all fields) #2 "kidney function" (MeSH terms) OR "non-diabetic" (all fields) #3 #1 AND #2
Embase	#1 "non-alcoholic fatty liver disease"/exp OR "NAFLD"/exp OR "liver failure"/exp #2 "chronic kidney disease"/exp OR "CKD"/exp #3 #1 AND #2
Cochrane Library	#1 "non-alcoholic fatty liver disease": ti, ab, kw OR "NAFLD": ti, ab, kw OR "liver failure": ti, ab, kw (word variations have been searched) #2 "chronic kidney disease": ti, ab, kw OR "CKD": ti, ab, kw (word variations have been searched) #3 #1 AND #2

ti, ab, kw – terms in the title, abstract or keyword fields; exp – exploded indexing term.

Screening

All of the information relevant to the subjects and the research was recorded into a standardized database. It included the information about the study's setting, primary outcome evaluation, treatment mode, duration, categories, statistical analysis, information source, and qualitative and quantitative evaluation, as well as the first author's surname and the total number of subjects.

The "Risk of Bias Tool" from the Cochrane Reviewer Manger v. 5 (https://training.cochrane.org/online-learning/core-software/revman/revman-5-download) was used to assess the methodology's robustness. The screening process was carried out by 2 authors (YC and WB).

Data extraction

Outcomes to be evaluated from the included studies were the incidences of CKD in NAFLD subjects compared with controls. Data collected from each study had been collected in separate forms by 2 authors working separately (QS and FL); then, the extracted data were compared and evaluated by a 3rd author (KW). Extracted items from each study were authors, year of publication, country of the study, total number of included subjects, number of interventional groups, numbers of the control group, the final conclusion, and outcomes related to the meta-analysis criteria. Next, studies were categorized into subgroup sections according to the measured outcomes from each study.

Data synthesis and analysis

Odds ratios and 95% CIs were determined dichotomously in the statistical analysis utilizing the random effects model. To begin with, the I^2 index was measured from 0% to 100%, whereas the heterogeneity scale included 0%, 25%, 50%, and 75%, representing no, low, moderate, and high levels of heterogeneity, respectively. If the value of I^2 was greater than 50%, the random effect was prioritized over the fixed influence. The fixed model is suitable for use with studies with a high degree of similarities and with low heterogeneity. In the current study, all analyses were carried out using the random model. Forest plots were generated and they showed p-values and I^2 of different subgroup analyses. Since a value of $p < 0.05$ was required to draw any conclusions, we used a subgroup analysis on the first dataset. The publication bias was assessed with the Begg's test and visual examination of funnel plots. The Reviewer

Manager, v. 5.4.1 (The Cochrane Collaboration, Copenhagen, Denmark), was used for the statistical analysis with two-tailed p-values.

Bias risk in the criteria for assessment

The examination of the criteria reveals 3 distinct types of prejudice. In other words, the risk of bias were rated from low (when all quality parameters were met) to moderate (when some of the quality parameters were met but not all) to high (when none of the quality criteria was met or included). The examination of the paper revealed similar anomalies.

Two authors (DM and FL) reviewed the publications independently to evaluate the risk of bias, and a 3rd author (NW) assessed the criteria in case the initial check results were not identical from the 2 authors.

Results

Among the 1618 unique reports, the current meta-analysis included 19 studies[10–28] published between 2008 and 2021 that matched the inclusion criteria. The study groups in these papers consisted of 1,111,046 subjects in total; 310,804 were diagnosed with NAFLD, and 800,242 were non-NAFLD (Table 2).

NAFLD compared to non-NAFLD

The relationship between NAFLD and CKD incidence in diabetic and non-diabetic subjects compared with the control group was assessed, including a total of 18 clinical trials which compared the incidence of CKD in NAFLD subjects to the control group (non-NAFLD). As shown in Fig. 2–5, the incidence of CKD is highly significant ($p < 0.001$) in NAFLD subjects compared to controls (OR: 1.95; 95% CI: 1.65–2.31).

Diabetic subjects

Five studies compared the incidence of CKD between NAFLD and non-NAFLD for diabetic subjects, while 6 studies assessed the impact of diabetes on the incidence of CKD in NAFLD subjects compared with non-diabetic NAFLD subjects as control. The impact of diabetes as comorbidity significantly increased the incidence of CKD in non-diabetic subjects with NAFLD ($p < 0.001$; OR: 1.79; 95% CI: 1.35–2.38) compared with non-NAFLD. Diabetes acts as an independent risk factor for CKD, as proven by a significant increase in CKD incidence for diabetic compared with non-diabetic NAFLD subjects ($p = 0.01$; OR: 1.82; 95% CI: 1.15–2.88).

Non-diabetic subjects

Only 3 studies compared the non-diabetic NAFLD group with the non-NAFLD group of non-diabetic

Table 2. Characteristics of the studies included in the meta-analysis

Study	Year	Country	Total	NAFLD	Non-NAFLD
Adams et al. [11]	2005	USA	402	201	201
Targher et al. [25]	2008	Italy	2103	1421	682
Campos et al. [15]	2008	USA	197	123	74
Chang et al. [17]	2008	South Korea	8329	2516	5813
Hwang et al. [19]	2010	South Korea	1361	748	613
Targher et al. [24]	2010	Italy	160	80	80
Athyros et al. [14]	2010	Greece	720	210	510
Park et al. [21]	2011	USA	562	66	496
Sirota et al. [23]	2012	Italy	11,469	4179	7290
Targher et al. [26]	2012	Italy	343	182	161
Xia et al. [27]	2012	China	1141	477	664
Musso et al. [20]	2012	Italy	80	40	40
Ahn et al. [12]	2013	South Korea	1706	545	1161
El Azeem et al. [10]	2013	Egypt	738	268	470
Park et al. [22]	2019	USA	1,032,497	262,619	769,878
Zhang et al. [28]	2020	China & USA	11,844	4273	7571
Akahane et al. [13]	2020	Japan	3725	1154	2571
Chen et al. [18]	2020	China	29,797	29,797	–
Cao et al. [16]	2021	China	3872	1905	1967
Total			1,111,046	310,804	800,242

NAFLD – non-alcoholic fatty liver disease.

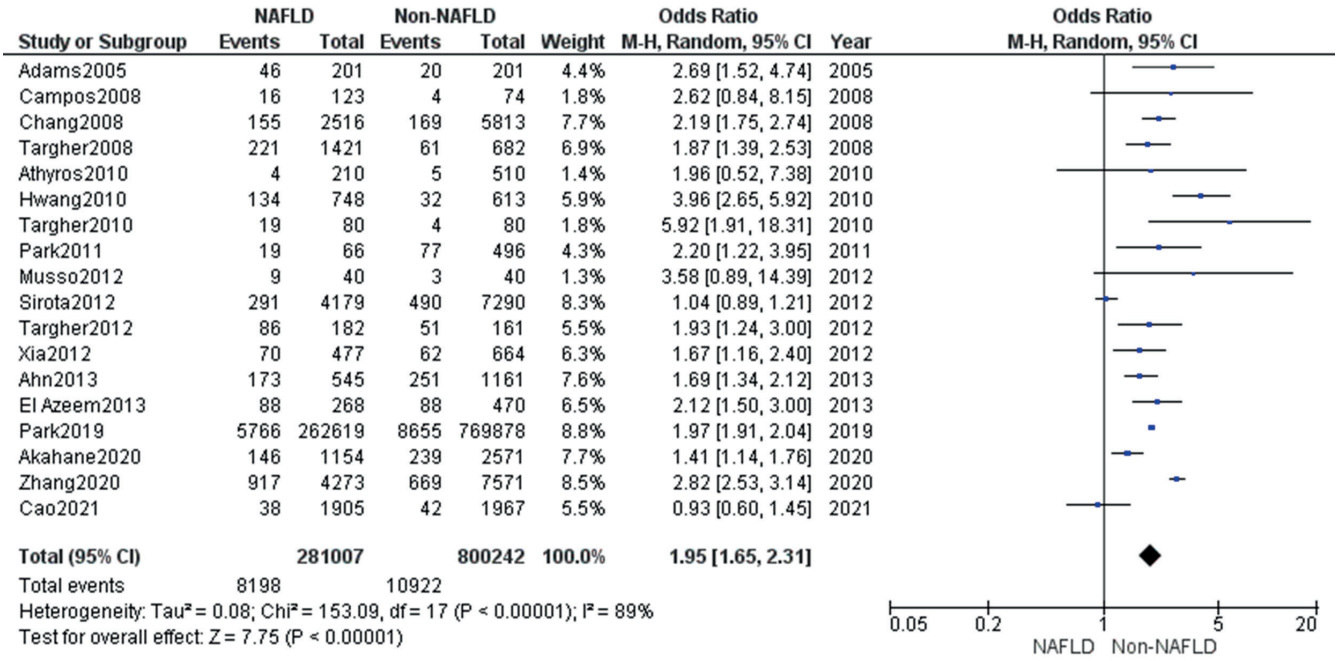

Fig. 2. A forest plot illustrating the impact of non-alcoholic fatty liver disease (NAFLD) compared to non-NAFLD groups on the incidence of chronic kidney disease (CKD)

95% CI – 95% confidence interval; df – degrees of freedom.

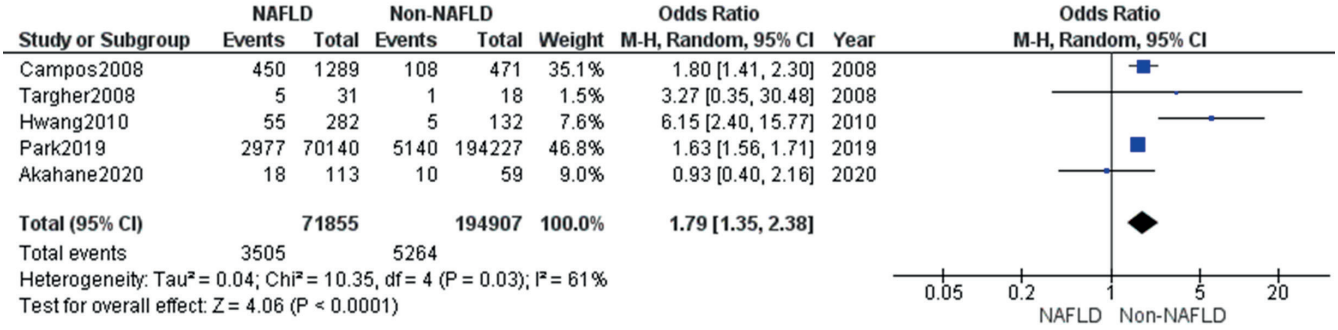

Fig. 3. A forest plot illustrating the impact of diabetic non-alcoholic fatty liver disease (NAFLD) compared to non-NAFLD groups on the incidence of chronic kidney disease (CKD)

95% CI – 95% confidence interval; df – degrees of freedom.

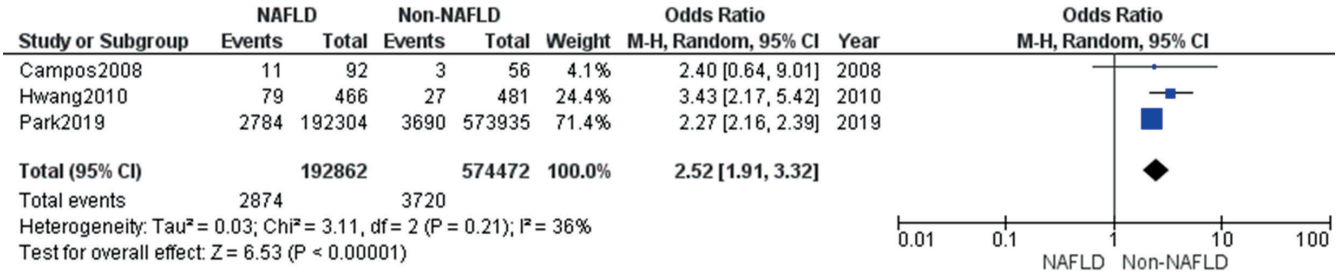

Fig. 4. A forest plot illustrating the impact of non-alcoholic fatty liver disease (NAFLD) compared to non-NAFLD groups on the incidence of chronic kidney disease (CKD)

95% CI – 95% confidence interval; df – degrees of freedom.

subjects. In addition, the incidence of CKD was significantly higher in the NAFLD group compared with non-NAFLD non-diabetic subjects ($p < 0.001$; OR: 2.52; 95% CI: 1.91–3.32).

The Begg's test p-values were statistically non-significant for included studies,[10–28] but these values were variable. The p-value related to the studies comparing NAFLD with non-NAFLD subjects was $p = 0.37$. In addition, for the analysis

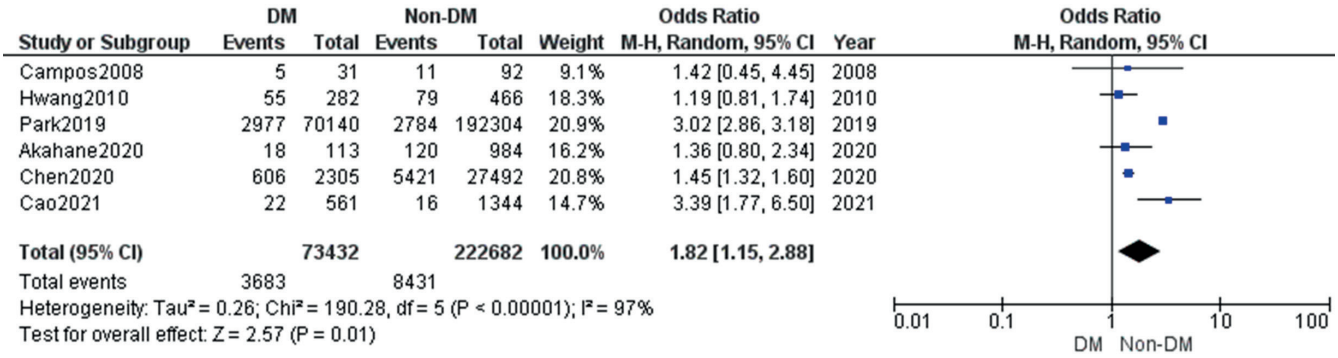

Fig. 5. A forest plot illustrating the impact of non-alcoholic fatty liver disease (NAFLD) subjects with and without diabetes on the incidence of chronic kidney disease (CKD)

95% CI – 95% confidence interval; df – degrees of freedom.

comparing diabetic subjects with control the p-value was 0.82, while for the analysis comparing non-diabetic subjects with control it equalled 0.9. The Begg's test for analysis comparing diabetic with non-diabetic subjects showed p = 0.72. On the other hand, a visual examination of funnel plots showed the presence of publication bias as supported with asymmetric distributions of studies (Fig. 6).

We found that no single study had sufficient data in all 7 categories. Throughout the quality spectrum, the procedures of the included studies varied greatly. The quality of the studies used in this meta-analysis ranged widely. The randomized trial was determined to have insufficient methodological tools.

Discussion

The aim of the study was to measure and assess the relationship between NAFLD and CKD incidence in both diabetic and non-diabetic subjects compared with controls.

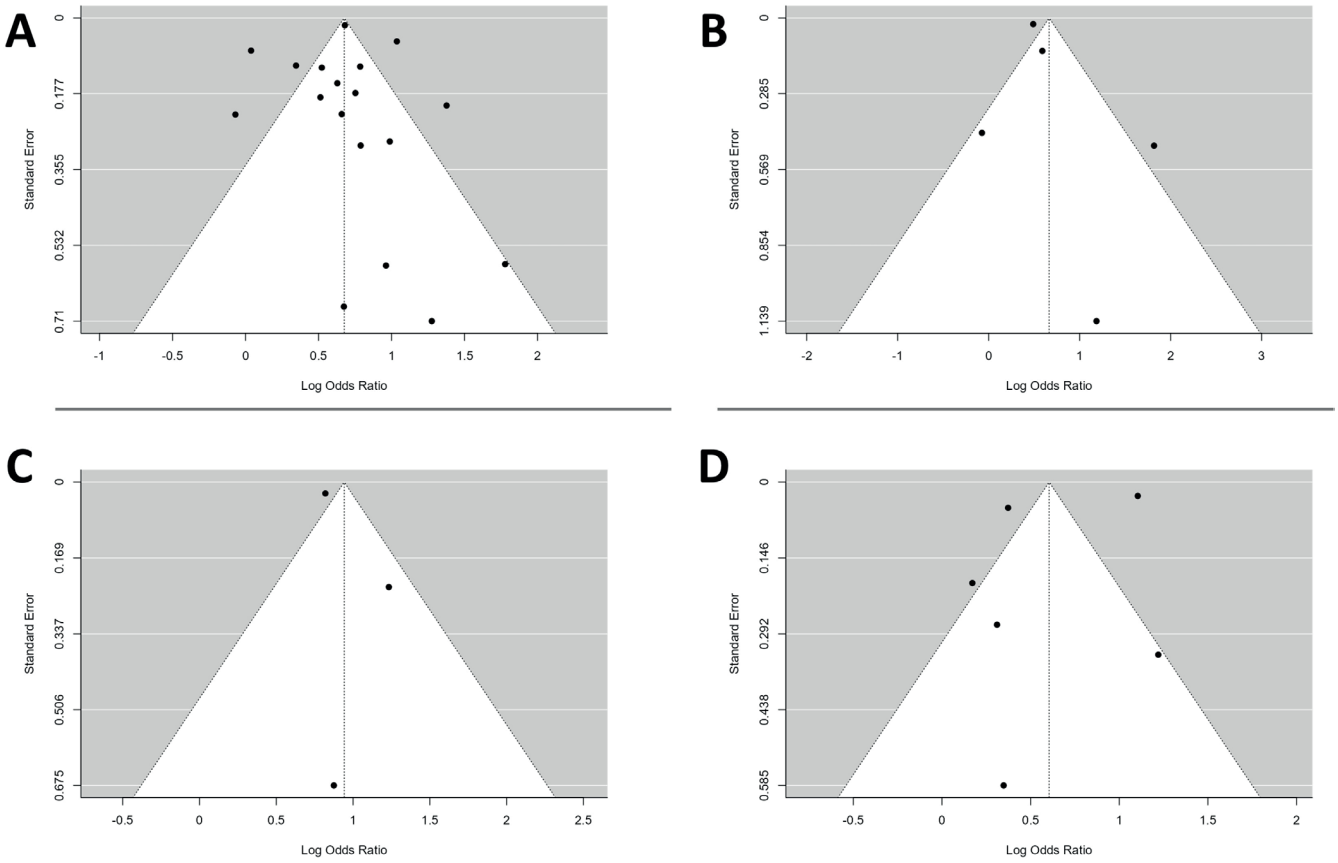

Fig. 6. Funnel plot showing the publication bias for non-alcoholic fatty liver disease (NAFLD) compared to non-NAFLD groups (A), diabetic group compared to control group (B), non-diabetic group compared to control group (C), and diabetic group compared to non-diabetic group (D)

Findings showed that the incidence of CKD is significantly higher in NAFLD subjects compared with controls. The diabetic non-NAFLD subjects showed significantly increased incidence of CKD compared to the non-diabetic subjects with NAFLD (OR: 1.79; 95% CI: 1.35–2.38). In addition, the incidence of CKD was significantly higher in the NAFLD group compared to the non-NAFLD non-diabetic subjects. Diabetes acts as an independent risk factor for CKD, as proven by a significant increase in incidence in diabetic compared with non-diabetic NAFLD subjects. However, because some of the included studies had a small sample size (3 studies had a sample size of less than 200 subjects), a careful analysis of the results is required, implying the necessity for further trials to confirm the current findings; such research could possibly have a substantial effect on the assessment of the intervention impact. The heterogeneity was high for the compared studies, hence subgroup analyses were performed to provide strong evidence for the final conclusion.

Hwang et al. findings imply that NAFLD is associated with an increased frequency of microalbuminuria in persons with prediabetes and newly diagnosed diabetes.[19] This correlation appears to hold even after controlling for potential confounders, such as age, gender, race/ethnicity, education, smoking status, and the presence of other Adult Treatment Panel III (ATP III)-defined components of the metabolic syndrome. Non-alcoholic fatty liver disease may play a role in mediating the elevated risk of CKD in subjects with microalbuminuria. However, as our investigation was limited to people with diabetes or prediabetes, we did not examine the influence of NAFLD on microalbuminuria in subjects with normal glucose levels.

Ahn et al. showed that NAFLD is substantially linked with CKD in the South Korean population aged 50 years or older.[12] The link between NAFLD and CKD remained statistically significant after analyzing for age, sex, current smoking, abdominal obesity, aspartate aminotransferase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (GGT), hypertension, diabetes mellitus, hypertriglyceridemia, and low high-density lipoprotein (HDL).

Over a million of Americans are predicted to have ESRD by 2015,[29] as the incidence of CKD continues to skyrocket. In addition to progressing to ESRD, CKD is also a major risk factor for CVD, and most persons with CKD die from CVD before they acquire ESRD. As a result, a lot of effort is being put into identifying potential causes of CKD that can be addressed by lifestyle changes. Non-alcoholic fatty liver disease is a growing risk factor for end-stage liver disease and CVD: the frequency of NASH as the major rationale for liver transplantation has risen from 1.2% to 9.7% in the last decade, becoming the 3rd most prevalent cause for liver transplantation in the USA.[30]

The key findings of our analysis are the following: NAFLD was associated with an increased prevalence and incidence of CKD. In addition, these associations remained statistically significant in diabetic and non-diabetic individuals, as well as in studies adjusting for traditional risk factors for CKD, and were independent of whole body/abdominal obesity and insulin resistance.

Limitations

Many publications were left out of the current meta-analysis because they did not meet the inclusion criteria, which introduced a substantial amount of bias into the study. There was also a considerable uncertainty regarding how to incorporate factors such as gender and race into the analysis. Analyses based on data from previous studies may be flawed due to information gaps. Twenty papers were included in the meta-analysis, 3 of which were very small (under 200 participants). Lost data and unpublished studies may contribute to the problem of influence bias. Studies differed in the average weight of their subjects.

Conclusions

This meta-analysis showed that the incidence of CKD is highly significant in NAFLD subjects compared with controls. The diabetic non-NAFLD subjects showed a significantly increased incidence of CKD compared to the non-diabetic subjects with NAFLD (OR: 1.79; 95% CI: 1.35–2.38). In addition, the incidence of CKD was significantly higher in the NAFLD group compared with non-NAFLD non-diabetic subjects. Diabetes acts as an independent risk factor for CKD, as proven by a significant increase in incidence for diabetic compared with non-diabetic NAFLD subjects. The results of our meta-analysis study did not show any correlation with demographic variables, such as participants' race or gender. Additional research is needed to validate these findings or significantly increase confidence in the effect evaluation because of the small sample sizes in several of the studies included in the meta-analysis.

ORCID iDs

Yueqiao Chen https://orcid.org/0000-0001-7801-4347
Wenjie Bai https://orcid.org/0000-0002-4962-6771
Dewen Mao https://orcid.org/0000-0001-9438-9325
Fuli Long https://orcid.org/0000-0002-1353-5886
Na Wang https://orcid.org/0000-0001-5913-145X
Kejing Wang https://orcid.org/0000-0001-7090-314X
Qinglan Shi https://orcid.org/0000-0001-5719-8757

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Serum TBK1 levels are correlated with inflammation, optic nerve sheath diameter and intracranial pressure in severe traumatic brain injury patients under deep sedation

Shuilong Zhuang[1,A,E,F], Qing Xu[2,D,F], Youfu Li[1,B,C], Ke Hu[1,C,E], Zhengcai Xia[1,B,C], Lin Zhang[1,C,E]

[1] Department of Critical Medicine, Yiyang Central Hospital, China
[2] Department of Neurosurgery, Yiyang Central Hospital, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Shuilong Zhuang
E-mail: zhuangshuilongzsl@163.com

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Abstract

Background. Severe traumatic brain injuries (STBIs) cause 1/3–1/2 of trauma-related deaths. Tumor necrosis factor (TNF) receptor-associated factor NF-κB activator (TANK)-binding kinase 1 (TBK1) is a biomarker associated with inflammation, while inflammation is a key promoter of the TBI process.

Objectives. To investigate the clinical significance of TBK1 in STBI patients.

Materials and methods. The present prospective observational study included a total of 95 STBI cases diagnosed from October 2019 to October 2021. The values for optic nerve sheath diameter (ONSD) were determined under deep sedation using 2-dimensional gray scale ultrasound. Intracranial pressure (ICP) was also measured. Serum levels of TBK1 and inflammatory factors such as C-reactive protein (CRP), interleukin (IL)-1β and IL-6 were evaluated with enzyme-linked immunosorbent assay (ELISA). Clinical variables including pathological type, Glasgow Coma Scale (GCS) score, sequential organ failure assessment (SOFA) score, and Acute Physiology and Chronic Health Evaluation II (APACHE II) score were recorded.

Results. The levels of TBK1 in the deceased patients were remarkably lower than in the patients who survived. The IL-1β and IL-6 were markedly elevated in deceased patients compared with survivors, and negatively correlated with serum levels of TBK1. The ONSD and ICP values were significantly higher in the deceased patients than in the patients who survived and were positively correlated with each other, while both were negatively correlated with TBK1 levels. Patients with lower TBK1 expression showed significantly lower GCS scores, higher SOFA and APACHE II scores, as well as a higher 1-month mortality rate. The Kaplan–Meier curve showed that patients with higher TBK1 levels had a higher 1-month survival rate compared with the patients with lower TBK1 levels. Only TBK1 and ONSD were independent risk factors for 1-month mortality in STBI patients.

Conclusions. Lower serum TBK1 levels are associated with higher inflammatory factors, higher ONSD and ICP levels, as well as a poorer prognosis in STBI patients.

Key words: inflammation, intracranial pressure, TBK1, optic nerve sheath diameter, severe traumatic brain injury

Cite as
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Background

It has been reported that globally an estimated 96 million people suffer from traumatic brain injuries (TBIs) every year.[1] Of these TBI cases, approx. 5.48 million are severe TBIs (STBIs), which are the cause of 1/3–1/2 of trauma-related deaths.[2]

Many proteins and genes are reported to be associated with TBIs. Inflammatory factors such as C-reactive protein (CRP), tumor necrosis factor (TNF)-α and interleukin (IL)-6 are increased in TBI patients, along with the activation of the inflammatory response in TBIs.[3] The factors related to oxidative stress, such as superoxide dismutase (SOD) and inducible nitric oxide synthase (iNOS) have also been reported to be elevated in TBIs.[4–6] Additionally, neuron-specific enolase (NSE) and S100β were found to be increased in TBI patients and were correlated with the prognosis.[7,8] However, despite these findings, new potential biomarkers for the prognosis of TBIs are needed.

The TNF receptor-associated factor NF-κB activator (TANK)-binding kinase 1 (TBK1) is a well-known 82-kDa protein with 729 amino acids.[9] It has been found that TBK1 plays an important role in a variety of diseases and bioprocesses, especially inflammation and autophagy. Moreover, TBK1 inhibits inflammation and insulin resistance by phosphorylating and inducing the degradation of inhibitory-κB kinase and nuclear factor-κB (NF-κB)-inducing kinase in adipose tissue.[10] In a recent research study, it was shown that the inhibition of TBK1 resulted in an increased polymorphonuclear neutrophil necroptosis in response to lipopolysaccharide (LPS) and subsequently augmented lung inflammation.[11] The TBK1 also induces autophagy in amyotrophic lateral sclerosis, cancer and other diseases.[12–14] Since the inflammatory response is activated in TBIs and the release of cytokines is one of the main causes of brain injury, we speculated that TBK1, which is also a key factor in inflammation, might be associated with the development of TBI. However, up to now, no study has reported on the role of TBK1 in TBIs.

Objectives

In the present research, we evaluated the clinical significance of TBK1 in STBI patients and the relationship between TBK1, inflammatory factors, optic nerve sheath diameter (ONSD), and intracranial pressure (ICP), as well as the predictive value of TBK1 in the prognosis of STBI patients. This study might provide new potential biomarkers useful in the prognosis of TBIs.

Materials and methods

Patients

The present prospective observational research included a total of 95 STBI cases diagnosed from October 2019 to October 2021. All patients were enrolled after meeting the following criteria: 1) patients diagnosed with TBIs by advanced imaging including magnetic resonance imaging (MRI) and computed tomography (CT) scans; 2) STBI defined as a patient with Glasgow Coma Scale (GCS) scores from 3 to 8; 3) patients who experienced a closed craniocerebral injury; 4) patients admitted within 24 h of the trauma. The exclusion criteria included: 1) patients with open craniocerebral injuries; 2) patients with severe complications such as fractures in other body regions; 3) patients with cancer or severe renal, liver or heart diseases; 4) patients who received surgery within 3 months before the start of the study; 5) patients who were predicted to die within 24 h; 6) patients with severe ocular trauma, optic neuritis, optic nerve tumors, and other ophthalmic diseases. All patients received a routine treatment. Decompression and continuous drainage procedures were performed in all patients. No do-not-resuscitate orders were implemented for legislative reasons. This study obtained the approval from the ethics committee of the Yiyang Central Hospital, China (approval No. YY2019018). Written informed consent was obtained from all participants. The study conformed to the principles outlined in the Declaration of Helsinki.

Measurement of ONSD and ICP

Optic nerve sheath diameter and ICP were measured within 24 h of admission. The ONSD value was determined with a 2-dimensional gray scale ultrasound, using a Philips IU-22 ultrasonic diagnostic apparatus (Philips, Amsterdam, the Netherlands) with a probe frequency of 3–9 MHz. All patients were under deep sedation during the evaluation. Briefly, the probe was lightly put on the eyelid of the patient, and cross-sectional and sagittal scanning were performed twice on each side for every patient (Fig. 1). The ONSD at 3 mm behind bilateral eyeballs was recorded. For the accuracy of ONSD, it should be measured perpendicularly to the long axis of the optic nerve sheath. Every patient received 2 evaluations by 2 independent physicians. The mean value of the 2 evaluations was regarded as the ONSD value for the patient.

The intracranial pressure was measured using a Codman ICP monitor (DePuy Synthes, Raynham, USA), as reported elsewhere.[15]

Measurement of TBK1 and inflammatory factors

Blood samples of all patients were collected within 24 h of admission. Serum levels of TBK1 and inflammatory factors were determined with the enzyme-linked

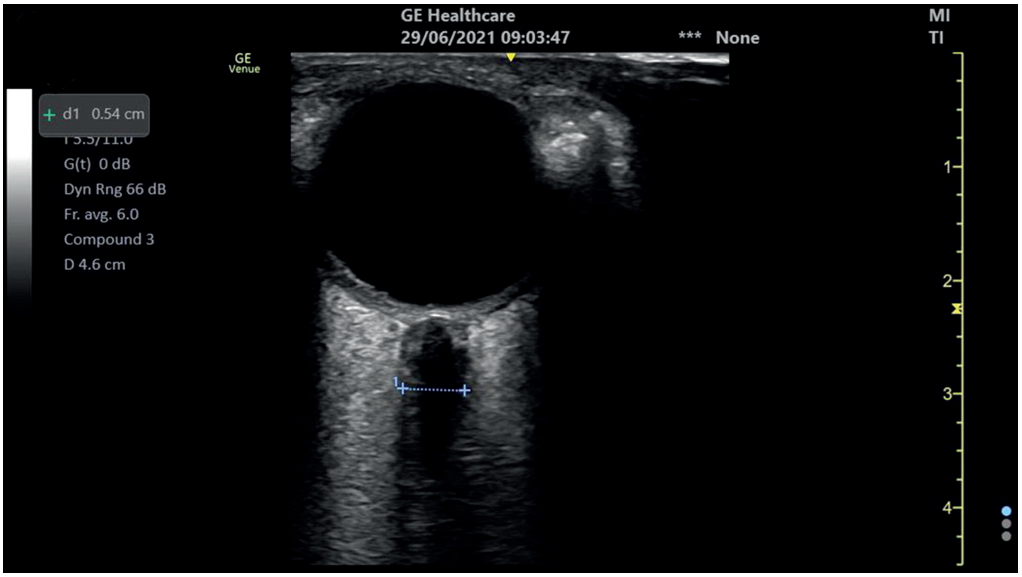

Fig. 1. Bilateral optic nerve sheath of a typical patient

immunosorbent assay (ELISA) method using the following kits according to the manufacturers' instructions: TBK1 kit (cat. No. MBS9427001; MyBioSource, San Diego, USA), CRP (cat. No. EK1316; Boster Bio, Pleasanton, USA), IL-1β (cat. No. ab214025; Abcam, Waltham, USA), and IL-6 (cat. No. ab178013; Abcam).

Data collection

Demographic data for all patients including age, sex, body mass index (BMI), and complications were collected. Clinical variables included pathological type, GCS score, sequential organ failure assessment (SOFA) score, and Acute Physiology and Chronic Health Evaluation II (APACHE II) score. All patients were followed up for 1 month and the 30-day mortality was analyzed. The survival duration was defined from the admission to death or the last follow-up appointment.

Statistical analyses

All normally distributed data were expressed as mean ± standard deviation (M ±SD) and non-normally distributed data were expressed as median (range). The distribution of the data was analyzed using the Kolmogorov–Smirnov test. Rates were compared using the χ^2 tests. The comparison between the 2 groups was conducted using unpaired t-tests or Mann–Whitney U tests for normally and non-normally distributed data, respectively. The Kaplan–Meier curve and log-rank test were used for survival analysis. A receiver operating characteristic (ROC) curve was used for the prediction of patient mortality by TBK1 and the cut-off value was selected using the Youden index. The Spearman's rank correlation coefficient was used to evaluate correlations due to the lack of normality of some variables. The logistic regression analysis was conducted for 1-month mortality of STBI patients using the backstepping method. The Hosmer–Lemeshow tests and Nagelkerke pseudo R^2 were used for the goodness-of-fit. All calculations were performed using SPSS v. 18.0 (SPSS Inc., Chicago, USA) and GraphPad v. 6.0 (GraphPad Software, San Diego, USA).

Results

Serum levels of TBK1 were downregulated in deceased STBI patients

The study included 95 STBI cases, of which 17 (17.89%) died within 28 days. The basic characteristics of all patients were listed in Table 1. The mean GCS ($p < 0.001$) score was markedly lower, while the SOFA ($p = 0.037$) and APACHE II scores ($p < 0.001$) were significantly higher in deceased patients compared with the survivors. No significant differences were found among the other indices.

The serum levels of TBK1 were determined. The levels of TBK1 in deceased patients were markedly lower than in the patients who survived ($p < 0.001$, Fig. 2).

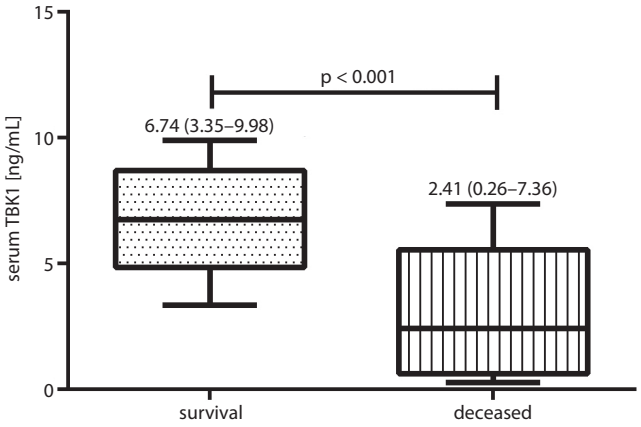

Fig. 2. Serum levels of TANK-binding kinase 1 (TBK1) in deceased and survivor severe traumatic brain injury (STBI) patients. Comparison between the 2 groups was conducted using the Mann–Whitney U test

Table 1. Basic characteristics of all STBI patients

Variables	All (n = 95)	Survival (n = 78)	Deceased (n = 17)	p-value*
Age [years]	50 (33–67)	50.5 (33–67)	49 (33–67)	0.880
Sex, male:female	62:33	51:27	11:6	0.921
BMI [kg/m^2]	23.97 (18.14–31.96)	25.13 (18.14–31.96)	22.96 (19.07–31.28)	0.088
TBI causes, n (%)				
Traffic accident	69 (72.63)	58 (74.36)	11 (64.71)	0.093
Fall	21 (22.11)	17 (21.79)	4 (23.53)	
Strike	5 (5.26)	3 (3.85)	2 (11.76)	
Pathological type, n (%)				
Intracranial hemorrhage	35 (36.84)	30 (38.46)	5 (29.41)	0.238
Severe contusion	24 (25.26)	20 (25.64)	4 (23.53)	
Subarachnoid hemorrhage	20 (21.05)	16 (20.51)	4 (23.53)	
Epidural or subdural hematoma	11 (11.58)	9 (11.54)	2 (11.76)	
Diffuse axonal injury	5 (5.26)	3 (3.85)	2 (11.76)	
Treatment strategy, n (%)				
Decompression surgery	95 (100)	78 (100)	17 (100)	1.000
Drainage treatment	95 (100)	78 (100)	17 (100)	
GCS score	6 (3–8)	7 (4–8)	5 (3–8)	<0.001
SOFA score	8.06 ±3.72	7.69 ±3.61	9.76 ±3.86	0.037
APACHE II score	16 (10–25)	15 (10–25)	21 (12–24)	<0.001

STBI – severe traumatic brain injury; BMI – body mass index; GCS – Glasgow Coma Scale; SOFA – sequential organ failure assessment; APACHE II – Acute Physiology and Chronic Health Evaluation II. * p-value was obtained by comparison between survival and deceased patients using unpaired t-test for normally distributed data or Mann–Whitney U test for non-normally distributed data or χ^2 test for rates. Normally distributed data were expressed as mean ± standard deviation (M ±SD). The non-normally distributed data were expressed as median (range). The counting data were expressed as number (rates).

Serum levels of TBK1 were correlated with serum inflammatory factors in STBI patients

To further investigate the role of TBK1 in STBI patients, the correlations between TBK1 and inflammatory factors were analyzed. It was found that IL-1β ($p < 0.001$) and IL-6 ($p = 0.028$) were elevated in deceased patients compared with survivors (Fig. 3). However, CRP showed no difference between the 2 groups. The Spearman's analysis showed that serum TBK1 levels were negatively correlated with the levels of IL-1β and IL-6, as well as SOFA and APACHE II scores. The TBK1 was positively correlated with the GCS scores (Table 2).

Table 2. Correlation between TBK1, inflammatory factors CRP, IL-1β and IL-6, as well as GCS, SOFA and APACHE II scores analyzed with Spearman's correlation analysis

Factors	Spearman's correlation	p-value
CRP	–0.148	0.152
IL-1β	–0.320	0.002
IL-6	–0.329	0.001
GCS score	0.415	<0.001
SOFA score	–0.259	0.011
APACHE II score	–0.480	<0.001

TBK1 – TANK-binding kinase 1; CRP – C-reactive protein; IL – interleukin; GCS – Glasgow Coma Scale; SOFA – sequential organ failure assessment; APACHE II – Acute Physiology and Chronic Health Evaluation II.

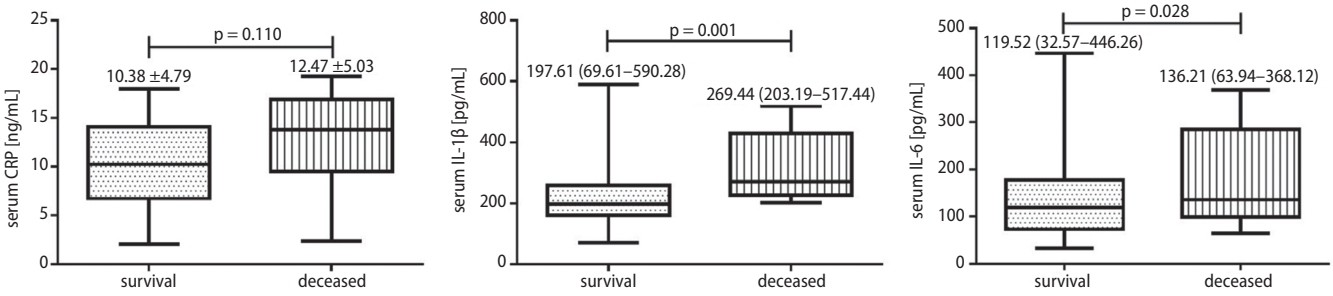

Fig. 3. Serum levels of the inflammatory factors: C-reactive protein (CRP), interleukin (IL)-1β and IL-6 in deceased and survivor severe traumatic brain injury (STBI) patients. Comparison between the 2 groups was conducted using the unpaired t-test or Mann–Whitney U test for normally and non-normally distributed data, respectively

Serum levels of TBK1 were correlated with levels of ONSD and ICP in STBI patients

Next, ONSD and ICP values were analyzed. As shown in Fig. 4A, the values of ONSD ($p < 0.001$) and ICP ($p = 0.001$) were significantly increased in the deceased patients compared to the patients who survived. Additionally, the ONSD values were positively correlated with ICP values using the Spearman's analysis ($p = 0.012$; Fig. 4B). We divided all patients into TBK1 high expression and low expression groups according to the mean value of TBK1 (6.10 ng/mL). The GCS scores were used as a hierarchical variable during comparison. It was found that patients with lower TBK1 levels showed higher ONSD and ICP levels ($p = 0.024$ and $p = 0.006$, respectively; Table 3). The Spearman's analysis found that serum levels of TBK1 were negatively correlated with ONSD (Spearman's correlation: −0.206, $p = 0.044$) and ICP (Spearman's correlation: −0.294, $p < 0.004$). These results indicated that serum TBK1 levels and ONSD could predict the alteration in ICPs in STBI patients.

Table 3. ONSD and ICP in STBI patients with different expression of TBK1

Variables	High TBK1 (n = 50)	Low TBK1 (n = 45)	p-value*
ONSD [mm]	4.20 ±0.40	4.40 ±0.48	0.024
ICP [mm H_2O]	178.77 ±57.91	216.78 ±73.82	0.006

ONSD – optic nerve sheath diameter; ICP – intracranial pressure; STBI – severe traumatic brain injury; TBK1 – TANK-binding kinase 1.
* Comparison was made using the unpaired t-test for normally distributed data between the 2 groups.

Relationship between TBK1 and clinical outcomes and prognosis in STBI patients

We analyzed the clinical outcomes in STBI patients with different expression levels of TBK1. The GCS scores were used as a hierarchical variable when comparing continuous data using a t-test. It was found that patients with lower TBK1 expression showed significantly lower GCS scores ($p < 0.001$), higher SOFA ($p = 0.014$) and APACHE II ($p < 0.001$) scores, as well as higher 1-month mortality ($p < 0.001$; Table 4). Furthermore, the Kaplan–Meier curve showed that the patients with higher TBK1 levels had a longer 1-month survival compared to the patients with lower TBK1 levels ($p = 0.007$; Fig. 5A). The ROC curve showed that TBK1 has the potential to predict 1-month mortality of STBI patients using a cutoff value <4.59 ng/mL with an area under the curve (AUC) = 0.850, a sensitivity of 76.47%, and a specificity of 78.12%. Additionally, the logistic regression analysis was conducted to identify factors that showed significant differences in univariate analysis. The Hosmer–Lemeshow tests showed that the goodness-of-fit ($p = 0.633$) and the Nagelkerke pseudo R^2 values were 0.443 and 0.557, respectively, indicating that the goodness-of-fit was acceptable. It was found that only TBK1 and ONSD were independent risk factors for 1-month mortality in STBI patients ($p < 0.001$ and $p = 0.014$, respectively; Table 5).

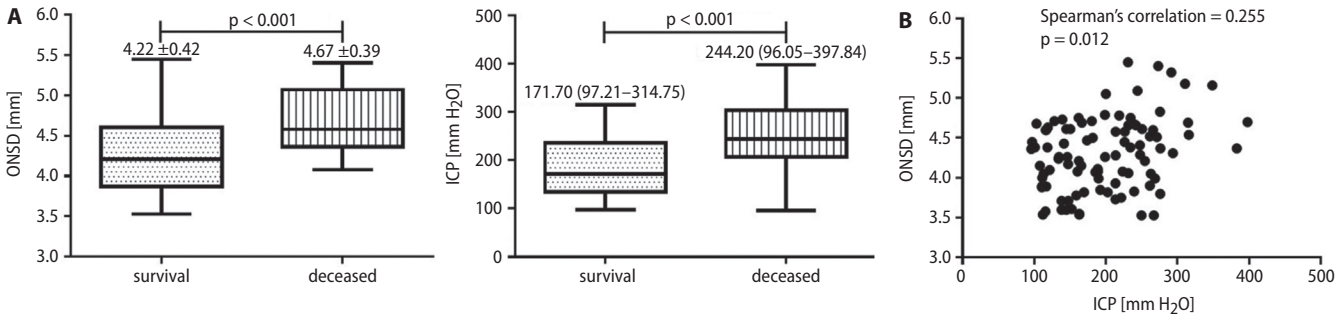

Fig. 4. A. Optic nerve sheath diameter (ONSD) and intracranial pressure (ICP) in deceased and survivor severe traumatic brain injury (STBI) patients. Comparison between the 2 groups was conducted using the unpaired t-test or Mann–Whitney U test for normally and non-normally distributed data, respectively; B. Correlation between ONSD and ICP in all STBI patients calculated using the Spearman's correlation

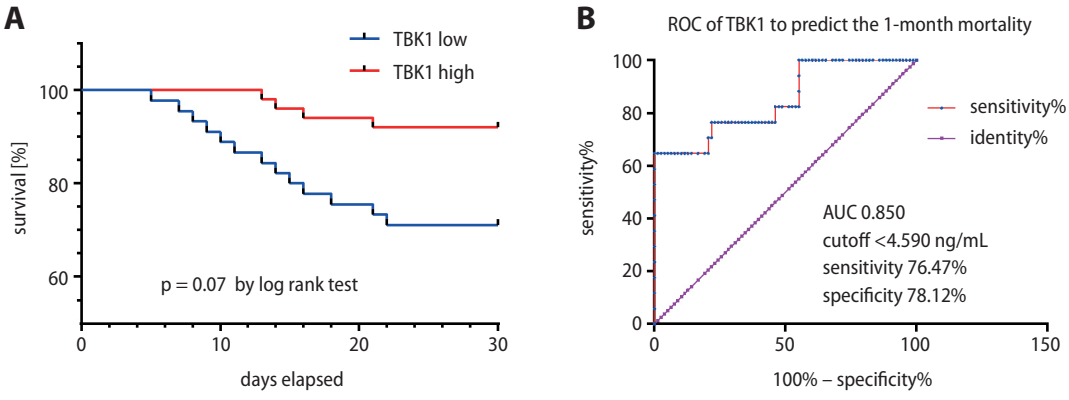

Fig. 5. A. The Kaplan–Meier curve for patients with different expression levels of TANK-binding kinase 1 (TBK1); B. Receiver operating characteristic (ROC) curve was used to predict the 1-month mortality using TBK1

AUC – area under the curve.

Table 4. Relationship between TBK1 and clinical outcomes and prognosis of STBI patients

Variables	High TBK1 (n = 50)	Low TBK1 (n = 45)	p-value*
Age [years]	49.00 ±10.75	49.75 ±10.34	0.729
Male:female	35:15	27:18	0.138
BMI [kg/m^2]	23.55 (18.14–31.96)	24.76 (18.79–31.90)	0.744
TBI causes, n (%)			
Traffic accident	36 (72.00)	33 (73.33)	0.593
Fall	12 (24.00)	9 (20.00)	
Strike	2 (4.00)	3 (6.67)	
Pathological type, n (%)			
Intracranial hemorrhage	20 (40.00)	15 (33.33)	0.494
Severe contusion	14 (28.00)	10 (22.22)	
Subarachnoid hemorrhage	9 (18.00)	11 (24.44)	
Epidural or subdural hematoma	5 (10.00)	6 (13.33)	
Diffuse axonal injury	2 (4.00)	3 (6.67)	
GCS score	5 (3–8)	7 (5–8)	<0.001
SOFA score	7.18 ±3.76	9.04 ±3.46	0.014
APACHE II score	20 (10–25)	15 (10–21)	<0.001
1-month mortality, n (%)	4 (8.00)	12 (26.67)	<0.001

TBK1 – TANK-binding kinase 1; STBI – severe traumatic brain injury; BMI – body mass index; GCS – Glasgow Coma Scale; SOFA – sequential organ failure assessment; APACHE II – Acute Physiology and Chronic Health Evaluation II. * p-value was obtained by comparison between survival and deceased patients using the unpaired t-test for normally distributed data or Mann–Whitney U test for non-normally distributed data χ^2 test for rates. The normally distributed data were expressed as mean ± standard deviation (M ±SD). The non-normally distributed data were expressed as median (range). The counting data were expressed as number (rates).

Table 5. Logistic regression for risk factors of 1-month mortality in STBI patients

Variables	Wald	OR	95% CI	p-value
GCS score	1.422	0.675	0.354–1.288	0.233
SOFA score	0.101	0.952	0.702–1.291	0.750
APACHE II score	0.635	1.120	0.848–1.479	0.426
TBK1	13.241	0.507	0.352–0.731	<0.001
CRP	0.945	0.913	0.759–1.097	0.331
IL-1β	1.614	1.004	0.998–1.011	0.204
IL-6	2.232	0.992	0.982–1.002	0.135
ONSD	6.018	8.837	1.550–50.395	0.014
ICP	0.674	1.006	0.992–1.019	0.412

STBI – severe traumatic brain injury; GCS – Glasgow Coma Scale; SOFA – sequential organ failure assessment; APACHE II – Acute Physiology and Chronic Health Evaluation II; TBK1 – TANK-binding kinase 1; CRP – C-reactive protein; IL – interleukin; ONSD – optic nerve sheath diameter; ICP – intracranial pressure; OR – odds ratio; 95% CI – 95% confidence interval.

Discussion

There are many factors influencing the prognosis of TBI patients. However, new factors associated with TBI are still needed. In the present study, we demonstrated that serum TBK1 levels were decreased in STBI patients and were correlated with increased inflammation, ONSD, ICP, as well as a poor prognosis.

The TBK1 is a protein that plays an important role in many diseases, inflammation and organ injuries. In TBK1-knockdown mice, the decreased expression of TBK1 significantly increased neuroinflammation by the activation of receptor-interacting protein kinase 1 (RIPK1) activity in aging human and animal models, while an increased expression of TBK1 inhibited neuroinflammation.[16] In high-fat diet (HFD)-induced obesity, a deficiency in TBK1 facilitated the inflammation through the regulation of AMP-activated protein kinase (AMPK) signaling, while the overexpression of TBK1 suppressed the inflammation by attenuating NF-κB activity in adipose tissue.[10] In pancreatic inflammation, the inhibition of TBK1 inhibited autophagy and upregulated the inflammatory response.[17] In TBK1-deficient mice, an increased susceptibility to LPS-induced lethality and immune cell

infiltrates in multiple tissues were observed.[18] Additionally, TBK1 and IL-1β were reported to induce autophagy in macrophages, and TBK1 was essential for IL-1β-induced autophagy, indicating the relationship between TBK1, autophagy and inflammation.[19] All these studies indicated that TBK1 plays a key role in inflammation and can suppress the inflammation in bioprocesses, including neuroinflammation. In this study, we found that serum TBK1 levels were negatively correlated with serum levels of inflammatory factors. Increased inflammatory factors were found in TBI patients in many studies.[20–22] Generally, the inflammatory process is activated during TBIs, which may in turn accelerate brain injury.[23] We also found that lower TBK1 levels predicted a poor prognosis for STBI patients, indicating that TBK1 can influence the progression of TBIs, which might be associated with the inflammatory response.

The relationship between ONSD and ICP has been noticed in several studies, including studies on TBIs. Legrand et al. demonstrated that the mean ONSD value was significantly higher (7.8 ±0.1 mm) in the deceased TBI patients compared to survivors (6.8 ±0.1 mm), and lower values of ONSD predicted a better 6-month survival.[24] In another study, Sekhon et al. found that each 1-millimeter increase in ONSD was associated with a twofold increase in hospital mortality in TBI patients, while ONSD was independently associated with increased ICPs within 48 h of admission.[25] Young et al. observed that ONSD was positively correlated with ICPs, and pediatric patients with an ONSD > 6.1 mm needed careful ICP monitoring.[26] In our research, we also observed that ONSD and ICP levels were elevated and positively correlated in the deceased STBI patients, which is consistent with the abovementioned findings. Interestingly, serum levels of TBK1 were negatively correlated with the values of ONSD and ICP. However, the meaning of this phenomenon requires more studies to confirm.

Limitations

The present study has several limitations. First, we only included a small sample of the study population. Secondly, only a few inflammatory factors were detected and more inflammatory factors could have been tested. Thirdly, we did not test the levels of TBK1 in cerebrospinal fluid. Finally, we excluded patients who were expected to die within 24 h of admission, which might have introduced bias into our study.

Conclusions

This study demonstrated that lower serum TBK1 levels were associated with increased inflammation, higher ONSD and ICP levels, as well as a poorer prognosis in STBI patients. The presented research provides more clinical evidence for TBK1 in TBIs.

ORCID iDs

Shuilong Zhuang https://orcid.org/0000-0003-0790-1074
Qing Xu https://orcid.org/0000-0002-2484-6217
Youfu Li https://orcid.org/0000-0002-8758-1383
Ke Hu https://orcid.org/0000-0001-6531-5386
Zhengcai Xia https://orcid.org/0000-0001-7474-1379
Lin Zhang https://orcid.org/0000-0001-5536-7422

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Drug-related problems among community-dwelling elderly with ischemic stroke in China

Lulu Tian[A–F], Juan Wu[A–F], Zhan Qi[A–F], Shijing Qian[A–F], Sha Zhang[A–F], Danfei Song[A–F], Beilei Chen[A–F], Deqiu Zhu[A–F]

Department of Pharmacy, Tongji Hospital, School of Medicine, Tongji University, Shanghai, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Deqiu Zhu
E-mail: deqiuzhu48@gmail.com

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Conflict of interest
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Abstract

Background. Ischemic stroke incidence is increasing amongst elderly patients in China; this is closely associated with drug-related problems (DRPs).

Objectives. To evaluate the influencing factors of DRPs among elderly patients with a history of ischemic stroke in the Chinese community and the role clinical pharmacists play in providing solutions.

Materials and methods. This study was conducted in 2 community health service centers in Putuo District, Shanghai, China, between December 2018 and June 2019. Demographics and clinical characteristics of the 130 selected patients were collected. Drug-related problems were classified using the Pharmaceutical Care Network Europe (PCNE)-DRP V8.03 classification system. The number, types, causes, interventions, and status of DRPs were then analyzed.

Results. The average number of DRPs per patient was 1.3, corresponding to 256 causes. "Treatment effectiveness P1" was identified as the most common problem (75.0%). The main causes were "drug selection C1" (33.2%) and "patient-related C7" (30.9%). Antihypertensive drugs, statins, aspirin, and Chinese patent medicines were the top 4 drugs for DRPs. Age, unintentional medication discrepancy and medication compliance were independent predictors of DRPs. Pharmacists provided 339 interventions, mainly "at drug level I3" (38.9%) and "at patient level I2" (30.7%). Most of the interventions (85.5%) were accepted by the patients and 65.9% of the problems were solved.

Conclusions. The number, types and etiology of DRPs in elderly patients with ischemic stroke in our community are diverse and treatment effectiveness is the main cause of their occurrence. Clinical pharmacists play an important role in providing interventions for major causes of DRPs.

Key words: community, ischemic stroke, drug-related problem, treatment effectiveness, clinical pharmacists

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Background

Chronic diseases have become the main cause of death for the Chinese elderly, with stroke identified as the leading cause.[1,2] Stroke can be divided into ischemic stroke and hemorrhagic stroke, with the former accounting for about 70% of strokes.[3] Ischemic stroke patients have a high rate of recurrence, mortality and disability. Secondary prevention can effectively reduce the recurrence and mortality. Effective secondary prevention strategies include dietary modification, exercise, as well as the use of aspirin, statins and antihypertensive agents.[4,5] However, it has been reported that few follow-up patients with ischemic stroke present good compliance with secondary prevention for 1 year after discharge from hospital, and only 1/2 of the patients adhere to secondary prevention medication.[6,7]

Quality of life has also been researched in relation to ischemic attacks. Hohmann et al. evaluated the impact of pharmaceutical care on health-related quality of life in patients after a transient ischemic attack or ischemic stroke.[8] In addition, ischemic stroke and problems related to drug use, selection and dose, duration of treatment, and results of follow-up of drug use were also examined.[9] In the study by Hohmann et al., a 61-year-old patient was diagnosed with acute ischemic stroke due to sudden numbness and thrombolysis in the right upper extremity, and was hospitalized in the stroke unit. This case was evaluated according to the Simple Object Access Protocol (SOAP) .[10] In a review by Poels et al., the relationship between the use of immune checkpoint inhibitors and atherosclerotic cardiovascular disease was analyzed. It has been stated that the incidence of atherosclerotic cardiovascular disease increases in pathologies such as cardiac problems, ischemic stroke and coronary artery disease due to the use of immune checkpoint inhibitors.[11] In a randomized controlled study, parameters of patients with ischemic attack and stroke who received pharmacist intervention were compared. The intervention consisted of a focused drug review, motivational interview, and 3 follow-up phone calls. Clinical outcomes were reported as cardiac death, ischemic stroke or acute myocardial infarction.[12]

In recent years, DRPs in the elderly have been the focus of research in China. In one study, possible DRPs were followed up for 1 year in 184 elderly patients hospitalized in the geriatric clinic. The percentage of elderly patients who had problems related to at least 1 drug was determined as 34.5%. It was observed that the most important reason for potential DRPs was drug selection.[13] In another study, 291,944 drug prescriptions given to 10,643 patients were examined and the rate per patient according to the drugs used was reported as 3548 DRPs. The authors reported that noteworthy problems were related to treatment efficacy (39.9%) and drug doses (47.0%).[14] In yet another study, data of elderly patients over 60 years of age who were using at least 5 medications were compared, and it was stated that DRP was caused by economic reasons and deficiencies related to the correct drug protocols.[15] In a study comparing systemic blood pressure values, a total of 525 DRPs were detected. It was reported that the efficacy of treatment in the detected problems was resolved with drug changes (48.76%). More than 90% of the patients accepted these interventions.[16] Additionally, the elderly patients often take multiple medications and have more comorbidities, which can lead to DRPs.[17] Pharmacological databases from different provinces in China were compared. Patients over 64 years of age have the highest mortality rates, according to the databases. When the death rates were evaluated in terms of gender, the rate was higher in men than in women.[18] More specifically, in studies related to DRPs, problems in ambulatory patients,[19] neurological problems,[20] respiratory problems, and cardiac problems have been reported.[21,22]

A drug-related problem (DRP) is defined as an event or circumstance involving drug therapy that actually or potentially interferes with desired health outcomes.[23] Many studies showed that DRPs can increase morbidity, length of hospitalization, mortality and medical expenditures, and cause a heavy burden on patients and society.[24–26]

The majority of previous studies on DRPs for patients with ischemic stroke concerned patients in the acute phase of ischemic stroke treated in a hospital. As a member of the multidisciplinary care team (MDT), the pharmacist participated in the treatment process from admission to discharge, and there were 0.32–1.8 DRPs on average. The acceptance rate of pharmacist's recommendations for doctors and patients reaches 89–94%.[27–29] However, research on DRPs in patients with a history of ischemic stroke in the community was seldom reported. The authors stated that the elderly with chronic diseases in the community generally were taking many types of medications (more than 5 types). The medication compliance was poor, and multiple referrals between clinics led to problems, such as repeated medications.[17,30] After receiving acute treatment in hospitals, patients who have had an ischemic stroke usually receive drug therapy in the community. Currently, they are out of the supervision of the MDT. Therefore, it is very important to investigate the DRPs for patients in the community with a history of ischemic stroke, as well as the intervention effect of clinical pharmacists on DRPs.

Objectives

The primary objective of the study was to evaluate DRPs and analyze the factors influencing them among Chinese community-dwelling elderly who have had an ischemic stroke. The secondary objective was to assess the interventions provided by clinical pharmacists.

Materials and methods

Study design

Data on drug-related problems among community-dwelling elderly who have had an ischemic stroke were prospectively collected from December 2018 to June 2019. The research subjects were elderly who have had an ischemic stroke and were treated at either Cao Yang Community Health Service Center or Gan Quan Community Health Service Center. Both institutions are class 1 hospitals located in Putuo District, Shanghai, China. The Health Service Centers have 6 community health service stations and nursing homes, covering more than 220,000 citizens. The study was conducted by a pharmacist team from Shanghai Tongji Hospital, China, with support from the 2 Health Service Centers. The study was approved by the Medical Ethics Committee of Shanghai Tongji Hospital, Tongji University School of Medicine (Shanghai Tongji Hospital Ethics Committee, approval No. 2018-055). The study was carried out in accordance with the Declaration of Helsinki. All enrolled patients signed an informed consent form.

The inclusion criteria were: 1) age ≥65 years; 2) history of ischemic stroke; and 3) regular (>28 days) taking of more than 5 drugs. Exclusion criteria were: 1) severe or end-stage disease; 2) mental disorder or severe cognitive dysfunction; or 3) inability to complete the questionnaire.

Data collection

A total of 8 general practitioners from the 2 Health Service Centers received the training to assist in the collection of data. There were 6 clinical pharmacists who received different training, including the Pharmaceutical Care Network Europe-drug-related problem (PCNE-DRP) classification system, medication therapy management for ischemic stroke and inappropriate medications evaluation for older adults in China. Among them, 3 were senior clinical pharmacists (with more than 5 years of experience and a clinical pharmacist qualification certificate) and 3 were junior clinical pharmacists (with less than 2 years of experience). Patient demographics and clinical information were collected using one-to-one interviews, patient medical records and hospital information systems. Patient medication compliance was evaluated according to the 8-item Morisky Medication Adherence Scale (MMAS-8 ≥ 6 means high medication compliance, MMAS-8 < 6 means low medication compliance).

PCNE-DRP classification

Drug instructions, clinical guidelines and drug databases, such as UpToDate (https://www.wolterskluwer.com/en/solutions/uptodate) and Micromedex (https://www.micromedexsolutions.com), were used to identify DRPs and propose interventions. Drug-related problems were classified according to the PCNE-DRP V8.03 classification system. This system has 5 primary domains, namely Problems (P), Causes (C), Planned Interventions (I), Intervention Acceptance (A), and Status of the DRP (O). The implementation plan is shown in Fig. 1. Drug-related problems were investigated and classified by a clinical pharmacist, reviewed by a senior clinical pharmacist, and then re-investigated and reclassified by another senior clinical pharmacist to reach a consensus. If there was a variation in classification, the final decision would be determined through group discussion.

Statistical analyses

A patient may have multiple medication problems, and each problem can have more than 1 cause and can lead to more interventions. As a result, statistical analyses were carried out separately at patient and drug level. Data were analyzed with IBM SPSS software v. 20.0 (IBM Corp., Armonk, USA). Categorical variables were described as frequencies or percentages, and continuous variables were described as mean ± standard deviation (M ±SD) if the data passed the normal distribution test. Medians and quartiles (quartile 1 (Q1), quartile 3 (Q3)) were used for skewed data.

The incidence of DRPs with various influencing factors were analyzed using binary logistic regression. All the reported variables were independent. For all continuous variables greater than 0, the Box–Tidwell test was conducted to check the linear relationship between explanatory variables and the logit of the response variables. For all interaction variables, p-value > 0.05/22 = 0.0023 is considered a linear relationship. The absence of multicollinearity among independent variables was checked using linear regression. For all variables, the tolerance was >0.1 and the variance inflation factor (VIF) was <10. No multicollinearity was found. Studentized residuals after regression were used to check the assumption of the lack of strong influential outliers. Four cases had higher studentized residuals and these cases were checked and determined to be reasonable. Therefore, those cases were included in the regression. Basic assumptions for conducting the logistic regression were met.

The binary logistic regression used the "Forward LP" method. The likelihood ratio test (LR test) was used to evaluate whether the calculated model with added variables was statistically significant. The Hosmer–Lemeshow test and Nagelkerke's R^2 were used to check the goodness-of-fit. The Wald coefficient test was conducted for each regression coefficient.

Results

Baseline characteristics

As shown in Fig. 1, a total of 134 (considering that contact with 2 was lost and 2 died) elderly patients who had

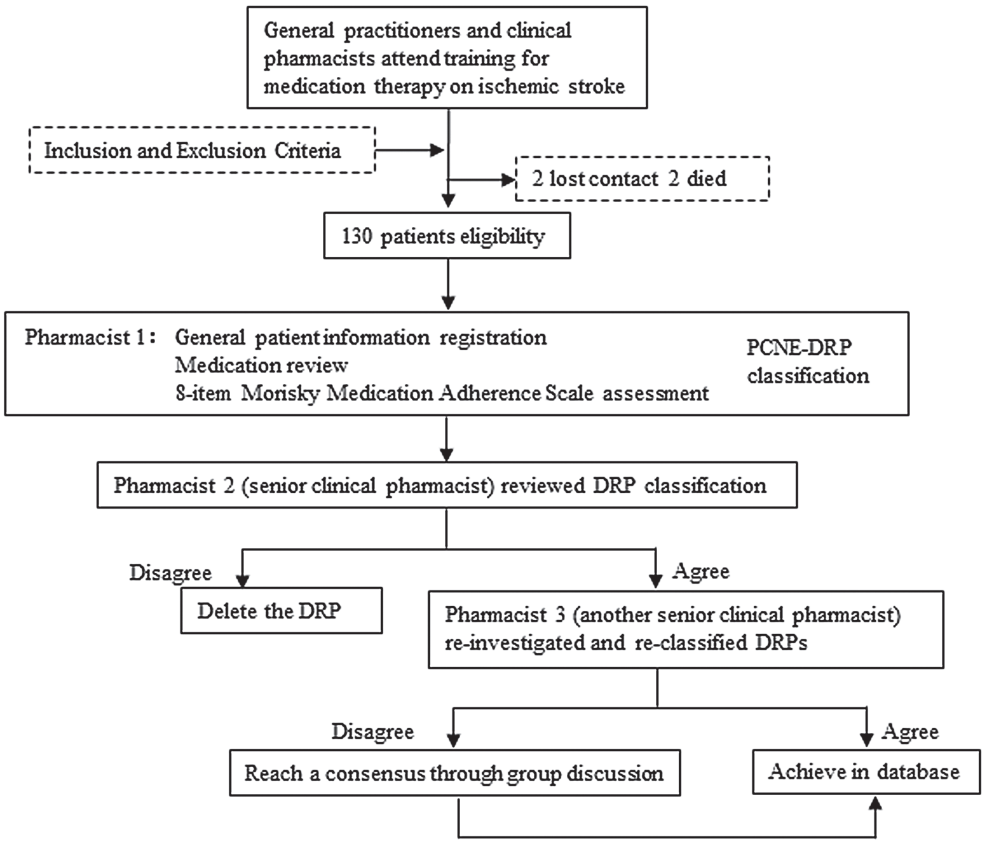

Fig. 1. Flowchart of patient enrollment and drug-related problems classification

PCNE-DRP – Pharmaceutical Care Network Europe-drug-related problem.

ischemic stroke were initially recruited. Of these, contact with 2 patients was lost and 2 patients died, and therefore 130 patients were enrolled. The clinical characteristics of the enrolled patients were shown in Table 1. The median age of the subjects (Q1, Q3) was 76.5 years (Q1, Q3: 70, 83) and 54.6% (71/130) were male. Of the subjects, 25.4% (33/130) had a history of drug allergy and the average body mass index (BMI) was 23.7 ±3.6 kg/m^2. There were 9.2% (12/130) current smokers, 15.4% (20/130) current drinkers, 69.2% (90/130) were married and lived with family, and 16.9% (22/130) were educated at primary school or below. The most common comorbidities were hypertension (86.9%, 113/130), atrial fibrillation or coronary heart disease (69.2%, 90/130), hyperlipidemia (57.7%, 75/130), skeletal diseases (49.2%, 64/130), and sleep disorders (46.9%, 61/130). The median (Q1, Q3) number of daily prescription drug kinds was 7 (Q1, Q3: 6, 9), mean daily intake of oral drugs was 15 tablets (Q1, Q3: 8, 23), and mean daily intake of oral dietary supplements was 0 tables (Q1, Q3: 0, 1). Of the patients, 26.9% (35/130) had unintentional medication discrepancies due to referrals between clinics. The median points of MMAS-8 were 5.5 (Q1, Q3: 3.5, 7).

Indentification of drug-related problems

As shown in Table 2, all 130 patients had DRP reported in their files. A total of 164 DRPs were found, averaging 1.3 per patient. Of these, 75.0% (123/164) were related to "treatment effectiveness P1", 12.8% (21/164) to "treatment safety P2" and 12.2% (20/164) were related to "other problems P3". There were 256 causes, and the top 3 were "drug selection C1" (33.2%, 85/256), "patient related C7" (30.9%, 79/256) and "other C8" (21.9%, 56/256). The pharmacists provided 339 planned interventions, with an average of 2.1 for each DRP. Interventions were mainly made "at drug level I3" (38.9%, 132/339), followed by "patient level I2" (30.7%, 104/339) and "prescriber level I1" (18.6%, 63/339). The top 3 drugs associated with DRPs were antihypertensive drugs (22.0%, 36/164), statins (18.9%, 31/164) and aspirins (15.9%, 26/164), which accounted for more than 1/2 of all DRPs. The 4th-ranked drugs associated with DRPs were Chinese patent medicines (7.3%, 12/164). The distribution of the causes for these 4 types of DRPs is shown in Table 3. The most common cause of antihypertensive DRPs was "no or inappropriate outcome monitoring C8.1", while the most common cause of aspirin DRPs was "patient uses/takes less drug than prescribed or does not take the drug at all C7.1". Both C8.1 and C7.1 were the main causes of statin DRPs. The main cause of Chinese patent medicine DRPs was "dosage regimen not frequent enough C3.3".

Analysis of factors associated with the incidence of drug-related problems

The regression coefficient, Wald test, p-value, and odds ratio (OR) values are listed in Table 4. The χ^2 for LR test was 11.688, degrees of freedom (df) was 3, and the p-value

Table 1. Demographics and clinical characteristics of participants (n = 130)

Characteristics		Value
Age, median (Q1, Q3) [years]		76.5 (70, 83)
Gender, male, n (%)		71 (54.6)
Drug allergy history, n (%)		33 (25.4)
BMI[a], M ±SD [kg/m^2]		23.7 ±3.6
Smoking status, n (%)	current smoker	12 (9.2)
	ex-smoker	13 (1.0)
	non smoker	105 (80.8)
Drinking status, n (%)	current drinker	20 (15.4)
	ex-drinker	16 (12.3)
	non drinker	94 (72.3)
Living status, n (%)	married and living with family	90 (69.2)
	unmarried/divorced/widowed and living alone	11 (8.5)
	unmarried/divorced/widowed and living with family	29 (22.3)
Education level, n (%)	primary school or below	22 (16.9)
	junior high school and above	108 (83.1)
Comorbidities, median (Q1, Q3), n		6 (5, 8)
Comorbidity, n (%)	hypertension	113 (86.9)
	atrial fibrillation or coronary heart disease	90 (69.2)
	hyperlipidemia	75 (57.7)
	skeletal disease	64 (49.2)
	sleep disorder	61 (46.9)
	diabetes	58 (44.6)
	digestive disease	56 (43.1)
	urinary disease	47 (36.2)
	renal disease	31 (23.8)
	depressive and anxiety neurosis	20 (15.4)
	tumor	17 (13.1)
	infectious disease	16 (12.3)
	COPD	13 (10.0)
	blood disease	9 (6.9)
	Parkinson's disease	2 (1.5)
	others	39 (30.0)
Daily prescription drug kinds, median (Q1, Q3), n		7 (6, 9)
Daily oral drugs, median (Q1, Q3), number of tablets		15 (8, 23)
Daily oral dietary supplements, median (Q1, Q3), number of tablets		0 (0, 1)
Unintentional medication discrepancies due to referral[b], n (%)		35 (26.9)
MMAS-8 score, median (Q1, Q3)		5.5 (3.5, 7)

M ±SD – mean ± standard deviation; BMI – body mass index; COPD – chronic obstructive pulmonary disease; MMAS-8 – 8-item Morisky Medication Adherence Scale; Q1 – 1st quartile; Q3 – 3rd quartile. Gender, drug allergy history, smoking status, drinking status, living status, education level, comorbidity, and unintentional medication discrepancies due to referral were categorical variables described as percentages. Age, BMI, comorbidities, daily prescription drug kinds, daily oral drugs, daily oral dietary supplements, and MMAS-8 score were continuous variables described as M ±SD, or medians and quartiles (Q1, Q3, as appropriate). [a] Obesity: BMI ≥ 28 kg/m^2; [b] unintentional medication discrepancies due to referral: number of patients have unintentional medication discrepancies between self-reported use of prescribed drugs and the medication record due to referrals between clinics; MMAS-8 ≥ 6 means high medication compliance, MMAS-8 < 6 means low medication compliance.

was 0 (<0.05). The Nagelkerke's R^2 of the model was 0.324, which shows a good fit. The Hosmer–Lemeshow test was conducted and the χ^2 was 11.688, and p-value of the Hosmer–Lemeshow test was 0.166 (greater than 0.05), meant that Hosmer–Lemeshow goodness of fit test result was statistically significant. The model can predict DRP incidence with 79.2% accuracy, as shown in Table 5.

Multivariate binary logistic analysis showed that patients' age, unintentional medication discrepancy and medication compliance were significantly correlated to DRPs. As shown

Table 2. Distribution of the types, causes and interventions for drug-related problems according to the PCNE-DRP classification V8.03

Domain	Code	Details	n	%
Types of problems	P1	treatment effectiveness	123	75.0
	P1.1	no effect of drug treatment	6	3.7
	P1.2	effect of drug treatment not optimal	95	57.9
	P1.3	untreated symptoms or indication	22	13.4
	P2	treatment safety	21	12.8
	P2.1	adverse drug event (possibly) occurring	21	12.8
	P3	other	20	12.2
	P3.1	problem with cost-effectiveness of the treatment	5	3.0
	P3.2	unnecessary drug treatment	8	4.9
	P3.3	unclear problem/complaint	7	4.3
Prescribing and drug selection-related causes	C1	drug selection	85	33.2
	C1.1	inappropriate drug according to guidelines/formulary	6	2.3
	C1.2	inappropriate drug (within guidelines but otherwise contraindicated)	11	4.3
	C1.3	no indication for drug	18	7.0
	C1.5	inappropriate duplication of therapeutic group or active ingredient	3	1.2
	C1.6	no or incomplete drug treatment in spite of existing indication	29	11.3
	C1.7	too many drugs prescribed for indication	18	7.0
	C2	drug form	1	0.4
	C2.1	inappropriate drug form (for this patient)	1	0.4
	C3	dose selection	28	10.9
	C3.1	drug dose too low	10	3.9
	C3.2	drug dose too high	5	2.0
	C3.3	dosage regimen not frequent enough	12	4.7
	C3.4	dosage regimen too frequent	1	0.4
	C4	treatment duration	4	1.6
	C4.1	duration of treatment too short	1	0.4
	C4.2	duration of treatment too long	3	1.2
Drug use-related causes	C6	drug use process	3	1.2
	C6.1	inappropriate timing of administration or dosing intervals	2	0.8
	C6.3	drug over-administered	1	0.4
	C7	patient-related	79	30.9
	C7.1	patient uses/takes less drug than prescribed or does not take the drug at all	31	12.1
	C7.2	patient uses/takes more drug than prescribed	3	1.2
	C7.3	patient abuses drug (unregulated overuse)	4	1.6
	C7.4	patient uses unnecessary drug	5	2.0
	C7.5	patient takes food that interacts	3	1.2
	C7.6	patient stores drug inappropriately	1	0.4
	C7.7	inappropriate timing or dosing intervals	17	6.6
	C7.9	patient unable to use drug/form as directed	15	5.9
	C8	other	56	21.9
	C8.1	no or inappropriate outcome monitoring (including TDM)	49	19.1
	C8.2	other cause	5	2.0
	C8.3	no obvious cause	2	0.8
Planned interventions	I0	no intervention	4	1.2
	I0.1	no intervention	4	1.2
	I1	at prescriber level	63	18.6
	I1.1	prescriber informed only	1	0.3
	I1.2	prescriber asked for information	5	1.5
	I1.3	intervention proposed to prescriber	56	16.5
	I1.4	intervention discussed with prescriber	1	0.3
	I2	at patient level	104	30.7
	I2.1	patient (drug) counselling	64	18.9
	I2.3	patient referred to prescriber	40	11.8
	I3	at drug level	132	38.9
	I3.1	drug changed to…	19	5.6
	I3.2	dosage changed to…	27	8.0
	I3.3	formulation changed to…	1	0.3
	I3.4	instructions for use changed to…	24	7.1
	I3.5	drug paused or stopped	31	9.1
	I3.6	drug started	30	8.8
	I4	other intervention or activity	36	10.6
	I4.1	other intervention	29	8.6
	I4.2	side effect reported to authorities	7	2.1

P – problems; C – causes; I – planned interventions; PCNE-DRP – Pharmaceutical Care Network Europe-drug-related problem; TDM – therapeutic drug monitoring. All items in the table were categorical variables described as percentages.

Table 3. Top 4 drug classes causing drug-related problems

Domain	Antihypertensive drugs		Statins		Aspirins		Chinese patent medicine	
	details	n	details	n	details	n	details	n
Types of problems	total	36	total	31	total	26	total	12
	P1.2 Effect of drug treatment not optimal	27	P1.1 No effect of drug treatment	2	P1.1 No effect of drug treatment	1	P1.2 Effect of drug treatment not optimal	7
	P2.1 Adverse drug event (possibly) occurring	6	P1.2 Effect of drug treatment not optimal	20	P1.2 Effect of drug treatment not optimal	14	P1.3 Untreated symptoms or indication	2
	P3.3 Unclear problem/ complaint	3	P1.3 Untreated symptoms or indication	1	P1.3 Untreated symptoms or indication	2	P2.1 Adverse drug event (possibly) occurring	1
			P2.1 Adverse drug event (possibly) occurring	2	P2.1 Adverse drug event (possibly) occurring	7	P3.2 Unnecessary drug treatment	2
			P3.1 Problem with cost-effectiveness of the treatment	2	P3.3 Unclear problem/ complaint	2		
			P3.2 Unnecessary drug treatment	1				
			P3.3 Unclear problem/ complaint	3				
Major causes	C8.1 No or inappropriate outcome monitoring (incl. TDM)	17	C7.1 Patient uses/takes less drug than prescribed or does not take the drug at all	9	C7.1 Patient uses/takes less drug than prescribed or does not take the drug at all	9	C3.3 Dosage regimen not frequent enough	3
	C1.6 No or incomplete drug treatment in spite of existing indication	6	C8.1 No or inappropriate outcome monitoring (incl. TDM)	8	C7.7 Inappropriate timing or dosing intervals	6	C1.7 Too many drugs prescribed for indication	2
	C7.1 Patient uses/takes less drug than prescribed or does not take the drug at all	5	C1.6 No or incomplete drug treatment in spite of existing indication	6	C1.6 No or incomplete drug treatment in spite of existing indication	4	C7.1 Patient uses/takes less drug than prescribed or does not take the drug at all	2
	C1.2 Inappropriate drug (within guidelines but otherwise contraindicated)	4	C7.9 Patient unable to use drug/form as directed	5	C1.2 Inappropriate drug (within guidelines but otherwise contraindicated)	3		

P – problems; C – causes; TDM – therapeutic drug monitoring. All items in the table were categorical variables counted in numbers.

Table 4. Forward stepwise regression for the influencing factors of DRPs

Variables	Regression coefficient	Wald χ^2	p-value	OR (95% CI)
Age	–0.061	4.528	0.033	0.941 (0.889–0.995)
Unintentional medication discrepancy	1.746	6.797	0.009	5.734 (1.543–21.311)
Medication compliance	–0.428	14.520	0.000	0.652 (0.523–0.812)
Constant	7.449	9.232	0.002	1718.704 (–)

OR – odds ratio; DRPs – drug-related problems; 95% CI – 95% confidence interval. A value of $p < 0.05$ is considered statistically significant. The Nagelkerke's R^2 of the model was 0.324. Age, unintentional medication discrepancy and medication compliance were influencing factors ($p < 0.05$) of DRPs obtained using binary logistic regression analysis.

in Table 4, OR of age was 0.941, which means that age had only little effect on the incidence of DRPs. Medication compliance was a protective factor (less DRP risk). Unintentional medication discrepancy was more likely to cause DRPs, since the OR of unintentional medication discrepancy was 5.734.

Acceptance of interventions and the status of DRPs

As shown in Table 6, a total of 85.5% (290/339) of the interventions were accepted, 66.1% (224/339) received interventions were implemented completely, and 65.9% ((81+27)/164) drug-related problems were partially or completely resolved.

Discussion

To the best of our knowledge, this is the first prospective study on DRPs in Chinese community-dwelling elderly who have had an ischemic stroke. We found that DRPs were common among elderly ischemic stroke patients

Table 5. Predicted correct percentage of the DRP incidence model

Observed		Predicted DRPs		Percentage correct (%)
		no	yes	
Observed DRP	no	29	16	64.4
	yes	11	74	87.1
Overall percentage		–	–	79.2

DRPs – drug-related problems.

Table 6. Acceptance of interventions and the outcomes of drug-related problem

Domain	Code	Detailed	n	%
Intervention accepted	A1	Total	290	85.5
	A1.1	intervention accepted and fully implemented	224	66.1
	A1.2	intervention accepted, partially implemented	24	7.1
	A1.3	intervention accepted but not implemented	14	4.1
	A1.4	intervention accepted, implementation unknown	28	8.3
Intervention not accepted	A2	total	5	1.5
	A2.1	intervention not accepted: not feasible	2	0.6
	A2.2	intervention not accepted: no patient consent	3	0.9
Other	A3	total	44	13.0
	A3.1	intervention proposed, acceptance unknown	40	11.8
	A3.2	intervention not proposed	4	1.2
Not known	O0	total	47	28.7
	O0.1	problem status unknown	47	28.7
Solved	O1	total	81	49.4
	O1.1	problem completely solved	81	49.4
Partially solved	O2	total	27	16.5
	O2.1	problem partially solved	27	16.5
Not solved	O3	total	9	5.5
	O3.1	problem not solved, lack of patient cooperation	3	1.8
	O3.3	problem not solved, intervention not effective	3	1.8
	O3.4	no need or possibility to solve problem	3	1.8

A – intervention acceptance; O – status of the drug-related problem (DRP). All items in the table were categorical variables described as percentages.

in the community, since 65.4% of patients had at least 1 DRP. The average incidence of DRPs per patient was relatively high (1.3) compared with previous studies. One study in Germany found the DRP incidence of 1.8 per person among ischemic stroke inpatients categorized by the hospital setting.[27] Another study identified the DRP incidence of 1.04 per person among hospitalized stroke patients using the Hepler–Strand classification,[28] and another identified the DRP rate of 0.32 among hospitalized ischemic stroke patients using the PCNE-DRP V8.0 classification system.[29] The differences in DRP may be due to the classification system utilized.

Chen et al. reported that the mean DRP rate of ischemic stroke patients hospitalized in China was 0.32. They stated that DRP rates were high, especially in patients with kidney failure (0.91) and liver failure (0.65). The authors found that drug selection and treatment safety were the 2 main problems encountered the most frequently. They reported that proton pump inhibitors, followed by cerebrovascular/nootropics and sedative-hypnotics were associated with DRPs, respectively (in this sequence of decreasing frequency). They also observed that the most common type of DRPs was "treatment safety P2" (60.2%),[29] likely because inpatients were monitored by MDT. In inpatients, it was easier to monitor the efficacy of the drug, and the potential safety hazards of the drug were less understood. Additionally, hospitalized patients were generally in the acute phase of ischemic stroke. There were numerous types of treatment drugs in the acute phase for hospitalized patients, such as the use of antibiotics and proton pump inhibitors, larger drug doses and multiple drug delivery modes. Furthermore, the use of injections also increased the risk of adverse drug events. Similarly, Hohmann et al. found problems related to DRPs in 105 patients (67.7%). They found the ratio of DRPs per patient to be 1.8 ±2.0. They stated that the main criteria related to DRPs were

drug type, indication and dosage.[27] Harris et al. conducted an evaluation of the Pharmacists Act on Care Transitions in Stroke service. As in other studies, correct drug use, dosage, treatment duration, and dispensing, as well as drug use parameters were evaluated. In a study in which 27 patients were evaluated, they detected 30 DRPs.[9] Apart from the DRPs in people who have had ischemic strokes, researchers have also examined the clinical causes of ischemic strokes. In a study that prospectively examined the effects of blood pressure on stroke in China, they observed that pulse pressure and mean arterial pressure were indicative criteria for stroke. It was also stated that mean arterial pressure is a more important criterion than pulse pressure in terms of stroke. They also found that blood pressure was slightly lower in patients with hypertension.[31] Tang et al. suggested that lobar microhemorrhages in the brain may affect the outcome of poststroke depression. It has been stated that lobar microhemorrhages in the brain are an important factor in the clinical course of depression in Chinese stroke patients.[32]

In this study, among all the DRPs in elderly patients in the community who have had an ischemic stroke, "drug selection C1" (33.2%) and "patient related C7" (30.9%) were the 2 main causes. For example, aspirin or statins in the secondary prevention strategies for ischemic stroke were not taken consistently by patients or the way of taking them was wrong. Patients increased or decreased antihypertensive drugs doses without pharmacist instruction. Some drugs used for alleviating symptoms were taken for a long time. Patients should stop taking those drugs according to pharmacist's instruction. However, in fact some patients did not stop taking since they liked those drugs. Correspondingly, pharmacists put forward suggestions for interventions mainly at the drug level (38.9%) and the patient level (30.7%). Based on these findings, the drugs most commonly associated with DRPs were antihypertensive drugs, followed by statins, aspirin and Chinese patent drugs. The main problem with antihypertensive drugs was that patients had poor blood pressure control. Some patients followed the regimen continuously when they were discharged from hospital. The regimen was not adjusted in time when the efficacy was poor or adverse reactions occurred. At the same time, some patients increased or decreased the dosage by themselves. Aspirin DRPs were mostly due to incorrect usage and dosage. For example, aspirin enteric-coated tablets were not taken on an empty stomach. Sometimes, the frequency of every day administration was automatically changed to every other day by patients since some patients were afraid of side effects. There were numerous statin-related DRPs, and a number of patients did were not taking statins every day. Some thought that the treatment was finished after discharge, some thought that their blood lipids were not high and there was no indication for statin use, and some stopped taking the medication or reduced its dose due to the fear of possible adverse effects.

In contrast with previous studies,[27–29] we found that the proportion of Chinese patent medicine DRPs (7.3%) was not low, which may be related to the preference of the Chinese elderly for selecting Chinese patent medicines. A considerable number of patients believed that Chinese patent medicines had less side effects. They tended to use *Ginkgo biloba* preparations instead of aspirin and take Danshen tablets in the long term. This study also showed that the specific causes for Chinese patent medicine DRPs were related to the inconsistent dosage or frequency of administration. Sometimes, drug safety of the Chinese patent medicines was not fully clarified by drug instructions, so patients, especially the older ones, used these drugs at will. In this case, DRPs such as potential drug–drug interactions and adverse reactions occurred frequently.

Patient's age, unintentional medication discrepancy and medication compliance were independent predictors of DRPs. Unintentional medication discrepancy was a risk factor that caused DRPs. Such discrepancies were usually caused by referral between different hospitals. Pharmacists can remind patients of drug change caused by referral to reduce DRPs.

The high rates of intervention acceptance (85.5%) and resolved problems (65.9%) were consistent with other study of in-hospital stroke patients.[13–15] This indicated that the community and home pharmaceutical care led by clinical pharmacists could be fully trusted by patients. The full-course pharmaceutical care model (hospital–community–home) is worth promoting.

This study may have 2 potential effects. Elderly ischemic stroke patients in the community could fully understand and improve compliance with the secondary prevention strategies for ischemic stroke thanks to patient explanations and targeted medication education by clinical pharmacists. Extending pharmaceutical services provided by clinical pharmacists from hospital to community and even the patient's home is worthy of reference by peers.

Limitations

The sample size was small and there might be deviations in the results. Moreover, no control group was set, so we were unable to compare the influence with or without clinical pharmacists on DRPs among elderly ischemic stroke patients in Chinese communities. The economic aspects of drugs were not investigated.

Conclusions

This study indicates that DRPs are very common in elderly ischemic stroke patients in Chinese communities, since 2/3 (65.4%) of the study subjects had at least 1 DRP. The average incidence of 1.3 DRPs per patient is relatively high in comparison to previous research. The major

type of problem was "treatment effectiveness P1" (75%). The rates of intervention acceptance (85.5%) and solved problems (65.9%) are quite high. The number of DRPs was increased due to unintentional medication discrepancy due to referral. Meanwhile, patients' medication compliance can help decrease the DRPs.

ORCID iDs

Lulu Tian https://orcid.org/0000-0002-4969-1274
Juan Wu https://orcid.org/0000-0003-2013-4316
Zhan Qi https://orcid.org/0000-0003-2188-889X
Shijing Qian https://orcid.org/0000-0002-5236-4047
Sha Zhang https://orcid.org/0000-0002-6799-5300
Danfei Song https://orcid.org/0000-0002-0698-6910
Beilei Chen https://orcid.org/0000-0002-1596-481X
Deqiu Zhu https://orcid.org/0000-0002-8462-4501

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The relationship between *LRP5* rs556442 and rs638051 polymorphisms and mutations and their influence on bone metabolism in postmenopausal Xinjiang women with type 2 diabetes

Jun Li[1,D], Minli Song[1,C,D], Siyuan Li[2,A], Xiling Wang[2,B], Huirong Zhao[2,E], Zexin Hou[1,E,F]

[1] Department of Endocrinology and Metabolism, First Affiliated Hospital, Shihezi University School of Medicine, China
[2] Medical College, Shihezi University School of Medicine, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Jun Li
E-mail: xjlijun@163.com

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Abstract

Background. The Wnt/β-catenin signaling pathway plays a crucial role in bone development and metabolism. The low-density lipoprotein receptor-related protein 5 (LRP5), an important receptor in the Wnt signaling pathway, promotes the osteogenesis of osteoblasts and curbs bone resorption by osteoclasts.

Objectives. To determine the expression of *LRP5* polymorphisms (rs556442 and rs638051) and their relationship with bone mineral density (BMD) and bone metabolism markers in postmenopausal patients with type 2 diabetes mellitus (T2DM) in Xinjiang, China.

Materials and methods. According to dual-energy X-ray (DEXA) and oral glucose tolerance test (OGTT) results, 226 postmenopausal women from Xinjiang were divided into the following groups: normal glucose tolerance (NGT) + normal bone mass group (group A), NGT + abnormal bone mass group (group B), T2DM + normal bone mass group (group C), and T2DM + abnormal bone mass group (group D).

Results. Femoral neck BMD was lower in group B women with the AG/GG genotype (mutant type) compared to women with the AA genotype (wild-type) at rs556442. Alkaline phosphatase (ALP) levels were lower in group D women with the AG/GG genotype (mutant type) compared to women with the AA genotype (wild-type) at rs556442 and rs638051. The factors influencing BMD (lumbar spine vertebrae 1–4 (L1–L4)) were triglyceride (TG) levels, body mass index (BMI), menopausal transition age, and age for rs556442 patients, and TG levels and menopausal transition age for rs638051 patients in group D. The factors affecting BMD (hip) were TG levels, BMI and age for rs556442 patients, and TG levels and age for rs638051 patients.

Conclusions. The *LRP5* gene mutations are linked to bone metabolism disorders in postmenopausal women with T2DM and abnormal bone mass. High BMI and TG were positively associated with BMD, while increased age and menopausal transition age were negatively associated with BMD.

Key words: type 2 diabetes, bone metabolism, postmenopausal women, *LRP5* polymorphism

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Background

Increased blood glucose levels caused by insufficient insulin secretion or islet cell dysfunction in type 2 diabetes mellitus (T2DM) affect a number of metabolic processes in the human body. Genetic susceptibility is of great importance in T2DM.[1] This condition not only affects the quality of life and survival status of patients but also causes considerable economic and psychological burden.[2,3]

Osteoporosis (OP) is a common chronic complication in T2DM patients that is related to a decrease in estrogen levels in postmenopausal women. Goldshtein et al. showed that postmenopausal women with T2DM have an increased risk of osteoporotic fractures.[4]

The low-density lipoprotein receptor-associated protein 5 (LRP5) is a transmembrane receptor protein belonging to the low-density lipoprotein (LDL) receptor family. The *LRP5* gene is located on chromosome 11q12–13 and encodes an accessory receptor for the Wnt ligand 5.[5] The *LRP5* promotes the production of insulin, which is conducive to islet signal transduction and bone formation of osteoblasts.[6] It has been found that bone mineral density (BMD) is increased in mice with elevated *LRP5* expression.[7] In addition, *LRP5* is involved in lipid metabolism.[8] Recently, the relationship between the *LRP5* rs41494349 gene polymorphism and OP in postmenopausal women with T2DM was reported.[9] However, the relationship between *LRP5* rs556442 and rs638051 gene polymorphisms and bone metabolism in postmenopausal women with T2DM in Xinjiang remains unknown. Therefore, we aim to fill this research gap and lay the foundation for the prevention and treatment of OP in postmenopausal women with T2DM in Xinjiang.

Objective

The study aimed to explore the expression of *LRP5* polymorphisms rs556442 and rs638051, and their relationship with BMD and bone metabolism markers in postmenopausal patients with T2DM in Xinjiang.

Materials and methods

Research subjects

A total of 226 naturally postmenopausal women treated in the First Affiliated Hospital of Xinjiang Medicine School in Xinjiang, China, were enrolled into the study. Type 2 diabetes mellitus was diagnosed based on the 1999 World Health Organization (WHO) recommendations, and BMD was measured based on the 1994 WHO recommendations. Subjects were divided based on the oral glucose tolerance test (OGTT) and dual-energy X-ray (DEXA) results for BMD. Group A patients had a normal glucose tolerance and normal bone mass (50 patients), and group B consisted of patients with NGT and abnormal bone mass (49 patients). The T2DM patients with normal bone mass were constituted group C (47 patients), and T2DM patients with abnormal bone mass group D (80 patients).

This research complied with the Declaration of Helsinki. Approval from the Ethics Committee of the First Affiliated Hospital of Shihezi University School of Medicine, China, was obtained (approval No. 2015-125-01). We explained the risks, benefits and goals of the study to each participant. Those who agreed to participate signed a written informed consent form.

Data acquisition

General descriptive data of the subjects were collected, and body mass index (BMI) and waist-to-hip ratio (WHR) were calculated (Table 1). The subjects fasted for 8–10 h and antecubital blood was collected the next morning. Triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), fasting plasma glucose (FPG) calcium (Ca), and alkaline phosphatase (ALP) levels, as well as other indices were measured using an automatic biochemical analyzer (bs-280; Mindray, Shenzen, China). Using high-performance liquid chromatography (Bio-Rad D10; Bio-Rad, Hercules, USA), the level of glycosylated hemoglobin (HbA1c) was determined. Bone mineral density was analyzed in the lumbar spine and femur using the DEXA method. The detection of the rs556442 and rs638051 polymorphisms of the *LRP5* gene was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

Table 1. Comparison of baseline data among groups using Welch analysis of variance (ANOVA)

Variable	Group A (n = 50)	Group B (n = 49)	Group C (n = 47)	Group D (n = 80)	Welch F	p-value
Age [years]	59.54 ±9.50	65.61 ±12.00[a]	60.30 ±8.49[b]	69.28 ±8.10[abc]	17.503	<0.001
Menopausal transition age [years]	15.14 ±10.00	19.00 ±9.55[a]	13.49 ±8.68[b]	22.24 ±7.75[abc]	13.157	<0.001
BMI [kg/m^2]	27.70 ±3.56	24.80 ±4.38[a]	26.19 ±3.77	24.84 ±3.55[a]	7.634	<0.001
WHR	0.84 ±0.14	0.89 ±0.01[a]	0.91 ±0.06[a]	0.91 ±0.07[a]	5.461	0.002

Data are presented as mean ± standard deviation (M ±SD). BMI – body mass index; WHR – waist-to-hip ratio. [a] age, menopausal transition age, BMI and WHR of groups B and D, and WHR of group C compared with group A ($p < 0.001$); [b] age and menopausal transition age of groups C and D compared with group B ($p < 0.001$); [c] age and menopausal transition age of group D compared with group C ($p < 0.001$).

Determination of single nucleotide polymorphism sites

According to the general principles for selecting single nucleotide polymorphisms (SNPs), we consulted the relevant literature and selected the SNPs related to the *LRP5* gene. Then, we selected functionally related and important sites, such as missense mutations, which change the amino acid sequence and affect protein function. The best locus for the functional region of the *LRP5* gene according to the National Center for Biotechnology Information (NCBI) website was chosen (https://www.ncbi.nlm.nih.gov/gene/?term=LRP5+and+human).[10,11] In this study, two SNPs of the *LRP5* gene were selected – rs556442 and rs638051.

DNA extraction

Five milliliters of antecubital blood were collected from each subject in ethylenediaminetetraacetic acid (EDTA)-coated tubes. The DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and stored at –80°C. The concentration and purity of DNA were assessed using a NanoDrop 2000 UV spectrophotometer (Thermo Fisher Scientific, Waltham, USA), and the absorption of nucleic acids at 260 nm was quantified.

Gene sequencing

Primers for amplification and single base extensions were designed using Sequenom assay designer software v. 3.0 (Sequenom, San Diego, USA). The primer sequences were as follows: rs556442, forward: 5'-GGGCAGCCAAGATCGAAC-3' and reverse: 5'-CGTCCACCCAGAACAGCTT-3'; rs638051, forward: 5'-CTTTGGGCAGTGGGCTTAG-3' and reverse: 5'-CACCCTCTGGACATAGCTCTGA-3'. Polymerase chain reaction (PCR) conditions involved 1) preheating the samples to 94°C for 4 min (1 cycle); 2) 94°C for 20 s (45 cycles); 3) 56°C for 30 s (45 cycles); 4) 72°C for 1 min (45 cycles); and 5) final extension at 72°C for 3 min (1 cycle). The PCR products were obtained using a 384-pad spectral chip (JL-PZY96BT; Yibaiju, Shanghai, China). Each PCR product was treated with shrimp alkaline phosphatase (SAP; Sequenom). The Iplex (Sequenom) single base extension reaction was performed. Resin (Xingruikebo, Shihezi, China) purification procedure was also performed using the mass array nanodispenser RS1000 spotter (Sequenom), and the extension product was moved to the 384-pad spectral chip.[12–14] The MALDI-TOF MS (Sequenom) was performed to detect rs556442 and rs638051 genotypes under different conditions.[15] After the above reaction steps, the chip was put into the mass spectrometer (Sequenom). The flight time of these ionized products depended on the quality of each allele, measured using mass spectrometer. The smaller the ion mass, the faster it arrives. Finally, the molecular weight and base type were determined according to the position of the simplicity peak to succeed in typing.[16]

Statistical analyses

The IBM SPSS v. 22.0 software (IBM Corp., Armonk, USA) was used to analyze the data. The data conforming to a normal distribution are presented as the mean ± standard deviation (M ±SD). General data and biochemical parameters were compared between the groups using independent sample t-tests. When the baseline data was homogeneous (age [years], menopausal transition age [years], BMI [kg/m^2], and WHR), one-way analysis of variance (ANOVA) testing was used to conduct intergroup comparisons. Otherwise, analysis of covariance (ACNOVA) was used. The χ^2 goodness-of-fit was determined using the Hardy–Weinberg equilibrium (HWE) test. Multiple linear regression was adopted to analyze the factors influencing BMD, and independent variables were selected using the best subset regression, which was based on adjusted R^2 values. A p-value <0.05 was considered statistically significant.

The results of the statistical tests are available as Supplementary data.

Results

There were statistically significant differences in menopausal transition age, BMI, and WHR within groups A, B and D ($p < 0.05$, Table 1).

The ACNOVA demonstrated that FPG and HbA1c levels were statistically higher in groups C and D compared to group A ($p < 0.01$). Bone mineral density (femoral neck) and BMD (lumbar spine vertebrae 1–4 (L1–L4)) were statistically lower in groups B and D compared to group A ($p < 0.01$, Table 2).

The genotype frequencies of the 2 loci were consistent with the HWE ($p > 0.05$). There were no significant differences in genotype frequency and gene frequency between the rs556442 and rs638051 loci on the *LRP5* gene ($p > 0.05$, Table 3).

At the rs556442 locus in the group B, femoral neck BMD of the AG/GG genotype was statistically lower than that of the AA genotype ($p < 0.05$). In group D, ALP level was statistically lower for the AG/GG genotype compared to the AA genotype ($p < 0.05$, Table 4). At the rs638051 locus in group D, ALP level was statistically lower for the AG/GG genotype compared to the AA genotype ($p < 0.05$, Table 5).

In group D (T2DM + abnormal bone mass), the best subset regression analysis was performed with BMD (L1–L4) and BMD (femoral neck) as the response variables and age (X1), menopausal transition age (X2), BMI (X3), WHR (X4), FPG (X5), HbA1c (X6), TG (X7), high-density lipoprotein (HDL)

Table 2. Comparison of biochemical indexes among groups after analysis of covariance (ANCOVA)

Variable	Group A (n = 50)	Group B (n = 49)	Group C (n = 47)	Group D (n = 80)	F	p-value
FPG [mmol/L]	5.39 ±1.58	5.08 ±0.58	8.24 ±3.00[ab]	7.57 ±2.36[ab]	28.861	<0.001
HbA1c [%]	5.73 ±0.80	6.10 ±0.77	7.73 ±1.21[ab]	7.94 ±1.51[ab]	52.059	<0.001
TG [mmol/L]	2.12 ±1.29	1.31 ±0.59[a]	2.07 ±1.27[b]	1.50 ±0.99[ac]	7.676	<0.001
HDL-C [mmol/L]	1.33 ±0.39	1.35 ±0.42	1.42 ±0.68	1.29 ±0.39	0.840	0.473
LDL-C [mmol/L]	2.66 ±0.89	2.56 ±0.82	2.99 ±1.09[b]	3.43 ±1.19[abc]	9.370	<0.001
Ca [mmol/L]	2.28 ±0.07	2.28 ±0.11	2.44 ±0.43[ab]	2.32 ±0.24[c]	4.511	0.004
P [mmol/L]	1.11 ±0.15	1.12 ±0.19	1.13 ±0.14	1.20 ±0.47	1.259	0.289
ALP [U/L]	78.72 ±17.54	82.77 ±27.30	69.33 ±18.85[ab]	75.99 ±19.54	3.495	0.016
BMD (L1–L4) [g/cm^2]	1.21 ±0.15	0.89 ±0.17[a]	1.17 ±0.17[b]	0.92 ±0.11[ac]	68.544	<0.001
BMD (femoral neck) [g/cm^2]	0.93 ±0.13	0.72 ±0.10[a]	0.96 ±0.12[b]	0.75 ±0.11[ac]	63.717	<0.001

Data are presented as mean ± standard deviation (M ±SD). [a] FPG of groups C and D, HbA1c ($p < 0.001$), TG of groups B and D ($p < 0.001$), LDL-C of group D ($p < 0.001$), Ca of group C ($p = 0.004$), ALP of group C ($p = 0.016$), and BMD (L1-L4) and BMD (femoral neck) of groups B and D ($p < 0.001$) compared with group A; [b] FPG, HbA1c and LDL-C of groups C and D ($p < 0.001$), TG of group C ($p < 0.001$), Ca of group C ($p = 0.004$), ALP of group C ($p = 0.016$), and BMD (L1-L4) and BMD (femoral neck) of group C ($p < 0.001$) compared with group B; [c] TG, LDL-C, BMD (L1-L4), and BMD (femoral neck) of group D ($p < 0.001$), and Ca of group D ($p = 0.004$) compared with group C. FPG – fasting plasma glucose; HbA1c – glycosylated hemoglobin; TG – triglyceride; HDL-C – high-density lipoprotein cholesterol; LDL-C – low-density lipoprotein cholesterol; Ca – calcium; P – phosphorus; ALP – alkaline phosphatase; BMD – bone mineral density; L1–L4 – lumbar spine vertebrae 1–4.

Table 3. Genotype and allele distribution frequencies of the *LRP5* gene rs556442 and rs638051 locus calculated using the χ^2 test (n (%))

SNP	Genotype/allele	Group A	Group B	Group C	Group D	χ^2 value	p-value
rs556442	AA	31 (62.0)	30 (61.2)	33 (70.2)	52 (65.0)	3.433	0.763
	AG	17 (34.0)	17 (34.7)	10 (21.3)	23 (28.8)		
	GG	2 (4.0)	2 (4.1)	4 (8.5)	5 (6.2)		
	A	79 (79.0)	77 (78.6)	76 (80.9)	127 (79.4)	0.172	0.982
	G	21 (21.0)	21 (21.4)	18 (19.1)	33 (20.6)		
rs638051	AA	17 (65.4)	15 (55.6)	18 (58.0)	30 (53.6)	3.054	0.812
	AG	6 (23.1)	11 (40.7)	10 (32.3)	20 (35.7)		
	GG	3 (11.5)	1 (3.7)	3 (9.7)	6 (10.7)		
	A	40 (76.9)	41 (75.9)	46 (74.2)	80 (71.4)	0.719	0.869
	G	12 (23.1)	13 (24.1)	16 (25.8)	32 (28.6)		

SNP – single nucleotide polymorphism. The AA (wild-type), AG/GG (mutant-type) and A/G (allele gene) of groups B, C and D were compared with those of group A.

Table 4. Comparison of biochemical indices and bone mineral density (BMD) between genotypes at the rs556442 locus in groups using t-test

Variable	Group B		t-value	p-value	Group D		t-value	p-value
	AA	AG/GG			AA	AG/GG		
FPG [mmol/L]	5.23 ±0.65	4.93 ±0.58	1.639	0.108	8.22 ±2.69	7.31 ±1.36	2.009	0.050
HbA1c [%]	6.08 ±0.79	6.03 ±0.43	0.286	0.776	7.76 ±1.67	7.68 ±1.36	0.217	0.828
TG [mmol/L]	1.29 ±0.95	1.53 ±0.72	–0.942	0.351	1.85 ±1.25	1.51 ±0.95	1.256	0.213
HDL-C [mmol/L]	1.36 ±0.37	1.33 ±0.35	0.282	0.779	1.31 ±0.43	1.24 ±0.28	0.878	0.383
LDL-C [mmol/L]	2.51 ±0.93	3.03 ±0.80	–2.010	0.050	3.13 ±1.20	3.51 ±0.92	–1.459	0.149
Ca [mmol/L]	2.28 ±0.12	2.28 ±0.07	0.147	0.884	2.29 ±0.17	2.27 ±0.11	0.636	0.527
P [mmol/L]	1.15 ±0.19	1.09 ±0.16	1.143	0.259	1.26 ±1.24	1.06 ±0.13	1.151	0.255
ALP [U/L]	82.20 ±29.99	85.00 ±19.46	–0.396	0.694	77.46 ±20.66	66.36 ±21.02	2.278	0.025*
BMD (L1–L4) [g/cm^2]	0.91 ±0.19	0.86 ±0.10	1.202	0.235	0.93 ±0.10	0.90 ±0.12	1.192	0.237
BMD (femoral neck) [g/cm^2]	0.75 ±0.10	0.68 ±0.08	2.571	0.013*	0.76 ±0.10	0.74 ±0.13	0.767	0.446

Data are presented as mean ± standard deviation (M ±SD). *$p < 0.05$, **$p < 0.01$. FPG – fasting plasma glucose; HbA1c – glycosylated hemoglobin; TG – triglyceride; HDL-C – high-density lipoprotein cholesterol; LDL-C – low-density lipoprotein cholesterol; Ca – calcium; P – phosphorus; ALP – alkaline phosphatase; L1–L4 – lumbar spine vertebrae 1–4.

Table 5. Comparison of biochemical indices and bone mineral density (BMD) between genotypes at the rs638051 locus in the group D using t-test

Variable	AA	AG/GG	t-value	p-value
FPG [mmol/L]	7.61 ±2.21	7.21 ±1.34	0.831	0.410
HbA1c [%]	7.59 ±1.12	7.33 ±1.13	0.863	0.392
TG [mmol/L]	1.53 ±0.66	1.76 ±1.16	–0.893	0.377
HDL-C [mmol/L]	1.28 ±0.33	1.14 ±0.25	1.767	0.083
LDL-C [mmol/L]	3.39 ±1.01	3.62 ±1.14	–0.801	0.427
Ca [mmol/L]	2.27 ±0.09	2.29 ±0.12	–0.711	0.480
P [mmol/L]	1.12 ±0.12	1.16 ±0.18	–0.963	0.341
ALP [U/L]	80.00 ±16.83	66.54 ±25.38	2.301	0.026*
BMD (L1–L4) [g/cm^2]	0.92 ±0.12	0.89 ±0.11	0.970	0.337
BMD (femoral neck) [g/cm^2]	0.75 ±0.11	0.74 ±0.11	0.339	0.736

Data are presented as mean ± standard deviation (M ±SD). * $p < 0.05$, ** $p < 0.01$. FPG – fasting plasma glucose; HbA1c – glycosylated hemoglobin; TG – triglyceride; HDL-C – high-density lipoprotein cholesterol; LDL-C – low-density lipoprotein cholesterol; Ca – calcium; P – phosphorus; ALP – alkaline phosphatase; L1–L4 – lumbar spine vertebrae 1–4.

(X8), LDL (X9), Ca (X10), phosphorus (P) (X11), ALP (X12), and genotype (X13) as independent variables.

The best subset regression analysis demonstrated that lower TG level and BMI, older age and higher menopausal transition age at the rs556442 locus were risk factors for a decreased BMD (L1–L4). Lower TG level and higher menopausal transition age at the rs638051 locus were risk factors for decreased BMD (L1–L4). Except for higher menopausal transition age, all variables were also risk factors for a decreased BMD (femoral neck, Table 6).

Discussion

In an aging population, the incidence rate of OP is increasing with each calendar year, and high disability rate among OP patients places a heavy financial burden on the society.[17] As confirmed in other studies, postmenopausal women with T2DM have a decreased bone mass due to a rapid decline in estrogen levels, putting them at high risk for OP.[18,19] Therefore, at the gene level, further study of the pathogenesis of OP can provide a theoretical basis for explaining the occurrence of T2DM with OP. Currently, it is unclear whether T2DM can lead to the reduction of BMD. However, an increasing number of studies have shown that T2DM can increase bone fragility,[20,21] and such fragility leads to an increase in T2DM with OP.[22,23] The pathogenesis of T2DM complicated by OP is multifactorial and influenced by genetic and environmental factors. The Wnt signaling pathway is crucial in the axis differentiation of multicellular organisms,[24] where LRP5 is a transmembrane receptor of the Wnt protein.[25] Regarding the Wnt signaling pathway, studies on *LRP5* gene polymorphisms and T2DM or OP have been published both in China and in other countries, but there are few reports on the relationship between *LRP5* gene polymorphisms and OP in postmenopausal women with T2DM.

This study found that the genotype and allele frequency distribution of the rs556442 and rs638051 loci of *LRP5* comply with the Hardy–Weinberg law of genetic balance. This indicates that the population selected for this study has relatively stable heritability and is a representative of the target population. The genotype distribution was dominated by the AA genotype, which was 64.6% and 57.1% for rs556442 and rs638051, respectively (Table 3). The wild-type homozygous genotype was the most common, the mutant heterozygous genotype was the 2nd most common, and the mutant homozygous genotype was the least common. Astiazar et al.[26] and Koay et al.[27] found that the *LRP5* gene mutation can reduce BMD and increase the incidence of OP. This study found that for rs556442 in group B, the BMD (femoral neck) of the AG/GG genotype (mutant) was lower compared to the AA genotype (wild-type, 0.68 ±0.08 compared to 0.75 ±0.10 g/cm^2, $p = 0.013$). At rs556442, ALP level for the AG/GG genotype (mutant-type) was lower in group D than in the AA genotype (wild-type, 66.36 ±21.02 compared to 77.46 ±20.66 U/L, $p = 0.025$; Table 4). In group D, at rs638051, ALP level for the AG/GG genotype (mutant-type) was lower compared to the AA genotype (wild-type, 66.54 ±25.38 compared to 80.00 ±16.83 U/L, $p = 0.026$). These findings suggest that mutations at rs556442 and rs638051 loci may be related to BMD and bone metabolism (Table 5). Wang et al. showed that *LRP5* gene polymorphisms are genetically linked to increases in blood lipid levels, BMI and obesity.[28] Through the best subset regression analysis, decreased BMI and TGs and higher menopausal transition age and age were found to be risk factors for decreased BMD, suggesting that higher BMI and TG levels are associated with a lower risk of abnormal BMD among diabetic patients, similar to the findings of Li et al.[29] Thus, postmenopausal women with dyslipidemia should receive screening to prevent the occurrence of OP.

Table 6. Best subset regression analysis of the influencing factors of bone mineral density (BMD) in type 2 diabetes mellitus (T2DM) patients

SNP	BMD	Variable	Adjusted R^2	β	t-value	p-value
rs556442	L1–L4	TG	0.247	0.043	3.773	<0.001
		BMI	–	0.008	2.479	0.014
		menopausal transition age	–	–0.004	–3.162	0.002
		age	–	–0.003	–2.002	0.047
		WHR	–	–0.146	–1.010	0.314
		Ca	–	0.091	1.862	0.064
		HbA1c	–	–0.011	–1.331	0.184
	femoral neck	TG	0.210	0.025	2.845	0.005
		BMI	–	0.005	2.142	0.033
		age	–	–0.003	–2.797	0.006
		menopausal transition age	–	–0.001	–1.333	0.184
		FPG	–	0.006	1.437	0.152
		HbA1c	–	–0.008	–1.093	0.275
		HDL-C	–	0.022	1.068	0.287
		LDL-C	–	–0.013	–1.399	0.163
		ALP	–	–0.001	–1.130	0.260
rs638051	L1–L4	TG	0.272	0.035	3.580	<0.001
		menopausal transition age	–	–0.005	–3.217	0.002
		FPG	–	0.009	1.683	0.094
		BMI	–	0.004	1.065	0.289
		age	–	–0.001	–1.013	0.313
	femoral neck	TG	0.196	0.213	2.077	0.039
		age		–0.002	–2.262	0.025
		WHR	–	0.279	1.526	0.129
		LDL-C	–	–0.018	–1.886	0.061
		BMI	–	0.004	1.496	0.137
		menopausal transition age	–	–0.002	–1.861	0.064
		ALP	–	–0.001	–1.627	0.106

SNP – single nucleotide polymorphism; WHR – waist-to-hip ratio; BMI – body mass index; TG – triglyceride; HDL-C – high-density lipoprotein cholesterol; LDL-C – low-density lipoprotein cholesterol; Ca – calcium; ALP – alkaline phosphatase; FPG – fasting plasma glucose; HbA1c – glycosylated hemoglobin; L1–L4 – lumbar spine vertebrae 1–4.

Limitations of the study

The adjusted R^2 in the best subset regression analysis model established for rs556442 and rs638051 was relatively low, which was considered to be related to the quantity and quality of the included independent variables.

In the future, our research group will consider investigating more important factors that may affect the occurrence and development of diseases, such as FINS, FCP, ISI, PINP, CTX, NTX, etc., which include many invasive independent variables with high diagnostic value, so as to further improve the value of the model.

Conclusions

To summarize, mutations at rs556442 and rs638051 loci of the *LRP5* gene are related to bone metabolism in postmenopausal women in Xinjiang. Therefore, postmenopausal women with T2DM should undergo screening and early intervention strategies for the prevention of OP.

Supplementary data

The results of the statistical tests are available as Supplementary data at https://doi.org/10.5281/zenodo.7196566. The package contains the following files:

Supplementary Table 1. Normality test of the variables presented in Table 1,2.

Supplementary Table 2. Homogeneity of variances test of the variables presented in Table 1,2.

Supplementary Table 3. Welch ANOVA of the statistical values presented in Table 1.

Supplementary Table 4. ANCOVA of the statistical values presented in Table 2.

Supplementary Table 5. Post hoc tests of the statistical values presented in Table 1.

Supplementary Table 6. Post hoc tests of statistical values presented in Table 2.

ORCID iDs

Jun Li https://orcid.org/0000-0002-4847-0509
Minli Song https://orcid.org/0000-0003-4696-0085
Siyuan Li https://orcid.org/0000-0002-9352-9645
Xiling Wang https://orcid.org/0000-0001-5342-9853
Huirong Zhao https://orcid.org/0000-0003-3353-2137
Zexin Hou https://orcid.org/0000-0001-7154-0424

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Inflammatory activation biomarker profile after marathon running and its impact on cardiovascular stress in amateur middle-aged male runners

Michał Kosowski[1,2,A–F], Katarzyna Swoboda[2,A–F], Jan Chmura[3,A–C,E,F], Dorota Kustrzycka-Kratochwil[4,A–C,E,F], John A. Todd[5,A–C,E,F], Ewa A. Jankowska[1,2,A–C,E,F], Krzysztof Reczuch[1,2,C,E,F], Piotr Ponikowski[1,2,A–F]

[1] Institute of Heart Diseases, Wroclaw Medical University, Poland
[2] Institute of Heart Diseases, University Hospital, Wrocław, Poland
[3] Department of Motor Ability and Sport Performance, University School of Physical Education, Wrocław, Poland
[4] Centre for Heart Diseases, 4th Military Hospital, Wrocław, Poland
[5] Singulex, Inc., Alameda, USA

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Michał Kosowski
E-mail: michal.kosowski@umw.edu.pl

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Abstract

Background. Cardiovascular safety of marathon running middle-aged amateurs remains unclear. We previously hypothesized that transient release of cardiac troponin I (cTnI) and N-terminal pro-B-type natriuretic peptide (NT-proBNP), in addition to an acute inflammatory response to exercise, may be the cause.

Objectives. To evaluate the effects of running a marathon on inflammatory biomarkers, and its impact on cardiovascular function.

Materials and methods. Thirty-three healthy male amateur runners aged ≥50 (mean age: 57 ±7 years) were enrolled in the study. Venous blood samples were obtained before the marathon, just after the race, and 2–4 days and 7 days after the marathon. Using novel single molecule counting (SMC) technology, we measured plasma concentrations of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α). White blood cell (WBC) count was measured using a certified hematology analyzer. The results were related to previous analyses on cardiovascular stress and endothelial function biomarkers. Transthoracic echocardiography (TTE) and cardiac magnetic resonance (CMR) were used to determine myocardial function.

Results. We observed a sharp rise of all studied biomarkers after the race, which subsequently normalized after 2–4 days and stayed within the normal range 7 days after the race. We found no correlation between inflammatory and cardiovascular stress biomarkers. Transthoracic echocardiography and CMR did not show ischemic or inflammatory myocardial damage.

Conclusions. Marathon running is associated with a sharp and significant rise in inflammatory and cardiovascular stress biomarkers. We found no connection between immune activation and cardiac biomarker release. Cardiovascular imaging showed no myocardial damage due to ischemia or inflammation.

Key words: inflammation, marathon, cardiovascular biomarkers, cardiovascular imaging, novel methods

Background

Exercise and physical activity have been proven to minimize cardiovascular risk. Regular aerobic activity triggers physiological adaptations that improve cardiorespiratory fitness, prevent the development of coronary artery disease and alleviate symptoms of already diagnosed cardiovascular disease. There is also evidence that suggests a reduction in the risk of other chronic diseases, including arterial hypertension, type 2 diabetes, depression, and some types of cancer.[1,2] However, the intensity of activity to achieve beneficial effects remains controversial. Some studies have shown that there may be a U-shaped relationship between exercise intensity, cardiovascular disease and mortality. Very intensive endurance activity may be associated with worse survival compared with low and moderate physical activity.[3–6]

The authors have already presented an evaluation of cardiovascular stress biomarkers in marathon running middle-aged amateurs – a group at potential risk of cardiovascular disease.[7] The race was associated with a sharp and significant, yet transient, rise in the levels of cardiac stress (cardiac troponin I (cTnI) and N-terminal pro-B-type natriuretic peptide (NT-proBNP)) and vascular (endothelin-1 (ET-1)) function biomarkers. The cause of these changes remains unclear and has been extensively investigated.

The fatigue induced by endurance exercise has been studied in terms of inflammation and immunological consequences.[8,9] An acute inflammatory response involving cytokine release has been observed after strenuous exercise, including marathon running.[10–12] Systemic inflammation has an established role in the atherosclerotic process and has been linked to myocardial and endothelial injury.[13–15]

Cardiovascular imaging studies, including cardiac magnetic resonance (CMR), show exercise- and inflammation-related features of myocardial dysfunction and a possible association between inflammatory mediators release and myocardial fibrosis.[16–18]

Objectives

In this study, we aimed to evaluate the inflammatory response to marathon running and possible correlations with cardiovascular stress biomarkers release and myocardial function, assessed with multimodality imaging (transthoracic echocardiography (TTE) and CMR).

Materials and methods

Study design and participants

The project was a prospective, observational study enrolling male recreational long-distance runners. The inclusion criteria were: age ≥50 years, male sex, completion of at least one full-distance (42.195 km) marathon, and regular physical activity. Any known cardiovascular disease (diagnosis and current treatment) was an exclusion criterion. The detailed protocol has been previously published.[7]

Venous blood samples drawn from the antecubital vein were obtained at the screening phase (V1), immediately after the run (V2), 72–96 h after the run (subgroup of 12 runners who underwent CMR study; V3), and 7 days after the marathon (V4). Plasma samples after centrifugation in ethylenediaminetetraacetic acid (EDTA) were aliquoted and stored at –80°C until the analyses were done. Complete and white blood cell (WBC) counts were performed immediately after the blood draw using standardized hospital methods.

All the participants were asked to refrain from endurance training for at least 2 days before V1, V3 and V4 blood draw.

To evaluate inflammatory activation, we chose to analyze WBC, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α), biomarkers that have been extensively studied in terms of myocardial damage pathophysiology. We have previously analyzed data on cardiovascular stress biomarkers (cTnI, NT-proBNP and ET-1).[7]

Plasma concentrations of IL-6 and TNF-α were measured using a novel single molecule counting (SMC) technology (Erenna Immunoassay; Singulex, Inc., Alameda, USA). The assay was proven to be up to 2 orders of magnitude more sensitive than assays available commercially.

The 99th percentile upper reference limit (URL) was 7.2 pg/mL and 4.2 pg/mL for IL-6 and TNF-α, respectively. The URLs were evaluated against age- and sex-matched groups by the producer. White blood cell count was measured using a certified hematology analyzer. The 99th percentile URL for males aged ≥50 years was 10.0 × 10^3/μL.

Transthoracic echocardiography was performed using a commercially available ultrasound system (Vivid S6; GE Healthcare, Milwaukee, USA) at baseline (screening phase) and immediately after the run. The cardiac morphology and function measurements were carried out according to the American Society of Echocardiography and the European Association of Cardiovascular Imaging recommendations. Left ventricular (LV) longitudinal strain was assessed using 2-dimensional speckle-tracking technique.

Heart rate (HR) during the entire run was recorded in 1-minute intervals using chest band pulse meters. Training distances were self-reported by participants.

The group of 12 randomly chosen participants underwent CMR between the 2nd and 4th day after the marathon using Siemens Magnetom Aera 1.5 T scanner (Siemens AG, Munich, Germany). Late gadolinium enhancement (LGE) modality was used for the detection of myocardial scar formation and fibrosis. Cardiac edema was assessed using the short tau inversion recovery (STIR) method. Cardiac magnetic resonance follow-up was scheduled 3 months after the run.

Statistical analyses

Continuous variables with normal distribution were reported as mean ± standard deviation (M ±SD), and variables with skewed distribution were reported as median with interquartile range. Depending on the distribution type, the intergroup differences were compared using paired Student's t-test, Mann–Whitney U test or Wilcoxon test. Analysis of variance (ANOVA) was used to analyze the differences among group means. The Spearman's rank correlation test was used to calculate correlations. Factors determining biomarker level dynamics were defined using linear and logistic regression models. Statistica v. 10.0 (StatSoft Inc., Tulsa, USA) software was used to perform all analyses.

Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki. The Bioethics Committee of Wroclaw Medical University, Poland, reviewed and accepted the protocol of the study (approval No. KB-412/2014). All participants gave written informed consent for participation in the project.

Results

Participants

Initially, we have invited 50 Marathon Academy (Wrocław, Poland) initiative affiliates willing to participate in the 32nd Wroclaw Marathon (September 14, 2014) to be enrolled in the study. The number of subjects was limited to 50 in order to complete the whole protocol (as described above).

After the screening phase (physical examination, TTE and initial blood draw, 4–8 weeks before the run), we included 37 runners without cardiovascular disease. One subject was rejected due to moderate aortic regurgitation (bicuspid aortic valve) and 1 due to untreated hypothyroidism. Another 2 participants stated that they could not undergo all study procedures due to personal matters. Eventually, 33 healthy subjects (mean age: 57 ±7 years) were included.

The baseline parameters are presented in Table 1.

Table 1. Baseline characteristics

Parameter	Results (n = 33)
Age [years], M ±SD, min–max	57 ±7, 50–74
BMI [kg/m^2]	23.6 (21.9–25.7)
Resting HR [1/min]	57 (53–63)
Resting SBP [mm Hg]	120 (115–125)
Resting DBP [mm Hg]	73 (68–80)
Training and marathon run parameters	
Training intensity [km/month]	169 (74–201)
Long distance running experience [years]	6 (5–10)
Marathon total time [min]	250 (225–269)
Marathon mean HR [1/min]	149 (143–157)
Marathon maximum HR [1/min]	161 (157–172)

M ±SD – mean ± standard deviation; BMI – body mass index; HR – heart rate; SBP – systolic blood pressure; DBP – diastolic blood pressure.

Biomarkers

We observed a significant increase of the concentrations of all studied biomarkers (WBC, IL-6 and TNF-α) immediately after the marathon (all $p < 0.01$), which subsequently normalized (Fig. 1).

The IL-6 concentration was elevated immediately after the run above URL in all subjects. Peak IL-6 levels and absolute post-race increase correlated positively with peak WBC and TNF-α concentrations, as well as with their absolute increase.

The TNF-α levels were elevated above URL after the race in 19 (58%) subjects. The TNF-α levels after the run and absolute increase positively correlated with the other biomarkers.

White blood cell count was elevated above URL post-race in 30 (91%) subjects. Peak WBC count and absolute increase positively correlated with IL-6 and TNF-α.

Peak IL-6 concentration, WBC count, and absolute increase in IL-6 and TNF-α inversely correlated with finishing time.

Neither peak biomarker concentrations after the marathon nor their absolute increases were related to any cardiovascular stress biomarker level or any index of HR.

No specific parameter was found to determine the increase or peak level of any biomarker (logistic and linear regression models).

The analysis of the 12 selected participants in the CMR subgroup showed normalized biomarker concentration within 24–48 h after the marathon.

None of the participants reported any symptoms of infection or injury which could have contributed to increased biomarker concentrations.

All of the values of biomarker plasma concentrations, rates of abnormal biomarker levels and biomarker correlations are presented in Table 2–4.

Echocardiography and cardiac magnetic resonance

Both left and right ventricular size, and left ventricular global longitudinal strain (LVGLS) remained unchanged after the run and stayed within normal limits. We did not observe abnormal right ventricular function indices – tricuspid annular plane systolic excursion (TAPSE) and tricuspid annular plane systolic velocity (s'). A decrease in the left atrial

Table 2. Biomarker levels

Parameter					p-value						
n	V1	V2	V3	V4	V1 vs V2	V1 vs V3	V1 vs V4	V2 vs V3	V2 vs V4	V3 vs V4	ANOVA
IL-6 [pg/mL]											
n = 33	1.55 (1.3–2.11)	63.25 (39.72–75.03)	–	1.64 (1.3–2.02)	**<0.001**	–	0.734	–	**<0.001**	–	**<0.001**
n = 12	1.94 (1.33–2.12)	60.49 (44.14–82.89)	1.83 (1.31–2.87)	1.65 (1.29–1.80)	**0.002**	0.530	0.388	**0.002**	**0.003**	0.131	**<0.001**
TNF-α [pg/mL]											
n = 33	3.20 (2.85–3.78)	4.43 (3.80–5.49)	–	3.44 (2.80–3.88)	**<0.001**	–	0.514	–	**<0.001**	–	**<0.001**
n = 12	3.18 (2.84–3.90)	4.97 (3.83–5.50)	3.37 (2.83–4.34)	3.87 (3.20–4.02)	**0.009**	0.528	0.308	**0.034**	0.084	0.530	0.127
WBC [10^3/μL]											
n = 33	5.03 (4.47–6.15)	14.5 (12.21–16.71)	–	5.15 (4.38–6.15)	**<0.001**	–	0.492	–	**<0.001**	–	**<0.001**
n = 12	5.15 (4.27–6.32)	12.65 (11.57–15.51)	5.15 (4.82–5.54)	4.45 (4.32–6.18)	**0.002**	0.695	0.991	**0.002**	**0.023**	0.937	**<0.001**

Values in bold are statistically significant. V1 – baseline; V2 – marathon finish; V3 – 3–4 days after the marathon (n = 12); V4 – 7 days after the marathon; IL-6 – interleukin-6; TNF-α – tumor necrosis factor alpha; WBC – white blood cells. Data presented as median with interquartile range (Wilcoxon test and Friedman non-parametric analysis of variance (ANOVA) test).

Table 3. Rates of abnormal (above upper reference limit) biomarker levels

Parameter	V1 (n = 33)	V2 (n = 33)	V3 (n = 12)	V4 (n = 33)
hs-cTnI, n (%)	7 (21)	29 (88)	5 (42)	7 (21)
NT-proBNP, n (%)	3 (9)	22 (67)	3 (25)	3 (9)
ET-1, n (%)	5 (15)	31 (94)	4 (33)	4 (12)
TNF-α, n (%)	4 (12)	19 (58)	4 (33)	2 (6)
IL-6, n (%)	0	33 (100)	1 (8)	1 (3)
WBC, n (%)	0	30 (91)	0	0

V1 – baseline; V2 – marathon finish; V3 – 3–4 days after the marathon; V4 – 7 days after the marathon; hs-cTnI – high-sensitivity cardiac troponin I; NT-proBNP – N-terminal pro-B-type natriuretic peptide; ET-1 – endothelin-1; TNF-α – tumor necrosis factor alpha; IL-6 – interleukin-6; WBC – white blood cells.

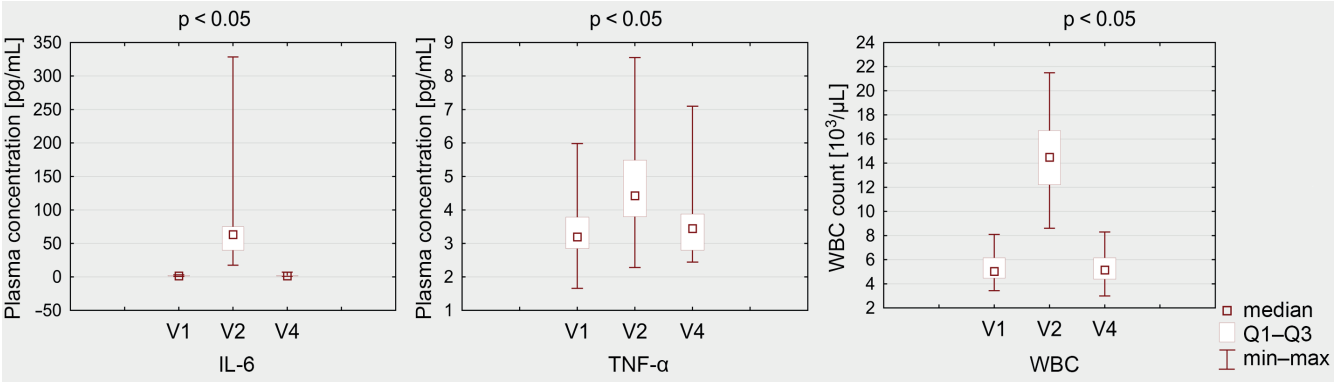

Fig. 1. Interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and white blood cell (WBC) concentrations measured before the marathon run (V1), immediately after (V2), and 7 days after (V4) in the whole studied group (n = 33). Data presented as median with interquartile (Q1–Q3) range and minimum–maximum values

volume index (LAVI) and an increase in the maximal right atrial volumes were found. A post-run reduction in peak velocity flow in early diastole (the E wave), peak velocity flow in late diastole caused by atrial contraction (the A wave) ratio and early diastolic mitral annular velocity (e') suggesting the deterioration of LV diastolic function were shown. However, E/e' septal and lateral ratios remained unaltered. We failed to demonstrate any correlations between echocardiographic parameters and inflammatory biomarker values. Echocardiographic data are presented in Table 5.

Both the CMR study carried out between the 2nd and 4th day after the marathon, and the follow-up study

Table 4. Spearman's rank correlation: post-race cardiovascular, inflammatory biomarker concentrations and total marathon time

Parameter	Peak hs-cTnI	Δhs-cTnI (V2–V1)	Peak NT-proBNP	ΔNT-proBNP (V2–V1)	Peak ET-1	ΔET-1 (V2–V1)	Peak TNF-α	ΔTNF-α (V2–V1)	Peak IL-6	ΔIL-6 (V2–V1)	Peak WBC	ΔWBC (V2–V1)	Total time
peak hs-cTnI	–	**0.92**	0.28	0.27	0.31	0.14	0.14	–0.03	0.26	0.26	–0.12	–0.09	0.24
Δhs-cTnI (V2–V1)	**0.92**	–	0.28	0.27	0.21	0.11	0.15	–0.07	0.24	0.24	–0.01	0.01	0.25
peak NT-proBNP	0.28	0.28	–	**0.97**	0.28	–0.03	0.23	0.02	0.18	0.19	0.06	0.12	0.26
ΔNT-proBNP (V2–V1)	0.27	0.27	**0.97**	–	0.27	–0.06	0.19	–0.02	0.16	0.17	0.01	0.10	0.23
peak ET-1	0.31	0.21	0.28	0.27	–	**0.72**	0.03	–0.12	0.02	0.02	–0.23	–0.13	0.19
ΔET-1 (V2–V1)	0.14	0.11	–0.03	–0.06	**0.72**	–	0.01	–0.22	–0.15	–0.16	–0.17	–0.11	0.29
peak TNF-α	0.14	0.15	0.23	0.19	0.03	0.01	–	**0.68**	**0.49**	**0.49**	**0.50**	**0.45**	–0.24
ΔTNF-α (V2–V1)	–0.03	–0.07	0.02	–0.02	–0.12	–0.22	**0.68**	–	0.33	**0.34**	**0.39**	**0.39**	**–0.35**
peak IL-6	0.26	0.24	0.18	0.16	0.02	–0.15	**0.49**	0.33	–	**1.00**	**0.44**	**0.39**	**–0.40**
ΔIL-6 (V2–V1)	0.26	0.24	0.19	0.17	0.02	–0.16	**0.49**	**0.34**	**1.00**	–	**0.43**	**0.39**	**–0.41**
peak WBC	–0.12	–0.01	0.06	0.01	–0.23	–0.17	**0.50**	**0.39**	**0.44**	**0.43**	–	**0.93**	**–0.35**
ΔWBC (V2–V1)	–0.09	0.01	0.12	0.10	–0.13	–0.11	**0.45**	**0.39**	**0.39**	**0.39**	**0.93**	–	–0.32
Total time	0.24	0.25	0.26	0.23	0.19	0.29	–0.24	**–0.35**	**–0.40**	**–0.41**	**–0.35**	–0.32	–

Values in bold are statistically significant with $p < 0.05$. hs-cTnI – high-sensitivity cardiac troponin I; NT-proBNP – N-terminal pro-B-type natriuretic peptide; ET-1 – endothelin-1; TNF-α – tumor necrosis factor alpha; IL-6 – interleukin-6; WBC – white blood cells; V1 – baseline; V2 – marathon finish.

conducted 3 months after the run did not reveal any abnormalities in cardiac dimensions or function. The STIR heart to skeletal muscle signal ratios were 1.74 (1.54–1.81) and 1.66 (1.58–1.73), respectively, for the initial and follow-up scan excluding myocardial edema. No myocardial fibrosis reflected by LGE was detected in any study.

Discussion

Our previous research using novel ultrasensitive laboratory assays showed a sharp but transient increase in cTnI, NT-proBNP and ET-1 levels in the setting of a marathon run.[7] The impact of this phenomenon on the circulatory system, as well as the cause and mechanism of cardiovascular stress biomarker release, remain unclear. Growing evidence linking immune response to strenuous exercise, a known effect of systemic inflammation on atherosclerosis and possible cardiac fibrosis, warranted further analyses.[8,9,13–15,17,18] This paper focused on studying the dynamics of inflammatory biomarker release, possible correlations with cardiovascular stress biomarkers and establishing whether marathon running provokes myocardial damage as assessed by imaging (TTE and CMR).

In 1902, Larrabee reported one of the first observations of exercise-induced increase in blood neutrophils among athletes who participated in the 1901 Boston Marathon.[19] The cytokine response to exercise was documented for the first time by Cannon and Kluger in the study where blood obtained from human subjects after exercise was injected into rats and caused an inflammatory response.[20]

Strenuous physical activity may trigger the same response as physical stress. The immunological response seen in the setting of trauma, sepsis, burn, etc., has a similar pattern to that caused by exercise. Local muscle damage due to prolonged exercise and systemic stress promotes cytokine production, originally released at the site of inflammation. Laboratory models show that the injection of TNF-α, IL-6 and IL-1 into laboratory animals induces an acute phase response. These cytokines are usually referred to as pro-inflammatory cytokines. The infusion of IL-6 alone can induce fever, but not shock, and cannot upregulate other inflammatory mediators.[21]

Since IL-6 and TNF-α release leads to hepatic production of C-reactive protein (CRP), which has established a role as a marker of increased cardiovascular risk, many studies have focused on the relationship between inflammation and cardiovascular response. Arterial inflammation in response to certain factors such as cholesterol, toxins, shear stress, and reactive oxygen species (ROS) causes endothelial dysfunction and thrombosis.[22] Rhabdomyolysis due to exertion triggers the release of von Willebrand factor from endothelium which, together with distention of vascular bed and reduced plasma volume, may provoke an imbalance between thrombosis and fibrinolysis.[23] Animal models, followed by case reports of athletes who died suddenly, showed interstitial fibrosis due to myocardial inflammation caused by endurance exercise.[13,24,25]

Table 5. Echocardiography

Parameter (n = 33)	Baseline	Post-race	p-value
Transthoracic echocardiography			
LVEDD [mm]	50 ±5	48 ±4	0.712
LVESD [mm]	31 ±4	30 ±4	0.386
RVEDD [mm]	27 ±4	27 ±3	0.523
IVS [mm]	12 ±2	12 ±1	0.142
PW [mm]	11 ±1	11 ±1	0.937
IVC [mm]	21 ±3	21 ±4	0.489
LVEF (%)	66 ±5	67 ±6	0.317
LVGLS (%)	−20.1 ±1.4	−19.9 ±1.5	0.721
TAPSE [mm]	24 ±4	24 ±5	0.722
TV s' [cm/s]	29.5 ±6.7	29.9 ±6.0	0.643
LA [mm]	37 ±3	35 ±4	**0.009**
LAVI [mL/m^2]	22 ±6	17 ±7	**<0.001**
LA min. volume [mL]	25 ±9	19 ±10	**<0.001**
LA max. volume [mL]	58 ±17	45 ±18	**<0.001**
RA min. volume [mL]	29 ±11	30 ±11	0.432
RA max. volume [mL]	49 ±12	53 ±12	**0.025**
E [m/s]	0.71 ±0.16	0.53 ±0.12	**<0.001**
A [m/s]	0.64 ±0.15	0.70 ±0.11	0.078
E/A	1.1 ±0.3	0.8 ±0.3	**<0.001**
e' septal [m/s]	0.09 ±0.03	0.07 ±0.02	**<0.001**
e' lateral [m/s]	0.12 ±0.04	0.10 ±0.03	**<0.001**
E/e' septal	8.0 ±2.5	8.1 ±2.6	0.837
E/e' lateral	5.8 ±1.6	5.3 ±1.3	0.134
Average E/e'	6.7 ±1.8	6.4 ±1.7	0.418

Values in bold are statistically significant. LVEDD – left ventricular end-diastolic diameter; LVESD – left ventricular end-systolic diameter; RVEDD – right ventricular end-diastolic diameter; IVS – intraventricular septum; PW – posterior wall; IVC – inferior vena cava; LVEF – left ventricular ejection fraction; LVGLS – left ventricular global longitudinal strain; TAPSE – tricuspid annular plane systolic excursion; TV s' – tricuspid annular plane systolic velocity; LA – left atrium; LAVI – left atrial volume index; RA – right atrium; E – peak velocity flow in early diastole (the E wave); A – peak velocity flow in late diastole caused by atrial contraction (the A wave); e' – early diastolic mitral annular velocity. Data presented as mean ± standard deviation (paired Student's t-test).

The release of TNF-α was well-described after physical activity and has been linked to a risk of cardiac dysfunction.[26–28] On the other hand, some studies emphasize the regulatory role of IL-6, which no longer should be considered a pro-inflammatory cytokine only. Despite reports suggesting a key role of IL-6 in destabilizing atherosclerotic plaques, this cytokine may also have anti-inflammatory properties and has been linked to exercise-related positive metabolic changes, adaptation to training, protection against ischemic myocardial injury, or even a reduced prevalence of arrhythmias among marathon runners.[29–31]

Papers on exercise-induced release of cardiovascular stress biomarkers were inconclusive in terms of the relationship between cardiovascular damage and acute immune response.[32–34] Our data show that the release of inflammatory, cardiovascular stress and endothelial function biomarkers after marathon running was rapid and sharp but transient. Despite potential BNP (and NT-proBNP) gene modulation via cytokine pathways, we did not observe any relationship between an increase of NT-proBNP and levels of WBC, IL-6 or TNF-α. Increased post-race cTnI levels, reflecting potential myocardial damage, were also independent of any inflammatory biomarker. Peak IL-6 concentration, WBC count and absolute increase in IL-6 and TNF-α inversely correlated with finishing time, suggesting that the intensity of exercise plays a role in inflammatory response. The same suggestions come from data obtained during the Copenhagen Marathon (1996–1998).[21] Table 6 depicts selected publications on exercise-induced inflammatory biomarker release.

Post-race echocardiography did not show any evidence for LV and right ventricular (RV) systolic dysfunction. These findings are in line with the majority of publications on this topic.[35–38] Nevertheless, some authors have reported transient RV systolic function impairment and increased estimated pulmonary vascular resistance following strenuous exercise.[39–41]

The interpretation of post-race changes in LV diastolic function should be made with caution, given the load

Table 6. Summary of selected publications on exercise-induced inflammatory biomarker release

Authors	Year	Findings
Ostrowski et al.[27]	1999	increase in plasma levels of TNF-α, IL-1 and IL-6 after a marathon (n = 10)
Nieman et al.[10]	2001	increase in plasma levels of IL-10, IL-1 receptor antagonist, IL-6, IL-8, and TNF-α after a marathon (n = 98)
Siegel et al.[23]	2001	increase in plasma levels of CRP and WBC count after a marathon (n = 55)
Jee and Jin[28]	2012	increase in plasma levels of CRP, TNF-α, soluble vascular cell adhesion molecule-1, serum E-selectin, and WBC after a 308-kilometer ultramarathon (n = 24)
Scherr et al.[32]	2011	increase in plasma levels of high-sensitivity cardiac troponin T, NT-proBNP, IL-6, and high-sensitivity CRP after a marathon with a normalization within 72 h (n = 102)
Santos et al.[34]	2016	increase in plasma levels of WBC, creatinine kinase, lactate dehydrogenase, IL-6, IL-10, IL-8, IL-12, CRP, and TNF-α after a marathon (n = 23)

TNF-α – tumor necrosis factor alpha; IL – interleukin; WBC – white blood cells; NT-proBNP – N-terminal pro-B-type natriuretic peptide; CRP – C-reactive protein.

dependence of diastolic parameters.[35–38,42] Since the duration of diastole is affected by HR, LV preload is altered by dehydration and redistribution of blood flow, and blood pressure changes modify LV afterload, the finding of a post-run decrease in E/A ratio and e' may not reflect the actual effort-induced impairment in LV filling, especially as it was not accompanied by corresponding changes in E/e' ratio.

Repeated CMR in a subgroup of 12 subjects did not confirm any abnormalties suggestive of ischemia- or inflammation-associated myocardial damage that could be linked with the race.

This is consistent with other authors' findings.[17,18]

Limitations

First, this was a single-center study that enrolled a relatively small but homogenous population, yet represented a group at potential risk of developing exercise-related adverse events. Second, the small sample size might have led to the underestimation of some statistical associations, as well as the inability to conduct parametric tests on all data and provide causality analysis. Third, post-race echocardiography was performed in the early recovery period and might not have reflected the exact cardiac response to exercise. Fourth, although widely accepted, the use of load-dependent methods could not adequately assess the effect of effort on LV ventricular diastolic function. Finally, due to the small random sample of 12 subjects who underwent a repeated CMR study, the generalizability of findings by this technique is limited.

Conclusions

Strenuous exercise, such as marathon running, is associated with a transient but significant increase in plasma concentrations of inflammatory and cardiovascular stress biomarkers. No correlation between immune activation, cardiac biomarker release or cardiac dysfunction was found. Cardiovascular imaging showed no permanent myocardial damage due to ischemia or inflammation. Given the growing number of recreational runners, further studies on the pathophysiology and clinical importance of these findings are warranted.

ORCID iDs

Michał Kosowski https://orcid.org/0000-0001-8911-5303
Jan Chmura https://orcid.org/0000-0002-9153-7983
Ewa A. Jankowska https://orcid.org/0000-0002-9202-432X
Krzysztof Reczuch https://orcid.org/0000-0002-1699-739X
Piotr Ponikowski https://orcid.org/0000-0002-3391-7064

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The effects of sorafenib in healthy and cisplatin-treated rats

Levent Demirtas[1,A–E], Mehmet Gürbüzel[2,A–D,F], Hilal Tahirler[3,A,B,E], Emin Murat Akbas[4,A,B,E], Ozhan Karatas[5,C], Yusuf Kemal Arslan[6,C]

[1] Department of Internal Medicine, Faculty of Medicine, Erzincan Binali Yıldırım University, Türkiye
[2] Department of Medical Biology, Faculty of Medicine, Erzincan Binali Yıldırım University, Türkiye
[3] Department of Internal Medicine, Gülhane Training and Research Hospital, University of Health Sciences, Türkiye
[4] Department of Endocrinology, Faculty of Medicine, Erzincan Binali Yıldırım University, Türkiye
[5] Department of Veterinary Pathology, Faculty of Veterinary Medicine, Sivas Cumhuriyet University, Türkiye
[6] Department of Biostatistics, Faculty of Medicine, Çukurova University, Adana, Türkiye

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Mehmet Gürbüzel
E-mail: mehmetgurbuzel@hotmail.com

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Abstract

Background. Sorafenib is a multikinase inhibitor currently used in the treatment of hepatocellular carcinoma, renal cell carcinoma and thyroid cancer.

Objectives. The literature on this agent is scarce. This study aimed to evaluate the effects of sorafenib when administered to both healthy and cisplatin-induced rats.

Materials and methods. The animals were divided into 4 groups: 1) control group that received 0.9% saline intraperitoneally (C); 2) group administered a single dose (7 mg/kg) of cisplatin (Cis); 3) a group administered 20 mg/kg of sorafenib for 7 days (Sor); 4) group administered 20 mg/kg of sorafenib followed by 7 mg/kg of cisplatin for 7 days (Cis+Sor). All animals were sacrificed 7 days after the completion of their treatment arm, and serum and tissue samples were taken.

Results. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and interleukin 38 (IL-38) levels were increased in the Sor and Cis+Sor groups compared to the control group. When compared with the control group, serum urea, creatinine, kidney IL-1β, and tumor necrosis factor alpha (TNF-α) levels did not change in the Sor group. When compared to the Cis group, the levels of these parameters decreased in the Cis+Sor group.

Conclusions. According to the data obtained, sorafenib caused liver toxicity when given to both healthy and cisplatin-induced rats. While sorafenib did not cause any significant changes in the kidneys when given to healthy rats, it had a healing effect in kidneys after stress induced by cisplatin.

Key words: IL-38, cisplatin, rat, nephrotoxicity, sorafenib

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Background

The molecular revolution that has taken place over the last 30 years has led to a significant increase in the knowledge regarding the etiology of cancer.[1] In the tumor development process, the misexpression of proto-oncogenes and tumor suppressor genes as a result of mutations is highly effective. The fact that almost all of the human genome sequences, including the sequences of oncogenes and tumor suppressor genes, have now been determined, has led to the development of targeted agents that aim to stop the proliferation and invasion of cancer cells by interfering with specific molecules that play a role in the mechanism of tumorigenesis; the same agents currently yield successful results in cancer treatment.[2] Transmission of signals received from cell surface receptors to transcription factors for the regulation of gene expression and activation of proteins in the apoptosis process occurs via the MAPK/ERK or alias RAS/RAF/MEK/ERK signaling pathway, whose components (such as Ras and B-RAF) are encoded by proto-oncogenes.[3,4] Thus, MAPK/ERK plays a key role in the signaling pathways involved in cell survival, proliferation and differentiation. Sorafenib, an orally administered bis-aryl urea, inhibits the tyrosine kinase activity of C-RAF and B-RAF (against both wild-type and V599E mutants).[3,5] Sorafenib, due to its multikinase inhibitor, suppresses FMS-like tyrosine kinase-3 (FLT3), platelet-derived growth factor receptor β (PDGFR-β), stem cell growth factor receptor (ScGFR or c-KIT), and vascular endothelial growth factor receptors (VEGFRs type 2 and 3).[2,3,5] Sorafenib has been clinically shown to be effective against hepatocellular carcinoma, renal cell carcinoma and thyroid cancer by increasing median survival rates.[6–8]

Cisplatin is a platinum compound used to treat solid tumors, such as bladder, colorectal, head and neck, lung, testicular, and ovarian cancers.[9] The most basic mechanism of action of cisplatin, which enters the cell and interacts with DNA, RNA and proteins, is to crosslink to purine bases in DNA molecules in order to form DNA-platinum adducts.[10,11] This binding causes DNA damage in the nucleus and mitochondria. The DNA damage is likely greater in rapidly proliferating cancer cells.[12] While apoptosis occurs as a result of damage to mitochondrial DNA, nucleotide excision repair (NER) and DNA mismatch repair (MMR) mechanisms in nuclear DNA take an active role in trying to repair the DNA damage. If the damage is beyond repair, the cell is again dragged into apoptosis.[11] Cisplatin has a more toxic effect than cisplatin analogs and causes serious side effects, such as nephrotoxicity, ototoxicity, hepatotoxicity, gastrointestinal toxicity, and peripheral neuropathy.[10,11] Cisplatin is mainly collected in the kidneys. It activates cytoprotective signaling pathways, such as p21, and signaling pathways such as MAPK, p53 and reactive oxygen species (ROS) which promote cell death. In addition, inflammation as a result of tumor necrosis factor alpha (TNF-α) induction and ischemia caused by vascular injury resulting in cell death can occur in renal tubular cells exposed to cisplatin. Due to the effect of renal tissue damage and vascular injury, the glomerular filtration rate (GFR) decreases, and ultimately, acute renal failure develops.[12]

Objectives

Information on how sorafenib works, both when given healthy subjects and in different stress situations, is very limited. This study aimed to investigate the effects of sorafenib on renal and liver tissues when administered to both healthy and cisplatin-induced rats. For this purpose, after general serum biochemical analyses, polymerase chain reaction (PCR) analyses were performed on the kidney, which is the tissue with the highest potential for cisplatin toxicity. In addition, histopathological evaluations were conducted on both liver and kidney tissues.

Materials and methods

Chemicals

Cisplatin (Cisplatin-Ebewe®, 100 mg/100 mL) used in the study was purchased from Liba Lab (Istanbul, Türkiye). Sorafenib (BAY 43-9006, Nexavar, 200 mg) obtained from Bayer Türk Kimya San. Ltd. Şti. (Istanbul, Türkiye), ketamine (Ketalar, 500 mg/10 mL) obtained from Pfizer Drug Co. (Istanbul, Türkiye) and xylazine (Xylazin Bio 2%, 20 mg/mL) from Bioveta (Ankara, Türkiye) were used.

Animals

Animal care and use were performed according to the Turkish National Animal Experiments Ethics Committee guidelines after the approval of Atatürk University's Animal Experiments Local Ethics Committee (approval No. 3125 from September 28, 2017). The 28 albino Wistar male rats weighing 200–220 g used in our study were purchased from Atatürk University Faculty of Medicine Experimental Application and Research Center, Erzurum, Türkiye. The animals were kept in plastic breeding cages with free access to a standard laboratory nutrient diet and tap water ad libitum. Controlled experimental conditions, such as a 12-hour light/dark cycle, 21 ±1°C temperature and relative humidity of approx. 60%, were maintained in the housing environments. The animals used in the experiment were divided into 4 groups, with 7 randomly selected rats in each group. The groups were as follows: C – control; Cis – cisplatin (7 mg/kg); Sor – sorafenib (20 mg/kg); and Cis+Sor – cisplatin (7 mg/kg) + sorafenib (20 mg/kg).

A single dose of 7 mg/kg cisplatin dissolved in 0.9% saline was administered intraperitoneally to the animals in the Cis and Cis+Sor groups. Sorafenib was administered at a dose of 20 mg/kg in the Sor and Cis+Sor groups. The sorafenib was administered orally for 7 days, with the first dose given 1 h after the administration of cisplatin. Only saline solution was administered intraperitoneally in the control group. At the end of the experimental period, an anesthetic cocktail (60 mg/kg ketamine + 7.5 mg/kg xylazine) dissolved in 0.9% saline was administered intraperitoneally, and then all of the animals were euthanized by exsanguination. The kidneys were taken from all the rats, washed with 0.9% saline and lysed with a homogenizer (IKA Ultra-Turrax® T25 basic homogenizer; IKA Werke, Staufen, Germany). Blood samples (~5 mL) were centrifuged at 2500 rpm for 15 min to obtain the serum (Eppendorf 5430R; Eppendorf, Hamburg, Germany). Homogenizations and serum samples were kept at –80°C for biochemical and PCR analyses.

Biochemical procedures

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and creatinine measurements were performed using an Olympus AU640 autoanalyzer (Olympus Corp., Kobe, Japan). The kinetic urease/glutamate dehydrogenase method was used to determine the urea level, and the uncompensated Jaffe method was used for creatinine. The ALT and AST levels were measured according to the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) using pyridoxal phosphate activation. Serum 8-hydroxy-deoxyguanosine (8-OHdG) and interleukin 38 (IL-38) measurements were also performed. Sandwich enzyme-linked immunosorbent assay (ELISA) kits (catalog No. SG-20424 and No. SG-21170, respectively) supplied by SinoGeneClon Biotech Co., Ltd. (Hangzhou, China) were used for quantitative measurement with a Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). The ELISA measurements were carried out in accordance with the manufacturer's instructions.

Total RNA purification and cDNA synthesis

Total RNA isolation was performed on kidney homogenizations using a GeneAll® Hybrid-RTM (cat No. 305-101; GeneAll Biotechnology, Seoul, South Korea) total RNA purification kit in accordance with the manufacturer's instructions. Complementary DNA (cDNA) was synthesized from total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA), according to the manufacturer's instructions. Concentration determinations of cDNA samples were obtained using the μDrop™ Plate integrated into the Multiskan Sky Microplate Spectrophotometer (Thermo Fisher Scientific).

Quantitative real-time PCR

Both reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR) procedures were performed using the QuantStudio™ 5 Real-Time PCR Instrument (Thermo Fisher Scientific). The SYBR green-based expression analysis in QuantiTect Primer Assays (Qiagen, Hilden, Germany) was used for quantitative analysis. The total volume used for the reactions was 20 μL (5 μL of cDNA, 12.5 μL of master mix and 2.5 μL of primer solution). The catalog and the National Center for Biotechnology Information (NCBI) reference sequence numbers of the primers used in this study are as follows: ACTB1 (Rn_Actb_1_SG): QT00193473 and NM_031144.3; IL-1β (Rn_Il1b_1_SG): QT00181657 and NM_031512.2; TNF-α (Rn_Tnf_1_SG): QT00178717 and NM_012675.3.

All samples were run under the same cycling conditions of 95°C for 15 min, 94°C for 15 s (40 cycles), 55°C for 30 s (40 cycles), and 72°C for 30 s (40 cycles). Melting curve analysis was performed to confirm the formation of amplification products immediately after the quantitative analysis. The cycling conditions for the melting curve analysis were 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The samples were normalized with the beta-actin 1 (*ACTB1*) gene. The obtained data were evaluated using the $2^{-\Delta\Delta CT}$ method.

Histopathological evaluation

After the necropsies of the rats, liver and kidney tissues were fixed in a 10% neutral-buffered formaldehyde solution for 48 h. After the tissues were subjected to the standard alcohol-xylol processes, they were embedded in paraffin blocks. The paraffin-blocked tissues were cut into 5-mm-thick sections using a microtome (Leica RM2145; Leica Instruments, Nußloch, Germany). Ten deparaffinized and randomly selected sections from each tissue sample were stained with hematoxylin and eosin (H&E). The histopathological evaluation was conducted by a pathologist who was blinded to the study groups using a light microscope (Zeiss Primo Star with an integrated Carl Zeiss Axiocam ERc 5s; Carl Zeiss AG, Oberkochen, Germany).

Statistical analyses

Statistical analyses of the data were performed using IBM SPSS v. 22.0 (IBM Corp., Armonk, USA). Continuous variables with normal distribution are reported as mean ± standard deviation (M ±SD). Variables that were not normally distributed are presented as median (interquartile range (IQR) (Q1–Q3)). The continuous variables were tested for the assumption of normality using the Shapiro–Wilk test. After, the homogeneity of the variances was tested using the Levene's test. One-way analysis of variance (ANOVA) was used to compare continuous variables between the groups when variables were normally distributed. After

ANOVA, Tukey's honestly significant difference (HSD) was performed as a post hoc test when the homogeneity assumption was met. Otherwise, the Games–Howell test was used as a post hoc test. When the variables did not show normal distribution, the Kruskal–Wallis (K–W) test was performed and the Dunn's test was used as a post hoc test. A value of $p < 0.05$ was considered statistically significant.

Results

Biochemical serum parameters

In the comparison between the groups, there was a statistically significant difference in urea levels ($F(3,24) = 21.9$, $p < 0.001$). The urea level in the Cis group was significantly higher than in all other groups. There was no difference between the control, Sor and Cis+Sor groups (Table 1–3).

The difference was significant in the comparison of creatinine between groups ($F(3,19) = 52.783$, $p < 0.001$). While the difference seen in the Sor group compared to the control group was insignificant, the differences between the control and Cis groups and the control and Cis+Sor groups were significant. Compared to the Cis group, the creatinine level in the Cis+Sor group was significantly lower (Table 1–3).

The differences in ALT levels between the groups were significant ($F(3,19) = 123.238$, $p < 0.001$). The differences between the control and Cis groups and Sor and Cis+Sor groups were not significant. The ALT level increased in the Sor group compared to the control group and the Cis group. Similarly, ALT levels in the Cis+Sor group were significantly increased compared to the control and Cis groups (Table 1–3).

The differences in AST levels between the groups were significant ($F(3,22) = 50.904$, $p < 0.001$). There was no difference between the control and Cis groups. The AST level in the Sor group increased compared to the control and Cis groups. Likewise, the increase in the Cis+Sor group was significant compared to both the control and Cis groups. In addition, the AST levels in the Cis+Sor group were significantly higher than in the Sor group (Table 1–3).

There was a significant difference in IL-38 levels between the groups (K–W statistics: 14.565, $p = 0.002$). There was no difference in the pairwise comparisons of the control and Cis groups and the Sor and Cis+Sor groups. The mean IL-38 levels in the Sor and Cis+Sor groups increased when compared with the control group. Likewise, the IL-38 levels in the Sor and Cis+Sor groups were significantly higher than in the Cis group (Table 1,2).

No difference was observed in the comparison of serum 8-OHdG levels between the groups ($F(3,20) = 1.068$, $p = 0.385$); therefore, a pairwise comparison could not be performed (Table 1–3).

qRT–PCR results

The IL-1β levels differed significantly between the groups ($F(3,20) = 9.138$, $p < 0.001$). The IL-1β expression level increased in the Cis group compared to the control group (3.6 ±1.4-fold). When compared with the Cis group separately, the levels were lower in the Sor and Cis+Sor groups (1.3 ±0.5-fold and 1.5 ±0.6-fold, respectively). There were no differences between the control, Sor and Cis+Sor groups (Table 1–3).

The TNF-α levels differed significantly between the groups ($F(3,18) = 39.507$, $p < 0.001$). The TNF-α expression levels were higher in the Cis and Cis+Sor groups when

Table 1. Comparison of the serum biochemical values and relative gene expression levels of the experimental animals

Parameters	Groups				ANOVA or K–W test results							
							post hoc test p-values					
	C	Cis	Sor	Cis+Sor	F value or K–W test statistics	p-value	C vs Cis	C vs Sor	C vs Cis+Sor	Cis vs Sor	Cis vs Cis+Sor	Sor vs Cis+Sor
Urea [mg/dL]	32.7 ±2.1	110.4 ±29.4	29.3 ±4.7	56.7 ±30.0	21.906*	<0.001	0.002	0.353	0.250	0.001	0.024	0.176
Creatinine [mg/dL]	0.22 ±0.01	0.89 ±0.22	0.19 ±0.03	0.43 ±0.03	52.783*	<0.001	0.008	0.248	<0.001	0.006	0.031	<0.001
ALT [u/L]	30.5 ±1.5	28.6 ±5.4	68.1 ±3.5	72.8 ±7.8	123.238*	<0.001	0.869	<0.001	<0.001	<0.001	<0.001	0.573
AST [u/L]	61.8 ±2.1	57.3 ±3.6	84.4 ±7.6	130.6 ±21.5	50.904*	<0.001	0.112	0.001	0.001	<0.001	<0.001	0.004
IL-38 [pg/mL]	137.9 (111.6–159.7)	124.6 (121.6–164.7)	437.1 (432.5–487.5)	396.2 (395.1–435.47)	14.565+	0.002	0.841	0.008	0.008	0.008	0.008	0.421
8-OHdG [ng/mL]	1.0 ±0.3	1.4 ±0.2	1.2 ±0.2	1.3 ±0.6	1.068	0.385	ns	ns	ns	ns	ns	ns
IL-1β (fold)	1.0 ±0.3	3.6 ±1.4	1.3 ±0.5	1.5 ±0.6	9.138**	<0.001	0.001	0.952	0.785	0.001	0.003	0.948
TNF-α (fold)	1.0 ±0.1	5.0 ±0.7	1.6 ±0.4	2.7 ±1.0	39.407*	0.001	0.010	0.063	0.010	0.004	0.009	0.052

The results were presented as mean ± standard deviation (M ±SD) for normally distributed data, and median (minimum–maximum) and interquartile range (IQR (Q1–Q3)) for non-normally distributed data. *Games–Howell test was performed as the post hoc test after ANOVA; **Tukey honestly significant difference (HSD) test was performed as the post hoc test after analysis of variance (ANOVA); +Dunn's test was performed as the post hoc test after the Kruskal–Wallis (K–W) test. ALT – alanine aminotransferase; AST – aspartate aminotransferase; IL – interleukin; TNF-α – tumor necrosis factor alpha; 8-OHdG –8-hydroxy-deoxyguanosine; ns – not significant; C – control group; Cis – group administered a single dose (7 mg/kg) of cisplatin; Sor – group administered 20 mg/kg sorafenib for 7 days; Cis+Sor – group administered 20 mg/kg sorafenib followed by 7 mg/kg cisplatin for 7 days.

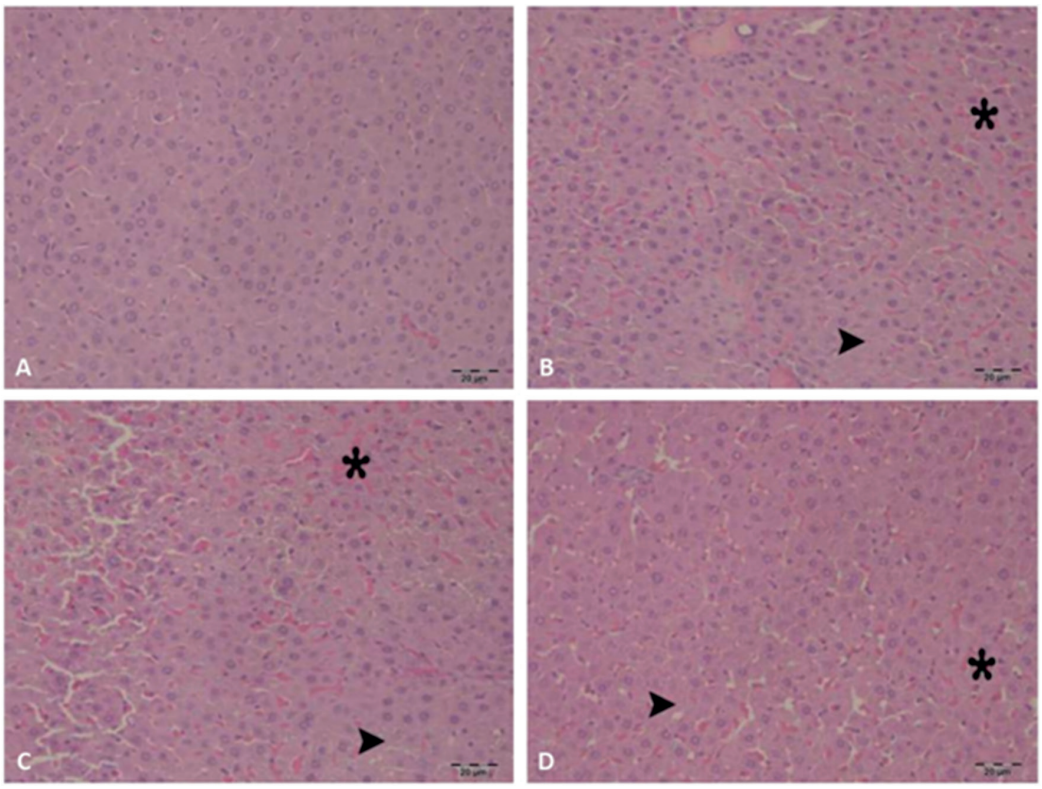

Fig. 1. Histopathological findings in liver tissues. A. Control group: normal histological appearance; B. Cis group: mild hemorrhagic areas (*) and necrotic hepatocytes (arrowhead); C. Sor group: severe hemorrhagic areas (*) and necrotic hepatocytes (arrowhead); D. Cis+Sor group: severe hemorrhagic areas (*) and necrotic hepatocytes (arrowhead); hematoxylin and eosin (H&E) staining

Cis – group administered a single dose (7 mg/kg) of cisplatin; Sor – group administered 20 mg/kg sorafenib for 7 days; Cis+Sor – group administered 20 mg/kg sorafenib followed by 7 mg/kg cisplatin for 7 days.

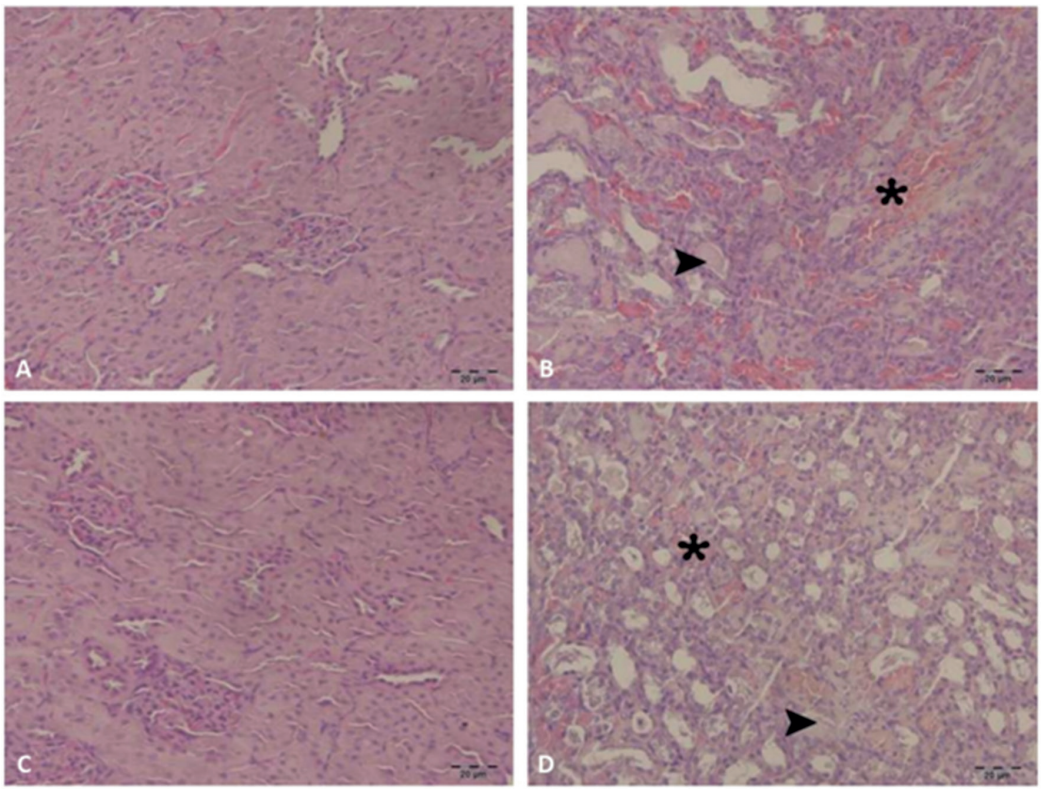

Fig. 2. Histopathological findings in kidney tissues. A. Control group: normal histological appearance; B. Cis group: severe hemorrhage in intertubular areas (*) and hyaline formations (arrowhead); C. Sor group: normal histological appearance; D. Cis+Sor group: moderate hemorrhage in intertubular areas (*) and hyaline formations (arrowhead); hematoxylin and eosin (H&E) staining

Cis – group administered a single dose (7 mg/kg) of cisplatin; Sor – group administered 20 mg/kg sorafenib for 7 days; Cis+Sor – group administered 20 mg/kg sorafenib followed by 7 mg/kg cisplatin for 7 days.

compared to the control group (5 ±0.7-fold and 2.7 ±1-fold, respectively). The difference between the control and Sor group was insignificant (1.6 ±0.4-fold). The expression level of the Cis + Sor group was significantly decreased compared to that of the Cis group (Table 1–3).

Histopathologic examination

The rat livers in the control group had a normal histological appearance. Mild hemorrhaging with mildly necrotic hepatocytes were observed in the Cis group.

Table 2. Results of normality tests

Parameters and groups		Shapiro–Wilk test		
		W	df	p-value
Urea	C	0.945	7	0.686
	Cis	0.835	7	0.089
	Sor	0.915	7	0.430
	Cis+Sor	0.841	7	0.102
Creatinine	C	0.869	7	0.183
	Cis	0.982	5	0.946
	Sor	0.852	6	0.163
	Cis+Sor	0.836	5	0.155
ALT	C	0.902	6	0.389
	Cis	0.868	5	0.260
	Sor	0.918	6	0.488
	Cis+Sor	0.906	6	0.413
AST	C	0.890	6	0.317
	Cis	0.914	6	0.466
	Sor	0.938	7	0.625
	Cis+Sor	0.919	7	0.465
IL-38*	C	0.897	5	0.395
	Cis	0.761	5	**0.037**
	Sor	0.882	5	0.319
	Cis+Sor	0.956	5	0.783
8-OHdG	C	0.887	5	0.341
	Cis	0.844	5	0.177
	Sor	0.924	7	0.499
	Cis+Sor	0.895	7	0.301
IL-1β	C	0.913	5	0.483
	Cis	0.940	5	0.667
	Sor	0.954	7	0.768
	Cis+Sor	0.892	7	0.287
TNF-α	C	0.826	5	0.131
	Cis	0.824	6	0.095
	Sor	0.954	5	0.766
	Cis+Sor	0.882	6	0.279

*Kruskal–Wallis tests were used for IL-38 since the data were not normally distributed according to the Shapiro–Wilk test. Analyses of variance (ANOVAs) were performed for other parameters. df – degrees of freedom; ALT – alanine aminotransferase; AST – aspartate aminotransferase; IL – interleukin; TNF-α – tumor necrosis factor alpha; 8-OHdG –8-hydroxy-deoxyguanosine; C – control group; Cis – group administered a single dose (7 mg/kg) of cisplatin; Sor – group administered 20 mg/kg sorafenib for 7 days; Cis+Sor – group administered 20 mg/kg sorafenib followed by 7 mg/kg cisplatin for 7 days. Statistically significant results are in bold.

Severe hemorrhages and necrotic hepatocytes were observed in the Sor and Cis+Sor groups (Fig. 1). The kidneys of the rats in the control and Sor groups had a normal histological appearance. Severe intertubular hemorrhages and hyaline formations were observed in the kidneys of the rats in the Cis group, while these formations were partially alleviated in the Cis+Sor group (Fig. 2).

Table 3. Results of the homogeneity of variance tests

Parameters	Levene's statistics	df1	df2	p-value
Urea	32.418	3	24	**<0.001**
Creatinine	9.995	3	19	**<0.001**
ALT	6.163	3	19	**0.004**
AST	16.839	3	22	**<0.001**
8-OHdG	3.507	3	20	**0.034**
IL-1β	1.814	3	20	0.177
TNF-α	4.216	3	18	**0.020**

According to Levene's test results, the Games–Howell test was used for urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), 8-hydroxy-deoxyguanosine (8-OHdG), and tumor necrosis factor alpha (TNF-α) variables, that did not show homogeneous distribution, and the Tukey's test was used for interleukin (IL)-1β, that showed homogeneous distribution. df – degrees of freedom. Statistically significant results are in bold.

Discussion

In this study, the effects of sorafenib were investigated in both healthy and cisplatin-stressed rats. For this purpose, serum parameters and changes in the expression levels were examined in the kidney tissue. It is known that cisplatin, a small molecule, accumulates in different cellular structures and cell types, including the cell membrane, cytosol, endoplasmic reticulum, mitochondria, nucleus, and lysosomes. This substance accumulates in many tissues and causes different types of toxicities.[13] However, it has been shown in many different studies that the most common toxic effect is nephrotoxicity.[12,13] According to the data, cisplatin increased urea and creatinine levels, while sorafenib did not cause any change. Statistically significant reductions in urea and creatinine levels were also noted when sorafenib was administered to cisplatin-induced animals. Compared to the control group, ALT, AST and IL-38 levels did not increase in the cisplatin group, but these parameters increased in rats given sorafenib. When sorafenib was given to the cisplatin-induced group, although there was no statistically significant difference in ALT and IL-38 levels, we observed that the AST level increased significantly in the Cis+Sor group compared to the group that was administered only sorafenib.

Increases in urea and creatinine levels indicate a reduction in renal functions, while increases in ALT and AST indicate liver damage. It was reported that the hepatotoxic and nephrotoxic effects of cisplatin vary according to the dose and time after administration.[14–16] Palipoch and Punsawad administered different doses of cisplatin to rats and recorded ALT, AST, BUN, and creatinine levels at 24 h, 48 h, 72 h, 96 h, and 120 h after administration.[14] The data indicate that after a single dose of cisplatin administration, ALT and AST decreased, while BUN and creatinine increased compared to the first recording times (24 h and 48 h) with the progression of time. Indeed, the liver is a dynamic organ, and the stress caused by cisplatin can be

expected to ease day by day. In addition, given that the kidneys are the main excretory organ in which cisplatin accumulates the most, the damage is likely to increase as time progresses. Similar data were obtained in our study. Serum ALT, AST and IL-38 values were found to be at the same level as those in the control group, when measurements were made 7 days after a single dose of cisplatin, while creatinine and urea values were found to be considerably high. The data indicate the presence of kidney damage even 7 days after cisplatin administration, similar to previous studies.[17,18] Previously, it was noted that sorafenib did not change BUN levels but decreased creatinine levels.[19–21] Our study is the first to show reduced levels of urea and creatinine in rats under stress by cisplatin.

It has also been reported that sorafenib increases ALT and AST, thereby inducing liver damage.[20,22] However, there is no information about how IL-38 levels progress with subsequent applications. The IL-38, a member of the IL-1 family with pro-inflammatory and anti-inflammatory effects, is expressed in different tissues, such as the heart, placenta, fetal liver, spleen, thymus, and tonsils, and is generally associated with rheumatic diseases.[23,24] It is not known precisely how this cytokine works under stress. There is no research in the literature on how the administration of sorafenib affects IL-38. However, in a study conducted in mice, it was noted that in the liver damage model induced by concanavalin A, IL-38 increased together with ALT and AST due to stress, thus showing a hepatoprotective effect.[25] Similarly, our data show that the IL-38 level is parallel to ALT and AST levels. In our analysis, it was observed that the level of 8-OHdG, which is a marker of oxidative DNA damage, increased in all groups compared with the control group, but these data were statistically insignificant.

In the 2nd part of our study, IL-1β and TNF-α expression levels in kidney tissue were examined. Similar to previous studies, IL-1β and TNF-α expression levels were increased in rats given only cisplatin.[17,18] The IL-1β and TNF-α expression levels did not change significantly in rats treated with sorafenib only. In addition, it was noted that IL-1β and TNF-α expression levels decreased when sorafenib was administered to rats that were given cisplatin. There are few studies in the literature reflecting the effects of sorafenib on cytokines when administered in healthy individuals or when used in cases of kidney damage or even any stress. Of these, sorafenib has been shown to reduce increased serum IL-1β and TNF-α levels in rats with adjuvant-induced arthritis.[26] It has been noted that the secretion of active cytokines, such as IL-6, IL-12 and TNF-α, decreased in dendritic cell cultures treated with sorafenib.[27] Sorafenib decreased the expression level of TNF-α in subcutaneous xenograft models of hepatocellular carcinoma in mice.[28] The results obtained from these studies are in line with our findings. Our data indicate that sorafenib reduces the levels of IL-1β and TNF-α induced by cisplatin.

Limitations

There are very few studies on how sorafenib works both alone and in case of any damage or stress. Although we present findings on how this substance affects the liver and kidneys, future studies should investigate how sorafenib affects other areas.

Conclusions

In this study, it was observed that when sorafenib was administered to both healthy and cisplatin-induced rats, it caused liver toxicity. When sorafenib was administered to healty rats, the kidneys did not show any changes in the parameters studied. It was also observed that when stress was induced with cisplatin, sorafenib had a mitigating effect on kidney parameters. Serum biochemical and PCR data were supported by histopathological evaluations. Considering that the information about sorafenib is still insufficient, we think that our findings will contribute to the currently available literature.

ORCID iDs

Levent Demirtas https://orcid.org/0000-0002-3951-5648
Mehmet Gürbüzel https://orcid.org/0000-0003-1524-0587
Hilal Tahirler https://orcid.org/0000-0002-5248-0654
Emin Murat Akbas https://orcid.org/0000-0002-3033-0073
Yusuf Kemal Arslan https://orcid.org/0000-0003-1308-8569
Ozhan Karatas https://orcid.org/0000-0002-2778-8059

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Sulforaphane reduces lipopolysaccharide-induced inflammation and enhances myogenic differentiation of mouse embryonic myoblasts via the toll-like receptor 4 and NLRP3 pathways

*Manfeng Wang[1,A,B,D,F], *Meiling Liu[1,E,F], Wanzhen Xu[2,C,F], Zongyan Teng[1,A,F], Xiaowei Wu[1,C,F], Lu Gan[1,B,F], Yina Zhang[1,A,F]

[1] Department of Geriatrics, Second Affiliated Hospital of Harbin Medical University, China
[2] Department of Neurosurgery, The First Hospital of Jilin University, Changchun, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
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Address for correspondence
Yina Zhang
E-mail: zhangyina12345@hrbmu.edu.cn

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* Manfeng Wang and Meiling Liu contributed equally to this work.

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Abstract

Background. Muscle loss and muscle weakness are manifestations of infection-induced sepsis, a condition that can lead to organ failure and death. Toll-like receptor 4 (TLR4) signaling and the NLRP3 inflammasome are involved in the inflammatory storm and the development of sarcopenia during sepsis. They are also potential targets for sepsis treatment.

Objectives. To explore the effects and molecular mechanisms of sulforaphane (SFN) on sepsis-associated inflammation and sarcopenia.

Materials and methods. Mouse C2C12 embryonic myoblasts were treated with lipopolysaccharide (LPS) to simulate sepsis-induced sarcopenia. Molecular mechanisms were investigated using quantitative real-time polymerase chain reaction (qRT-PCR), western blot, immunofluorescence, and enzyme-linked immunosorbent assay (ELISA).

Results. Sulforaphane significantly reduced the secretion of the inflammatory cytokine interleukin-1β (IL-1β) by C2C12 cells after LPS treatment, and inhibited the production of intracellular reactive oxygen species (ROS). It also increased the expression of E-myosin heavy chain, myosin ID heavy chain, and myogenin, and induced myogenic differentiation of LPS-treated C2C12 cells. Mechanistically, SFN reduced messenger ribonucleic acid and protein levels of TLR4, NLRP3, apoptosis-associated speck-like protein, and Caspase-1 in C2C12 cells, thereby inhibiting the inflammatory response and promoting myogenic differentiation. In addition, the TLR4 inhibitor TAK-242 induced myogenic differentiation in LPS-pretreated C2C12 cells in a similar manner.

Conclusions. Sulforaphane can reduce sepsis-induced inflammatory responses and enhance myogenic differentiation by regulating the TLR4 and NLRP3 inflammasome pathways.

Key words: sepsis, LPS, sulforaphane, C2C12, TLR4-NLRP3 pathway

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Background

Sepsis is a life-threatening organ dysfunction caused by an unbalanced response of the body to infection following severe trauma or surgery. It can develop into multiple organ failure and even lead to death.[1,2] Muscle tissue is the main target of damage during sepsis, and this leads to the development of sepsis-related sarcopenia and myasthenia.[3,4] Due to a lack of clear diagnostic markers and early clinical indications, sarcopenia often leads to severe respiratory muscle weakness and dysfunction, prolonged mechanical ventilation time, and other severe complications such as lung infection and lower extremity thrombosis. This leads to higher care costs and increases the risk of patient death.[5,6] Therefore, an in-depth study of the pathogenesis and prevention of septic sarcopenia and myasthenia has significance in clinical practice.

The pathogenesis of septic sarcopenia and myasthenia is complicated, and the exact molecular mechanisms are not clear. It is generally believed that the mechanisms of myogenic dysfunction caused by sepsis include muscle damage by inflammatory factors, mitochondrial dysfunction, hyperactivity of the NLRP3 inflammasome, release of inducible nitric oxide, and muscle cell apoptosis.[7–10] In recent years, new research has found that the reduced muscle production capacity caused by sepsis is also involved in the occurrence of sarcopenia.[11] Skeletal muscle cells express multiple toll-like receptors (TLRs) that recognize bacterial cell wall components, such as lipopolysaccharide (LPS). In sepsis, LPS expressed by Gram-negative bacteria binds specifically to TLR4 on the surface of skeletal muscle cells to initiate intracellular signaling, thereby mediating an inflammatory response and reducing protein synthesis.[12] The TLR4 activation upregulates autophagosome formation and expression of ubiquitin ligases atrogin-1 and muscle-specific RING finger protein-1 (MuRF1), which induces muscle protein hydrolysis and mediates muscle atrophy.[13] The development of sepsis is also accompanied by the activation of the NLRP3 inflammasome.[14] The NLRP3 is highly expressed in muscle and its activity has been found to significantly increase in myopathy.[15] Huang et al. determined from muscle morphology, organ weight, gene expression, and protein content of atrogin-1 and MuRF1, that NLRP3 knockout mice with sepsis had less muscle atrophy than wild-type sepsis mice.[16] Therefore, targeting TLR4 and NLRP3 may be a viable approach to treating sepsis-related sarcopenia and myasthenia.

Sulforaphane (SFN) is the hydrolyzed active product of glucosinolate that is extracted from cruciferous vegetables, such as broccoli.[17] Sulforaphane is known to have anti-inflammatory, anticancer and antioxidative stress properties.[18–20] Previous studies have shown that SFN treatment modulated the release of LPS-induced high mobility group protein B1, and thus reduced mortality in a mouse model of sepsis.[21] Moreover, SFN inhibited muscle atrophy and promoted the differentiation of C2C12 cells by increasing myosin ID heavy chain (myoD) and messenger ribonucleic acid (mRNA) levels, as well as through activating Akt/FoxO signaling.[22] Therefore, we hypothesized that SFN may alleviate the inflammation and sarcopenia caused by sepsis.

In this study, the mouse embryonic myoblast cell line C2C12 was treated with LPS to simulate sepsis-induced sarcopenia. Secretion of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and inducible nitric oxide synthase (iNOS) was measured by quantitative real-time polymerase chain reaction (qRT-PCR). The expression of IL-1β and the activation of nuclear factor kappa-B (NF-κB) were measured by western blot assay, whilst the effects of LPS on reactive oxygen species (ROS) were measured using a ROS kit. In addition, the effects of different concentrations of LPS on myoblast differentiation of C2C12 cells were also observed through the assessment of morphology. The expression of *myoD* and *myogenin* was measured using qRT-PCR and western blot assay. Meanwhile, the expression of E-myosin heavy chain (E-MHC) and myogenin was determined by immunofluorescence assay.

Objectives

To investigate the effect of SFN on sepsis-related sarcopenia and uncover its molecular mechanisms.

Materials and methods

Cell culture and reagents

Mouse embryonic myoblast C2C12 cell line was obtained from Shanghai Cell Resource Center of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) in a 37°C, 5% CO_2 incubator. Horse serum (Beyotime Biotechnology, Shanghai, China), LPS (Solarbio, Beijing, China), SFN (HY-13755; MedChemExpress, South Brunswick, USA), and TAK-242 (T125887; Aladdin-E, Shanghai, China) were also used. For cell differentiation experiments, the conditioned medium was replaced daily with fresh DMEM medium containing 2% horse serum after the cells adhered.

Quantitative real-time polymerase chain reaction

The RNA of treated C2C12 cells was extracted using TRIpure (BioTeke, Beijing, China), and then reverse-transcribed using Super M-MLV Reverse Transcriptase Kit (BioTeke) to obtain the corresponding complimentary deoxyribonucleic acid. The expression levels of mRNA

Table 1. Primers used in qPCR

Primer	Forward sequence	Reverse sequence
IL-6	ATGGCAATTCTGATTGTATG	GACTCTGGCTTTGTCTTTCT
TNF-α	CAGGCGGTGCCTATGTCTCA	GCTCCTCCACTTGGTGGTTT
iNOS	CACCACCCTCCTCGTTC	CAATCCACAACTCGCTCC
myoD	TCTATGATGACCCGTGTTTCG	TGCACCGCAGTAGGGAAGT
Myogenin	GAATGCAACTCCCACAGCG	AGGCAACAGACATATCCTCCA
TLR4	AGCAGGTGGAATTGTATCGC	TCAGGTCCAAGTTGCCGTTT
NLRP3	GAGTTCTTCGCTGCTATGT	ACCTTCACGTCTCGGTTC
ASC	TCTGGAGTCGTATGGCTTGG	TGCTTGCCTGTGCTGGTC
Caspase-1	CAGAACAAAGAAGATGGCACA	CCAACCCTCGGAGAAAGA
β-actin	CTGTGCCCATCTACGAGGGCTAT	TTTGATGTCACGCACGATTTCC

qPCR – quantitative polymerase chain reaction; IL – interleukin; TNF-α – tumor necrosis factor alpha; iNOS – inducible nitric oxide synthase; myoD – myosin ID heavy chain; TLR4 – toll-like receptor 4; ASC – apoptosis-associated speck-like protein.

in C2C12 cells were determined by use of the Exicycler™ 96 fluorescent quantitative PCR system (Bioneer, Daejeon, South Korea). Primer sequences of genes are listed in Table 1.

Western blotting

Total protein of the treated C2C12 cells was extracted using a protein extraction kit (Solarbio) and quantified using a bicinchoninic acid kit (Solarbio). A total of 40 μg of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyl difluoride (PVDF) membrane (Beyotime Biotechnology). The PVDF membrane was blocked with 5% skimmed milk for 1 h, and incubated with the corresponding primary antibodies overnight at 4°C. Primary antibodies used included: p-NF-κB p65 (WL02169; Wanleibio, Shenyang, China), NF-κB p65 (WL01980; Wanleibio), IL-1β (12507S; Cell Signaling Technology, Danvers, USA), myoD (ab16148; Abcam, Cambridge, UK), myogenin (ab1835; Abcam), TLR4 (WL00196; Wanleibio), NLRP3 (AG-20B-0014; Adipogen, San Diego, USA), apoptosis-associated speck-like protein (ASC) (67824T; Cell Signaling Technology), Caspase-1 (22915-1-AP; Proteintech, Wuhan, China), and β-actin (WL01845; Wanleibio). The PVDF membrane and horseradish peroxidase (HRP)-coupled secondary antibodies were incubated for 1 h at room temperature. Finally, the protein signal on the PVDF membrane was detected with an electrochemiluminescence developer (Haigene Bio, Harbin, China), and the optical density value of the target band was measured using a gel image processing system (Gel-Pro-Analyzer software; Media Cybernetics Inc., Silver Spring, USA).

Cell proliferation

The C2C12 cells in the logarithmic growth phase were cultured at a density of 3×10^4 cells/L in each well. After incubating overnight at 37°C in 5% CO_2, the cells were exposed to 0 μM, 1 μM, 5 μM, 10 μM, 20 μM, or 30 μM SFN for 6 h, 24 h, 72 h, or 120 h. Dimethyl sulfoxide (DMSO) was used as a solvent control. Each treatment group was repeated 5 times. To evaluate cell proliferation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was used. Each well of the microplate had 20 μL of MTT solution added (Beyotime Biotechnology), and plates were incubated at 37°C for 4 h. Then, the medium was removed and formazan dissolving solution was added to all wells. The MTT colorimetric value was obtained by measuring the absorbance at 570 nm with a microplate reader (TECAN, Männedorf, Switzerland).

Enzyme-linked immunosorbent assay

The IL-6 protein content in cell culture supernatant was evaluated using an IL-6 enzyme-linked immunosorbent assay (ELISA) detection kit (Wanleibio). First, a 96-well plate was coated overnight at 4°C with the IL-6 antibody (10 μg/mL). Samples and standards were then diluted and 100 μL was added to the wells and incubated at 37°C for 2 h. Then, the liquid was discarded from wells and 100 μL of capture antibody was added. Plates were washed 3 times with 300 μL phosphate buffered saline with Tween 20 (PBST; pH 7.3, 0.5% Tween 20) washing solution, and were soaked for 2 min each wash. The HRP-Streptavidin (100 μL), 3,3',5,5'-tetramethylbenzidine (TMB) chromogenic solution (100 μL) and TMB stop solution D (50 μL) were added to the wells sequentially. The absorbance was read at 450 nm in the microplate reader, and the linear regression curve of the standard product was drawn to calculate the concentration.

Cell morphology and immunofluorescence

For the assessment of cell morphology, the medium was replaced with DMEM containing 2% horse serum in order to induce the differentiation of the C2C12 cells

when they reached 70%–80% confluence. At the same time, different dosages of LPS or SFN were added to the cells. Morphology was observed and photographed at 0 h, 24 h, 72 h, and 120 h after the differentiation had commenced. For immunofluorescence, treated cells were fixed to a microscope slide with 4% paraformaldehyde for 15 min and then washed with PBS. Next, the cells were permeabilized with 0.1% Triton X-100 at room temperature for 30 min. Goat serum was added dropwise to the cells to block nonspecific binding of the antibodies, and slides were incubated at room temperature for 15 min. Sufficient amount of E-MHC antibody (1:50) and myogenin antibody (1:500) were added to the slides, and incubated overnight at 4°C. Fluorescent secondary antibody was added and incubated for 1 h at room temperature. Cell nuclei were stained by incubating the cells with 4',6-diamidino-2-phenylindole (DAPI) for 5 min, and slides were then sealed with an anti-fluorescence quencher. Images were collected under a fluorescence microscope (Olympus IX53; Olympus Corp., Tokyo, Japan).

Reactive oxygen species detection

A ROS assay kit (Beyotime Biotechnology) was used to quantify ROS levels in C2C12 cells. Diluted dichlorodihydroflourescein diacetate (DCFH-DA) was added to wells and incubated at 37°C for 20 min. Cells were washed 3 times with serum-free cell culture medium to remove the free DCFH-DA. Then, the cells were trypsinized and collected as a single cell suspension. The fluorescence intensity of DCFH-DA in the cell suspension was measured at an excitation wavelength of 500 nm and an emission wavelength of 525 nm.

Statistical analyses

The statistical analysis was carried out using GraphPad Prism v. 7.0 software (GraphPad Software, San Diego, USA). For the MTT assay, data were collected from 5 biological replications. The comparisons between the groups at different time points were performed using two-way analysis of variance (ANOVA) with the Tukey's post hoc analysis. Interactions between variables were also analyzed using Type III sums of squares, after testing the distribution and homogeneity of the data. Data in Fig. 1A are presented as median (interquartile range (IQR)) with each individual datum shown. For other experiments, data were collected from 3 biological replications and comparisons between the groups were performed using ordinary one-way ANOVA with the Holm–Sidak method used for post hoc analysis, after testing the distribution and homogeneity of data. Data in other figures are presented as individual datum. The value of $p < 0.05$ was considered statistically significant.

Results

Sulforaphane reduced the inflammatory response induced by lipopolysaccharide in mouse embryonic myoblasts

Initially, the effect of SFN on the viability of mouse embryonic myoblast cell line C2C12 was evaluated. The C2C12 cells were treated with SFN at different concentrations (1 μM, 5 μM, 10 μM, 20 μM, and 30 μM), and there was little difference in cell viability between the various concentrations after 6 h of treatment. After treatment for 24 h with increased SFN concentration, C2C12 cell viability clearly decreased. Furthermore, 120-hour treatment with 10 μM SFN resulted in lower viability compared to the control group. Nonetheless, the cell survival rate was still greater than 90% (Fig. 1A). Therefore, SFN concentrations of 1 μM, 5 μM and 10 μM were selected for subsequent experimental studies. Expression levels of IL-6 gene and protein in supernatant of C2C12 cells were significantly increased 6 h after the LPS treatment. However, 6 h of SFN treatment, especially at 10 μM, inhibited the increased expression levels of the *IL-6* gene (Fig. 1B) and IL-6 protein (Fig. 1C) induced by LPS. Therefore, 10 μM SFN was used for subsequent experiments.

Lipopolysaccharide increased the gene expression levels of TNF-α and iNOS in C2C12 cells. At the same time, 10 μM SFN was observed to reduce inflammation by significantly downregulating the expression of LPS-induced TNF-α (Fig. 1D) and iNOS (Fig. 1E). The activity of phosphorylated NF-κB protein in the LPS-treated cells was significantly higher than in the control cells, and was inhibited by SFN treatment (Fig. 1F). Lipopolysaccharide significantly increased IL-1β protein levels in the supernatant of C2C12 cells. However, after SFN treatment, IL-1β protein levels were significantly reduced (Fig. 1G). In addition, the ROS fluorescence-labeling assay showed that SFN significantly reduced the production of LPS-induced ROS (Fig. 1H,I). The above results suggest that LPS induces the production of multiple inflammatory mediators in mouse embryonic myoblasts, which is inhibited by SFN treatment.

Lipopolysaccharide inhibited the myogenic differentiation of mouse embryonic myoblasts

The differentiation of C2C12 cells was achieved by the addition of medium containing 2% horse serum. Compared with the control group, LPS treatment at multiple concentrations inhibited the formation of multinucleated myotubes that are normally observed during the myogenic differentiation. This manifested as a significant decrease in the mean myotube width. Notably,

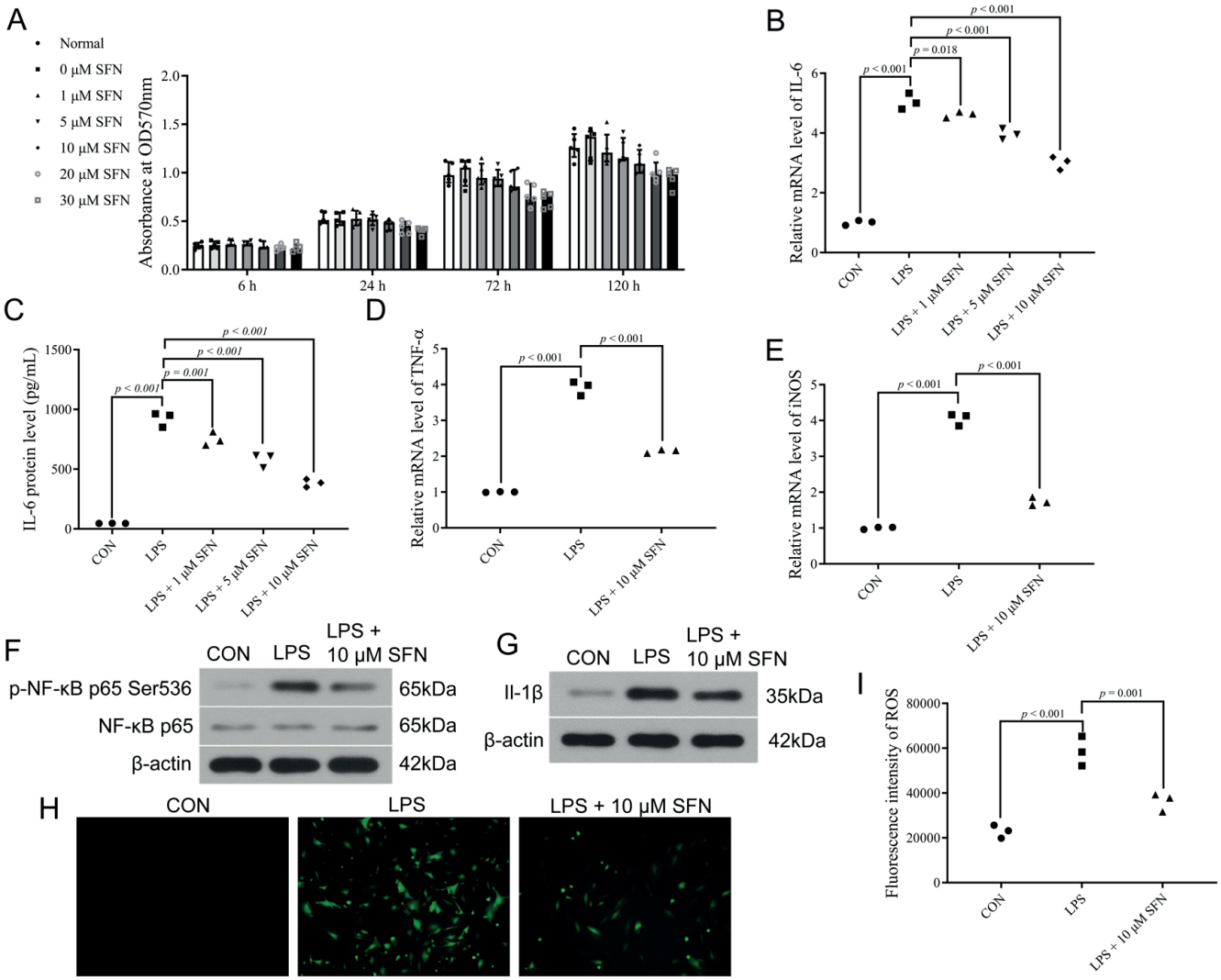

Fig. 1. Sulforaphane (SFN) reduced the lipopolysaccharide (LPS)-induced inflammatory response in C2C12 cells. A. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction (MTT) assay was performed after SFN treatment of C2C12 cells (0 μM, 1 μM, 5 μM, 10 μM, 20 μM, or 30 μM) for 6 h, 24 h, 72 h, or 120 h; B. C2C12 cells were treated with the following drugs: control (CON, equal volume of dimethylsulfoxide (DMSO)), 25 ng/mL LPS, 25 ng/mL LPS + 1 μM SFN, 25 ng/mL LPS + 5 μM SFN, and 25 ng/mL LPS + 10 μM SFN. Messenger ribonucleic acid (mRNA) levels of interleukin-6 (IL-6) were detected with quantitative real-time polymerase chain reaction (qRT-PCR); C. According to the grouping in B, expression levels of IL-6 protein in culture supernatants were detected using an enzyme-linked immunosorbent assay (ELISA) kit; D. C2C12 cells were treated with the following drugs: control (CON, equal volume of DMSO), 25 ng/mL LPS, and 25 ng/mL LPS + 10 μM SFN. The mRNA levels of tumor necrosis factor alpha (TNF-α) were detected using qRT-PCR; E. The mRNA levels of inducible nitric oxide synthase (iNOS) were detected using qRT-PCR; F. According to the grouping in D, expression levels of nuclear factor kappa-B (NF-κB) p65 and p-NF-κB p65 (Ser536) were detected with western blotting; G. Western blotting was used to detect the expression levels of IL-1β protein in the culture supernatants; H. Fluorescence images of intracellular reactive oxygen species (ROS) in each group; I. Fluorescence signal intensity of ROS

treatment with 1000 ng/mL LPS for 120 h had the most significant inhibitory effect on myogenic differentiation of C2C12 cells (Fig. 2A). Compared with the control group, LPS significantly reduced the expression of *myoD* (Fig. 2B) and *myogenin* (Fig. 2C), genes that are specific for myogenic differentiation in C2C12 cells. Compared with lower concentrations, LPS at a concentration of 1 μg/mL had the most significant impact on the downregulation of expression of these genes. Therefore, these results suggest that LPS inhibits the myogenic differentiation of mouse embryonic myoblast cell line C2C12. Subsequent experiments used 1 μg/mL LPS to inhibit myogenic differentiation.

Sulforaphane rescued inhibition of myogenic differentiation by lipopolysaccharide

It was found that SFN decreased the inhibitory effect of LPS on the formation of multinucleated myotubes. In C2C12 cells treated with LPS, 10 μM SFN treatment for 72 h or 120 h significantly restored myotube formation compared to cells treated with LPS alone (Fig. 3A). Compared with the control group, LPS significantly inhibited the expression of *myoD* and *myogenin*, both of which are genes that are specific to myogenic differentiation in C2C12 cells. However, SFN treatment increased

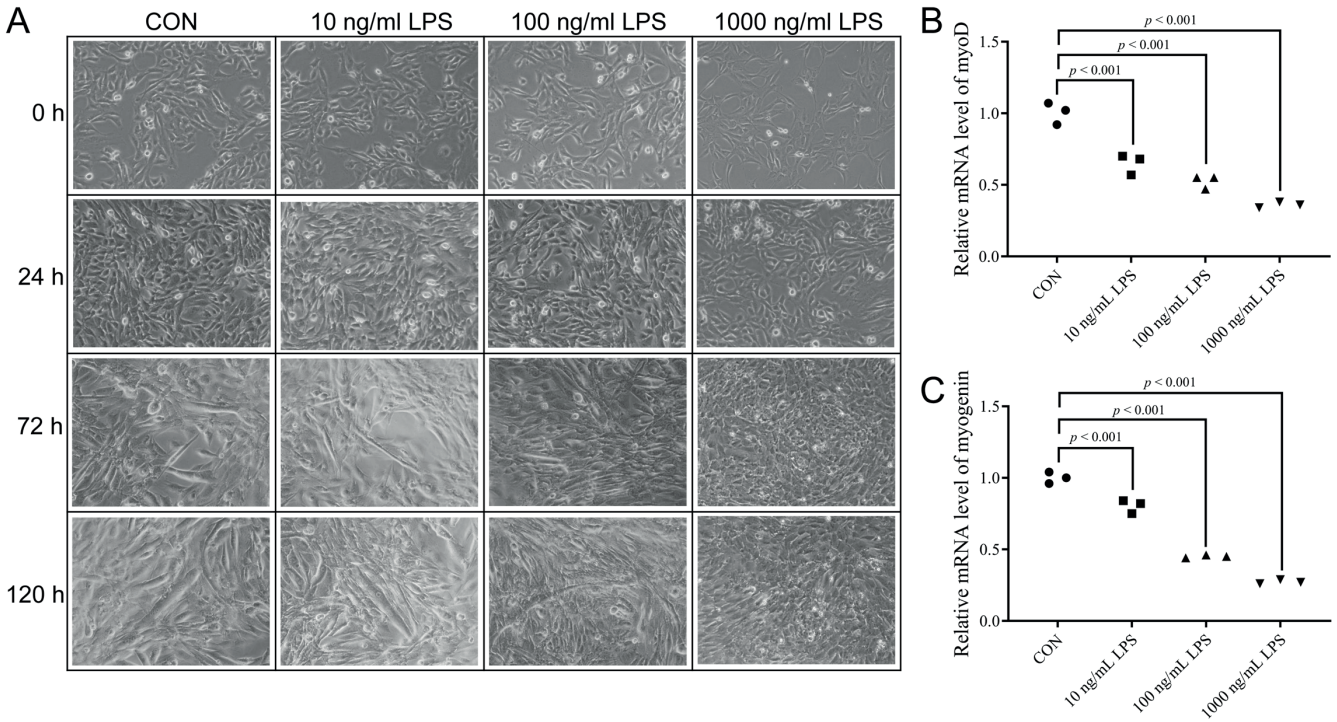

Fig. 2. Lipopolysaccharide (LPS) inhibited the myogenic differentiation of C2C12 cells. A. C2C12 cells were induced to differentiate in a conditioned medium containing 2% horse serum, and were treated with 0 ng/mL, 10 ng/mL, 100 ng/mL, or 1000 ng/mL of LPS. Images of cell morphology were taken at 0 h, 24 h, 72 h, and 120 h after the induction of differentiation; B. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the messenger ribonucleic acid (mRNA) levels of myosin ID heavy chain (myoD) after 72-hour differentiation of C2C12 cells; C. The mRNA level of myogenin was detected with qRT-PCR

CON – control.

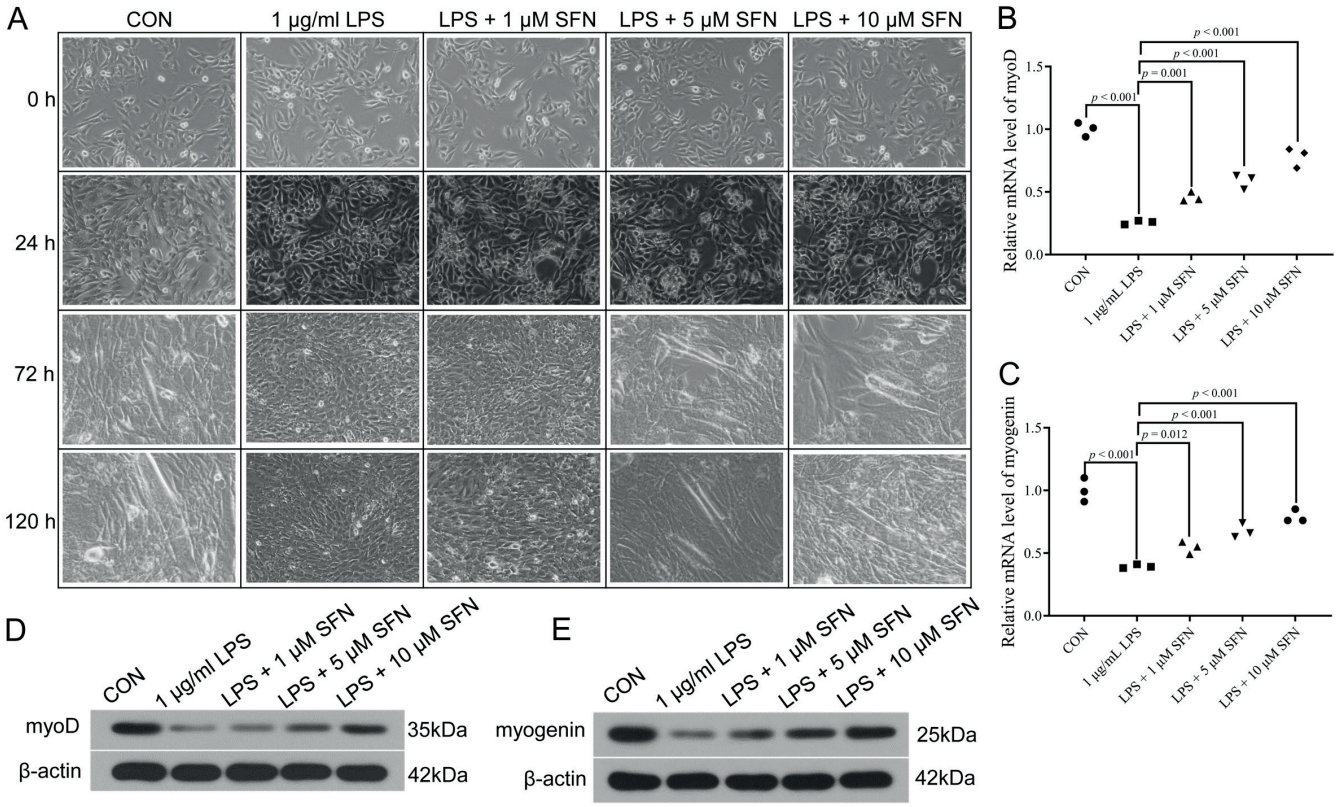

Fig. 3. Sulforaphane (SFN) rescued myogenic differentiation of C2C12 cells inhibited by lipopolysaccharide (LPS). A. C2C12 cells were treated with the following drugs during the differentiation process using 2% horse serum: control group (CON), 1 μg/mL LPS, 1 μg/mL LPS + 1 μM SFN, 1 μg/mL LPS + 5 μM SFN, and 1 μg/mL LPS + 10 μM SFN. Images of cell morphology were taken at 0 h, 24 h, 72 h, and 120 h after the induction of differentiation; B. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the messenger ribonucleic acid (mRNA) level of myosin ID heavy chain (myoD) after 72-hour differentiation of C2C12 cells; C. The mRNA level of myogenin was detected with qRT-PCR; D,E. Protein level of myoD (D) and myogenin (E) was detected with western blotting

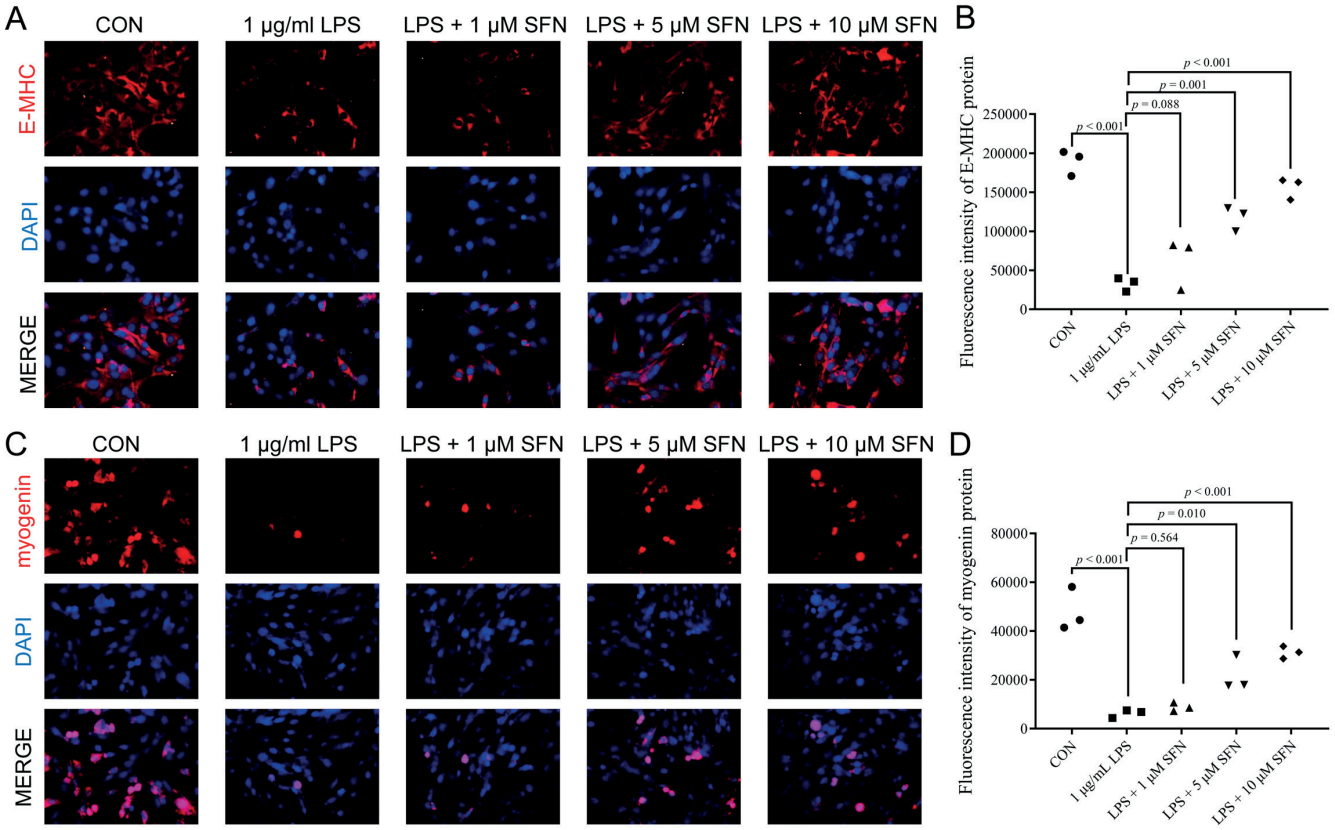

Fig. 4. Sulforaphane (SFN) increased the expression of E myosin heavy chain (E-MHC) and myogenin in differentiated C2C12 cells. A. C2C12 cells in each group (control group (CON), 1 μg/mL lipopolysaccharide (LPS), 1 μg/mL LPS + 1 μM SFN, 1 μg/mL LPS + 5 μM SFN, and 1 μg/mL LPS + 10 μM SFN) were induced to differentiate for 120 h, and immunofluorescence staining was used to detect the expression level of E-MHC. Nuclear staining was achieved with the use of 4′,6-diamidino-2-phenylindole (DAPI); B. The fluorescence intensity of E-MHC protein was quantified and analyzed; C. Immunofluorescence staining was used to detect the expression level of myogenin; D. The fluorescence intensity of myogenin protein was quantified and analyzed

the expression of *myoD* and *myogenin* compared with the LPS treatment group (Fig. 3B,C). Similarly, SFN increased the protein expression of myoD and myogenin in C2C12 cells treated with LPS (Fig. 3D,E). This indicates that SFN may ameliorate the inhibitory effects of LPS on myogenic differentiation.

The E-MHC is a landmark indicator that reflects the state of cell differentiation and the rate of cell fusion. Its expression increases with the extension of cell differentiation time. Immunofluorescence staining showed that LPS significantly reduced the expression of E-MHC during the differentiation process of C2C12 cells. However, after the addition of SFN (especially after the treatment with 10 μM SFN), the expression levels of E-MHC increased (Fig. 4A,B). Myogenin promotes the terminal differentiation of myoblasts into myotube muscle fibers. Immunofluorescence showed that LPS significantly reduced the expression of myogenin during the differentiation process of C2C12 cells. However, the treatment with SFN (especially 10 μM SFN) increased myogenin expression compared to the treatment with LPS alone (Fig. 4C,D). These results confirm that LPS inhibits myogenic differentiation, which is reversed by SFN treatment.

Sulforaphane regulated lipopolysaccharide-induced myogenic differentiation through the toll-like receptor 4 and NLRP3 signaling pathways

The C2C12 cells were treated with the TLR4 inhibitor TAK-242 to evaluate the role of TLR4 signaling in SFN therapy. Based on cellular morphology, it was shown that TAK-242 or SFN treatment of C2C12 cells significantly restored myotube formation, compared with LPS treatment (Fig. 5A). The treatment of C2C12 cells with LPS increased the gene and protein expression levels of TLR4 (Fig. 5B,C), NLRP3 (Fig. 5B,D), inflammation-related factor ASC (Fig. 5B,E), and apoptosis-related factor Caspase-1 (Fig. 5B,F). Compared with the LPS treatment group, TAK-242 and SFN reduced the gene and protein expression of TLR4, NLRP3, ASC, and Caspase-1. This indicates that LPS activates the TLR4 signaling pathway and causes the NLRP3 inflammasome cascade. These results also suggest that SFN may attenuate the activation of this inflammatory cascade that occurs through the TLR4 and NLRP3 signaling pathways.

It was also observed that LPS reduced the expression of myoD and myogenin to inhibit myogenic differentiation.

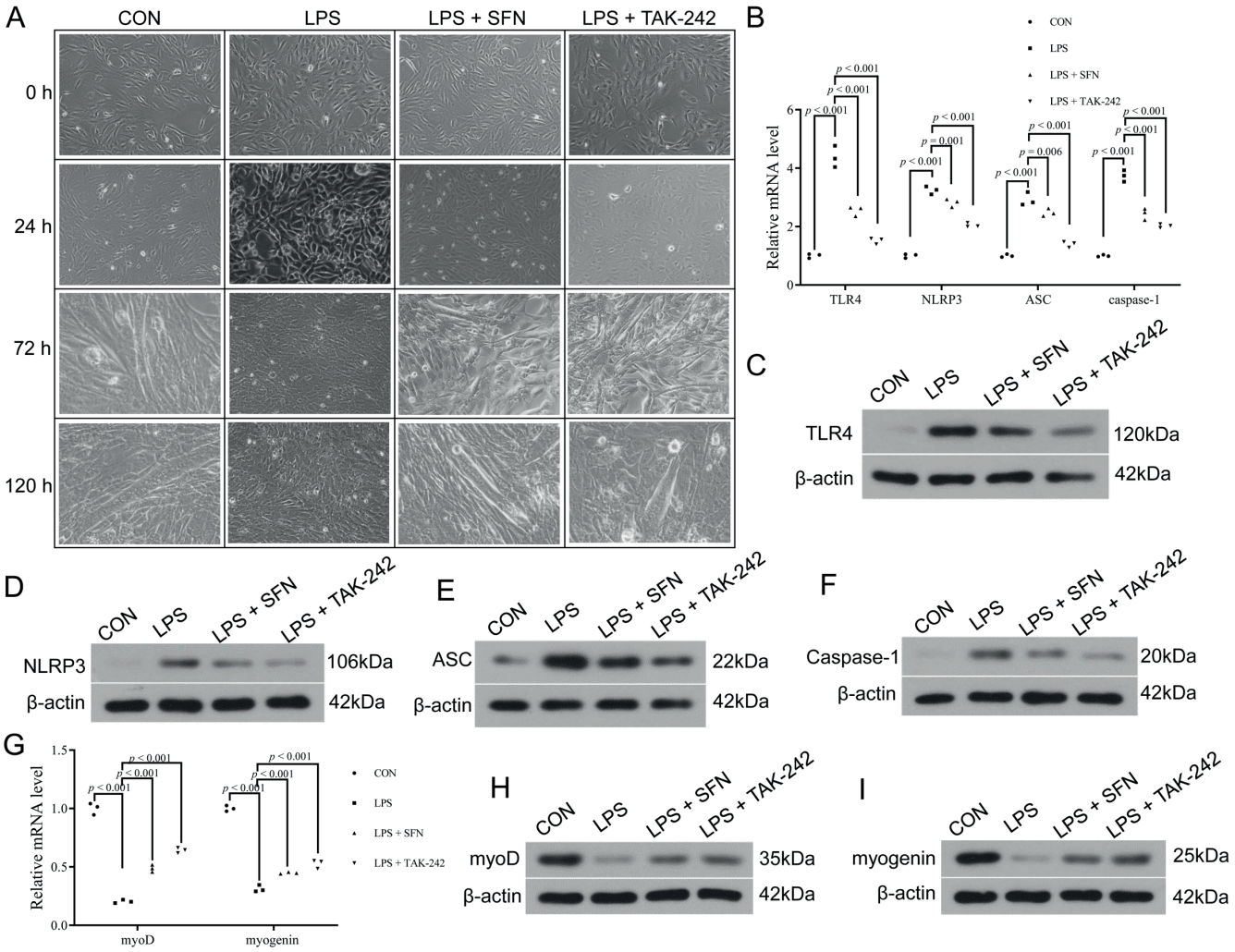

Fig. 5. Sulforaphane (SFN) regulated lipopolysaccharide (LPS)-induced differentiation through the toll-like receptor 4 (TLR4) and NLRP3 pathways in C2C12 cells. A. C2C12 cells were induced to differentiate in a conditioned medium containing 2% horse serum and treated with different concentrations of LPS (control (CON), 1 μg/mL LPS, 1 μg/mL LPS + 10 μM SFN, and 1 μg/mL LPS + 1 μM TAK-242). Images of cell morphology were taken at 0 h, 24 h, 72 h, and 120 h after the induction of differentiation; B. C2C12 cells treated with the aforementioned drugs were induced to differentiate for 72 h, and the messenger ribonucleic acid (mRNA) levels of TLR4, NLRP3, apoptosis-associated speck-like protein (ASC), and Caspase-1 were detected using quantitative real-time polymerase chain reaction (qRT-PCR); C–F. The protein levels of TLR4 (C), NLRP3 (D), ASC (E), and caspase-1 (F) were detected using western blotting; G. The mRNA levels of myosin ID heavy chain (myoD) and myogenin were detected using qRT-PCR; H,I. The protein level of myoD (H) and myogenin (I) were detected using western blotting

However, compared with the LPS treatment group, TAK-242 or SFN treatment significantly increased gene and protein expression of myoD and myogenin (Fig. 5G–I). This indicates that SFN alleviates the inhibition of myogenic differentiation induced by LPS, and is dependent upon silencing of the TLR4 signaling axis.

The immunofluorescence analysis of myogenic differentiation markers E-MHC and myogenin showed that LPS treatment significantly reduced their expression in C2C12 cells. However, compared with the LPS treatment, TAK-242 and SFN significantly increased the expression of E-MHC (Fig. 6A,B) and myogenin (Fig. 6C,D). These data indicate that the promotion of myogenic differentiation by SFN may be achieved by repressing the activation of the TLR4 pathway.

Discussion

Muscle tissue is the main target for sepsis injury, and muscular atrophy is a serious long-term complication frequently seen in patients with sepsis in the intensive care unit. Furthermore, muscular atrophy is an important cause of sepsis-related sarcopenia and myasthenia,[23,24] and occurs as a result of many factors. The increased expression of systemic inflammatory mediators is a prerequisite for the occurrence of skeletal muscle depletion,[25] while decreased musculogenic capacity and increased muscle proteolysis[26,27] are important determinants of skeletal muscle atrophy. Studies have shown that patients with sepsis exhibit high levels of pro-inflammatory cytokines that cause changes in the muscle microenvironment, abnormal levels of muscle fiber autophagy and oxidative stress damage, thereby accelerating muscle protein

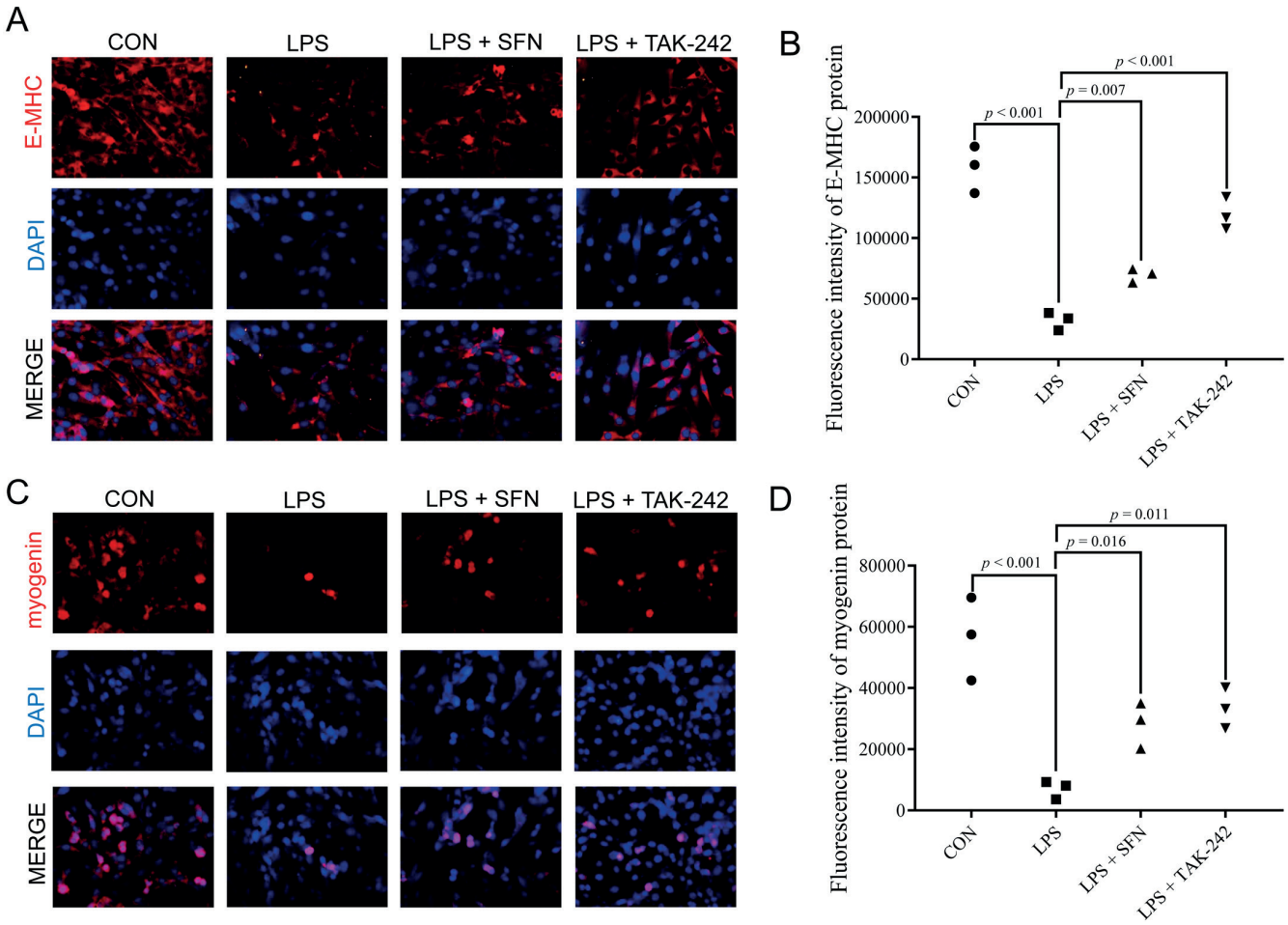

Fig. 6. Toll-like receptor 4 (TLR4) inhibitor increased the expression levels of E myosin heavy chain (E-MHC) and myogenin in differentiated C2C12 cells. A. C2C12 cells in each group (control (CON), 1 μg/mL lipopolysaccharide (LPS), 1 μg/mL LPS + 10 μM sulforaphane (SFN), and 1 μg/mL LPS + 1 μM TAK-242) were induced to differentiate for 120 h, and immunofluorescence staining was used to detect the expression levels of E-MHC. Nuclear staining was carried out using 4′,6-diamidino-2-phenylindole (DAPI); B. The fluorescence intensity of E-MHC protein was quantified and analyzed; C. Immunofluorescence staining was used to detect the expression levels of myogenin; D. The fluorescence intensity of myogenin protein was quantified and analyzed

degradation.[28–30] Under the conditions of sepsis, regeneration potential of activated myosatellite cells is impaired, and their proliferation and differentiation are inhibited, meaning that they cannot compensate for muscle loss. At present, the clinical treatment for sepsis-related sarcopenia and myasthenia is mainly aimed at appropriate nutrition and physical rehabilitation therapy. However, their efficacy is limited, and both have shown limitations in the repair of muscle loss before atrophy or depletion occurs. Therefore, to control the adverse effects of sepsis, the discovery of effective drugs and combinational therapies against multiple targets is urgently needed. Through a series of cytological experiments, we confirmed the effect of SFN in alleviating LPS-induced myoblast damage, which suggests that SFN may be a potential therapy for sepsis-related sarcopenia.

Sulforaphane is an isothiocyanate found in cruciferous vegetables such as broccoli and cabbage.[31] Sulforaphane has an anti-inflammatory activity and is therefore used in the treatment of various diseases.[32] Indeed, SFN has been shown to inhibit a variety of tumors by intervening in oxidative stress, inflammation, cell cycle, proliferation, apoptosis, and metastasis.[33,34] It has also been demonstrated to have a neuroprotective effect, and is used for preventing and treating various acute and chronic neurodegenerative diseases.[20] Furthermore, SFN can inhibit the expression of myostatin in porcine skeletal muscle satellite cells and stem cells in a dose-dependent manner.[35] Additionally, SFN increased the number and function of skeletal muscle stem cells, preventing age-related cardiac and muscle dysfunction.[36] Other studies have reported that SFN can prevent dexamethasone-induced muscle atrophy by regulating the Akt/FoxO1 axis in C2C12 myotubes.[22] However, the therapeutic efficacy of SFN has not been previously studied in the process of sepsis-related sarcopenia. Our study has demonstrated that SFN promotes LPS-induced myogenic differentiation of mouse myoblasts and further clarified that myogenic differentiation was achieved through the TLR4–NLRP3 signaling pathway. In the search for a therapeutic drug that could reverse the decline in skeletal muscle strength and quality that occurs in sepsis, SFN has demonstrated the potential

to reduce disabling muscular dystrophy and the incidence of sepsis mortality.

Toll-like receptor 4 was identified as the first human homologue of the *Drosophila Toll* gene and is well known as a receptor for LPS. In addition to being highly expressed in immune cells, TLR4 is also abundant in adipose tissue, liver and skeletal muscles.[37] The TLR4-mediated signal transduction activates NF-κB and promotes the production of pro-inflammatory cytokines, such as TNF-α and IL-6. These factors are well-known modulators of protein renewal in muscle and contribute to the development of muscle atrophy under various conditions. The NLRP3 inflammasome is a collection of cytosolic receptor proteins, including NLRP3, ASC and Caspase-1. Furthermore, the NLRP3 inflammasome is an important part of innate immunity, and is activated by pathogens, secreted toxins, crystals, and endogenous danger signals.[38] Studies have found that the regulation of the NLRP3 inflammasome alleviated the disease process and even blocked disease pathologies. For example, in NLRP3 knockout mice, the suppression of the inflammatory response contributed to the development of sarcopenia in aging mice and reduced muscle glycolysis.[39] Therefore, inhibiting the activation of the NLRP3 inflammasome could be an important strategy for the prevention and treatment of sarcopenia.[40] In sepsis patients, circulating LPS binds to TLR4 to activate the TLR4 signaling pathway. This leads to the release of a large amount of inflammatory mediators and the activation of the NLRP3 inflammasome.[8] Our data show that SFN inhibited LPS-induced cytokine release and expression of TLR4 and NLRP3 in C2C12 cells. We revealed that SFN may play a key role in improving septic sarcopenia and myasthenia by regulating the TLR4 signaling pathway and NLRP3 inflammasome activation.

Additionally, the concentration of circulating LPS usually increases in sepsis. The activation of the TLR4 signaling pathway by LPS led to the release of a large number of inflammatory factors and inhibited the proliferation of C2C12 cells. Lipopolysaccharide also downregulated the expression of *myoD* and *myogenin* in a dose-dependent manner, and inhibited the myogenic differentiation of C2C12 cells. Lipopolysaccharide binds to the TLR4 receptor on myoblast cells to activate the TLR4 signaling pathway and cause local inflammation. At the same time, LPS also causes the release of inflammatory mediators and activates the NLRP3 inflammasome, which is closely associated with the occurrence of sarcopenia. As an isothiocyanate, SFN exists in natural cruciferous vegetables and has potential anti-inflammatory effects. Indeed, SFN suppressed the release of inflammatory factors caused by LPS in C2C12 myoblasts, and restored cell proliferation and myogenic differentiation that was inhibited by LPS. Investigation of the underlying mechanisms revealed that SFN attenuated the expression of TLR4, NLRP3 and their related regulatory proteins in C2C12 myoblasts, which demonstrates the activation of the TLR4 and NLRP3 signaling pathways. This was similar to the effects brought about by the administration of the TLR4 inhibitor TAK-242. Therefore, dietary supplementation with vegetables containing SFN, such as broccoli and cabbage, may reduce sarcopenia and myasthenia in patients with sepsis through inhibiting the TLR4 and NLRP3 inflammasome pathways.

Limitations

Due to the instability of the animal model of sepsis, the study was not conducted in vivo. Further experiments are needed in future research.

Conclusions

Sulforaphane can improve the expression of myogenic factors myoD and myogenin by regulating TLR4 and NLRP3 signaling pathways, so as to ameliorate the inhibition of myogenic differentiation induced by LPS.

Supplementary data

The supplementary statistical data are available as Supplementary Tables (https://doi.org/10.5281/zenodo.7234725). The package consists of the following files:

Supplementary Table 1. Post hoc comparisons for MTT assay.

Supplementary Table 2. All statistical data for all figures.

ORCID iDs

Manfeng Wang https://orcid.org/0000-0002-7556-8739
Meiling Liu https://orcid.org/0000-0002-3418-7104
Wanzhen Xu https://orcid.org/0000-0001-6602-963X
Zongyan Teng https://orcid.org/0000-0002-4247-9609
Xiaowei Wu https://orcid.org/0000-0002-5725-9123
Lu Gan https://orcid.org/0000-0002-0731-004X
Yina Zhang https://orcid.org/0000-0001-5981-2626

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A miR-340/SPP1 axis inhibits the activation and proliferation of hepatic stellate cells by inhibiting the TGF-β1/Smads pathway

Ronghua Zhang[1,A,D], Meimei Wang[1,B,C], Hongjian Lu[1,B], Jingyao Wang[2,B], Xiangyang Han[1,B], Zhiyong Liu[3,C], Lin Li[1,C], Mingming Li[1,B,D], Xiaoli Tian[4,B], Shuang Chen[5,C], Guangling Zhang[2,A,E,F], Yanan Xiong[1,A,E], Jingwu Li[6,D,F]

[1] Hebei Key Laboratory for Chronic Diseases, School of Basic Medical Sciences, North China University of Science and Technology, Tangshan, China
[2] School of Clinical Medicine, North China University of Science and Technology, Tangshan, China
[3] Health Science Center, North China University of Science and Technology, Tangshan, China
[4] Gastroenterology Department, Tangshan Paraplegia Sanatorium, China
[5] Tianjin Key Laboratory of Early Druggability Evaluation of Innovative Drugs, Tianjin International Joint Academy of Biomedicine, China
[6] The Cancer Institute, Hebei Key Laboratory of Molecular Oncology, Tangshan People's Hospital, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Guangling Zhang
E-mail: zhanggl@ncst.edu.cn

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Abstract

Background. Hepatic fibrosis (HF) is a common pathological complication of liver cirrhosis which affects human health. It is well established that microRNAs (miRNAs) regulate the proliferation, activation and apoptosis of hepatic stellate cells (HSCs).

Objectives. To determine the function and molecular mechanism of miR-340-5p/secreted phosphoprotein 1 (SPP1) axis in HF and identify potential therapeutic targets.

Materials and methods. The HF model in cholestatic rats was induced by ligating the common bile duct. The histological sections of the liver tissues were stained with hematoxylin and eosin (H&E), Masson's trichrome or Sirius Red. The differential expression of mRNAs in the liver tissues was examined using the microarray analysis. The expression levels of miR-340-5p, SPP1, alpha-smooth muscle actin (α-SMA), Collagen I, phosphorylated Smad2 (p-Smad2), and p-Smad3 were determined using quantitative real-time polymerase chain reaction (qRT-PCR) or western blot. Cell proliferation was quantified using cell counting kit-8 (CCK-8) assays. The regulatory effect of miR-340-5p on SPP1 was determined with fluorescent reporter assay.

Results. The bile duct ligation (BDL) rat model was successfully induced, and SPP1 was upregulated in liver tissue from the BDL group compared to that of the sham group. The expression level of miR-340-5p was decreased in activated human primary normal fibroblasts (NFs) and activated LX-2 cells, and the mRNA and protein expression levels of SPP1 were increased in activated LX-2 cells. The SPP1 was the target of miR-340-5p, and the overexpression of SPP1 increased the proliferation of LX-2 cells, the expression of HF markers α-SMA and Collagen I, and key factors p-Smad2 and p-Smad3 (all $p < 0.05$). However, reverse results were obtained with the overexpression of miR-340-5p in LX-2 cells.

Conclusions. Our findings provide evidence that SPP1 targeted by miR-340-5p promotes LX-2 cell proliferation and activation through the TGF-β1/Smads signaling pathway. Therefore, miR-340-5p and SPP1 may be possible therapeutic targets for HF.

Key words: hepatic fibrosis, hepatic stellate cells, miR-340-5p, TGF-β1 signaling, secreted phosphoprotein 1

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Background

Hepatic fibrosis (HF) is a pathological complication of many liver diseases. It is generally associated with chronic hepatic inflammation and injury. In HF, hepatic stellate cells (HSCs) are activated to produce extracellular matrix (ECM), which becomes fibrous and accumulates.[1] Hepatic stellate cells are activated by several signalling pathways, including canonical tissue growth factor β1 (TGF-β1),[2] which is established in HF.[3] However, the complexity of HSC activation has limited the understanding of the regulatory mechanisms, which hinders the therapeutic options for HF.[4] Thus, clarifying this regulatory mechanism is essential for the development of effective antifibrotic therapy.

MicroRNAs (miRNAs) regulate the physiological and pathological process of fibrotic diseases by directly interfering with the expression of their functional target genes, especially genes affecting organs, such as liver, kidney, lung, or heart.[5] Hepatic stellate cells are activated and transformed by many miRNAs, highlighting miRNAs as potentially suitable targets for the treatment of HF. One example is miR-942, which mediates the activation of HSCs by downregulating bone morphogenic protein (BMP) and activin membrane-bound inhibitor (BAMBI) in human HF.[6] Also, miR-455-3p alleviates the activation of HSCs and HF by inhibiting the expression of heat shock transcription factor 1 (HSF1).[7] The miR-340-5p alleviates lung fibrosis by targeting the TGF-β/P38/ATF1 signaling pathway,[8] and the transplantation of bone marrow mesenchymal stem cell-derived exosomes containing miR-340-5p reduces endometrial fibrosis.[9] However, the effects of miR-340-5p in the process of HSC activation in human HF remain unknown.

In this study, a bile duct ligation (BDL) HF rat model was successfully induced and a microarray analysis was performed to identify genes involved in the pathophysiology of HF. One such gene highly upregulated in the liver of BDL rats was the secreted phosphoprotein 1 (*SPP1*) gene. Recent studies have revealed that *SPP1* is upregulated in various human fibrotic diseases,[10–12] and that several miRNAs, such as miR-539-5p,[13] miR-186[14] and miR-181c,[15] regulate *SPP1* expression. Therefore, we tested the possible involvement of the overexpression of *SPP1* in HF and its regulation by miR-340-5p.

Objectives

To determine the interactions between miR-340-5p and *SPP1* in HF and identify potential therapeutic targets.

Materials and methods

BDL rat model

The use of all animals was approved by the Laboratory Animal Ethics Committee of North China University of Science and Technology, Tangshan, China (approval No. LAEC-NCST-2020187). A total of 14 male Sprague Dawley rats (age: 8 weeks; weight: 210–260 g) (Beijing HFK Bioscience Co., Ltd., Beijing, China) were divided into the BDL group (n = 7) and sham group (n = 7), and maintained at 23 ±2°C with free access to water and food. Animals were anesthetized, and those in the BDL group underwent a common bile duct separation and ligation to establish the model, while the sham group rats underwent a laparotomy to separate the duct without ligation. Incisions were treated with penicillin, and rats were kept flat until the anesthesia subsided. On the 14th day after the operation, the rats were sacrificed by exsanguination through the abdominal aorta after anesthesia, the blood and liver tissues were collected by flash freezing in liquid nitrogen, and liver tissues were preserved in RNAlater™ Stable preservation solution of animal tissue RNA (Beyotime, Shanghai, China) at –80°C until used.

Serum enzymes

The Comprehensive Diagnostic Profile kit on a VetScan VS2 (Abaxis Inc. North America, Union City, USA) was used to determine the levels of liver injury markers, including serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bile acid (TBA), and total bilirubin (TBIL).[16]

Liver histology

Livers were paraffin-embedded, cut into 5-µm-thick sections, deparaffinized and rehydrated, followed by staining with hematoxylin and eosin (H&E; Beijing Yili Fine Chemicals Co., Ltd., Beijing, China), Masson's trichrome (PhyEasy™ Masson Staining Kit, PH1427; Phygene, Fujian, China) or Sirius Red (Picro Sirius Red Stain Kit, ab150681; Abcam, Cambridge, UK), or subjected to immunohistochemistry (Gibco, Thermo Fisher Scientific, Waltham, USA), according to the manufacturer's instruction. For immunohistochemistry, the sections were covered with prepared ethylenediaminetetraacetic acid (EDTA) solution (Wanleibio, Shenyang, China), and repaired under high pressure for 3 min. This was followed by applying peroxidase blocking agent (ReportBio, Hebei, China) for 10 min, and washing with phosphate-buffered saline (PBS). Subsequently, they were incubated with alpha-smooth muscle actin (α-SMA) primary antibodies (ab5694, 1:200; Abcam), then with a biotin-labeled secondary antibody (ab6721, 1:500; Abcam), and finally stained with diaminobenzidine tetrahydrochloride.

mRNA microarray and the determination of differentially expressed genes

Differential mRNA expression in the liver tissue of BDL and sham groups (n = 3) was analyzed using a Rat Gene Expression Microarray (Agilent Technologies, Santa Clara,

USA) with an 8 × 60K chip. The TIFF format image data file of the Agilent mRNA expression chip following hybridization scanning was preprocessed and analyzed using feature extraction software (method No. AG-GE-WL02-01-2012, data analysis method No. AG-GEDL00-01-2010; CapitalBio Technology, Beijing, China).

Cell culture

The use of human primary normal fibroblasts (NFs) was authorized by the Ethics Committee of Tangshan People's Hospital, China (approval No. RMYY-LLKS-2020-002).[17] The cells were cultured in Dulbecco's modified Eagle's F12 medium (DMEM/F12; Thermo Fisher Scientific) containing 10% epidermal growth factor, 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) (Minhai Biotechnology Co., Ltd., Beijing, China). The immortalized human hepatic stellate LX-2 cell line was obtained from Peking Union Medical College (Beijing, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Tianjin Meiji Chemical Co., Ltd., Tianjin, China) containing 1% P/S and 10% FBS. All cells were cultured and kept in an incubator with 5% CO_2 at 37°C. To further activate cells, they were treated with 10 ng/mL TGF-β1 (PeproTech, Inc., Cranbury, USA) for 24 h, as previously described.[18]

miRNA target prediction

The miRNA potential target genes were predicted using TargetScan (targetscan.org/vert_72/) and miRDB (https://mirdb.org/mirdb/index.html).[19]

Quantitative real-time polymerase chain reaction

TRIzol reagent (Invitrogen, Thermo Fisher Scientific) was used to extract the total RNA from tissues and cells following the manufacturer's protocol. NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific) was used to measure the total RNA concentration. Next, cDNAs were synthesized using an mRNA reverse transcription kit (Mei5 Biotechnology Co., Ltd., Beijing, China) or miRNA First Strand cDNA Synthesis kit (Sangon Biotech, Shanghai, China), with the latter kit using miRNA universal reverse primers. The Prime Script RT-PCR kit (TaKaRa, Kusatsu, Japan) and SYBR Select Master Mix (Thermo Fisher Scientific) were used for quantitative real-time polymerase chain reaction (qRT-PCR), and the reactions were performed on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Thermo Fisher Scientific). The qRT-PCR reaction conditions were set at 95°C for 3 min, and then 40 cycles at 95°C for 15 s, 60°C for 35 s and 72°C for 30 s. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression of the genes, with experiments being performed in triplicate. The mRNA expressions of Collagen I, α-SMA and SPP1 were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and miR-340-5p was normalized to U6, expression of GAPDH and U6 were set to 1. The primer sequences were as follows:

GAPDH forward
(F): 5'-CCGCATCTTCTTGTGCAGTG-3',
and GAPDH reverse
(R): 5'-TCCCGTTGATGACCAGCTTC-3';
U6 F: 5'-CTCGCTTCGGCAGCACATA-3';
miR-340-5p F: 5'-GCGGTTATAAAGCAATGAGA-3';
SPP1 F: 5'-GAGGTCTGCGTGAATCCCTA-3',
SPP1 R: 5'-GGAATGGCTGTAGTCGTCCA-3';
α-SMA F: 5'-ACTGCCTTGGTGTGTGACAA-3',
α-SMA R: 5'-CACCATCACCCCCTGATGTC-3';
Collagen I F: 5'-GGGCGAGTGCTGTGCTTT-3',
Collagen I R: 5'-GACCCATTGGACCTGAACC-3'.

Western blot analysis

Cell proteins were extracted with NP-40 Lysis Buffer (Beyotime), and bicinchoninic acid (BCA) assay (Beyotime) was used to determine protein concentration. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with 5% stacking gel and 10% separation gel was used to isolate proteins that were transferred to polyvinylidene fluoride (PVDF) membranes. Blots were blocked with 5% skimmed milk for 2 h and incubated with the corresponding primary antibody overnight at 4°C. On the following day, blots were treated with a secondary antibody at room temperature for 2 h and then developed using enhanced chemiluminescence (ECL) reagent (Applygen, Beijing, China). The protein expressions of Collagen I, α-SMA and SPP1 were normalized to GAPDH, while phosphorylated (p)-Smad2/3 were normalized to Smad2/3. Protein expression in the control group was set to 1. The antibodies used to probe the PVDF membranes were as follows: GAPDH (ab9485, 1:5000; Abcam), SPP1 (ab255435, 1:3000; Abcam), Collagen I (ab64883, 1:1500; Abcam), α-SMA (ab244177, 1:1000; Abcam), p-Smad2 (ab188334, 1:3000; Abcam), p-Smad3 (ab48054, 1:3000; Abcam), Smad2 (SRP 12209, 1:2000; Tianjin Saier Biotechnology Co., Ltd., Tianjin, China), and Smad3 (SRP 06283, 1:2000; Tianjin Saier Biotechnology Co., Ltd.).

Cell transfection

The following vectors were used in the present study: pcDNA3/miR-340-5p was used to overexpress miR-340-5p; pcDNA3/SPP1 was used to overexpress SPP1; shR-SPP1 was used to interfere with SPP1 expression; pcDNA3 was used as the negative control (NC) for pcDNA3/miR-340-5p and pcDNA3/SPP1; and pSilencer was used as NC for shR-SPP1. All the vectors were obtained from Tianjin Saier Biotechnology. The LX-2 cells, growing in a 96-well cell culture plates, were transfected with 0.25 μg DNA/well. The miR-340-5p inhibitor and control random sequence inhibitor (NC-inhibitor) (Zhongshi Gene Technology, Tianjin, China) were transfected into LX-2 cells at a final

concentration of 100 nM. All transfections were performed according to the Lipofectamine® 2000 protocol (Thermo Fisher Scientific).

Fluorescent reporter assay

Vector pcDNA3/EGFP-SPP1 3'UTR containing wild-type 3'-untranslated region (UTR) of SPP1 mRNA complementary to miR-340-5p sequence, and pcDNA3/EGFP-SPP1 3'UTR-MUT vector containing mutated 3'UTR of SPP1 mRNA complementary to miR-340-5p sequence, as well as pDsRed2-N1 vector (used as internal control of transfection), were purchased from Clontech Laboratories (Mountain View, USA). The LX-2 cells, growing in a 6-well cell culture plates, were transfected with: 1 µg pcDNA3 or pcDNA3/miR-340-5p, 1 µg pcDNA3/EGFP-SPP1 3'UTR or pcDNA3/EGFP-SPP1 3'UTR-MUT, and 0.1 µg pDsRed2-N1. After 48 h, the cells were treated with lysis buffer (Beyotime), and the fluorescence intensities of enhanced green fluorescent protein (EGFP) and red fluorescent protein (RFP) were determined using an F-4500 fluorescence spectrophotometer (Molecular Devices, San Jose, USA). The fluorescence intensity ratio of EGFP to RFP was calculated to determine the relative fluorescence intensity of the former. The pcDNA3 and pcDNA3/EGFP-SPP1 3'UTR transfection groups were the control for pcDNA3/miR-340-5 and pcDNA3/EGFP-SPP1 3'UTR, while the pcDNA3 and pcDNA3/EGFP-SPP1 3'UTR-MUT transfection groups were the control for pcDNA3/miR-340-5p and pcDNA3/EGFP-SPP1 3'UTR-MUT. The relative fluorescence intensity of EGFP was set to 1 in the control group.

Cell proliferation assay

The cell counting kit-8 (CCK-8) assay (Invitrogen, Thermo Fisher Scientific) was used to determine the proliferation of LX-2 cells. The cells were placed in 96-well plates with DMEM and a total of 3×10^3 cells per well. The pcDNA3/miR-340-5p, pcDNA3, miR-340 inhibitor, or NC-inhibitor were transfected individually with Opti-MEM (Tianjin Meiji Chemical Co., Ltd.), and Lipofectamine® 2000. A total of 100 µL of medium and 10 µL of CCK-8 reagent were added 24 h, 48 h or 72 h after transfection, and the absorbance at 450 nm was determined in each well after a 3-hour incubation.

Statistical analyses

The SPSS v. 26.0 software (IBM Corp., Armonk, USA) was used to analyze the experimental data and GraphPad Prism software (v. 8.0; GraphPad Software, San Diego, USA) was used to present the results. The unpaired Student's t-test bootstrap was used for comparisons between the 2 groups and the one-way analysis of variance (ANOVA) bootstrap followed by the least significant difference (LSD) test or Dunnett T3 post hoc test was used for multiple comparisons among the 4 groups. The Welch's correction was used when the homogeneity of variance assumption was not met, and the data description of statistical test results are shown in Supplementary Tables 1–6 (https://doi.org/10.5281/zenodo.7115492). Seven rats were used for the induced BDL rat model. The other experiments were performed in triplicate. Representative results are shown as mean ± standard deviation (M ±SD), with $p < 0.05$ considered statistically significant.

Results

BDL-induced HF rat model and differential gene expression

Using the BDL rat model, the characteristics of HF were determined. The results showed that the daily growth rate of the BDL group was significantly lower than controls, and the liver wet weight, body mass ratio, ALT, AST, TBA, and TBIL were significantly increased (Table 1). Significant histological changes and deposition of Collagen I were detected in liver tissue sections of BDL rats using H&E, Sirius Red and Masson's trichrome staining when compared to controls (Fig. 1A). In addition, the upregulation of α-SMA in the central venous and portal regions of BDL rat liver tissue sections was observed with immunohistochemical analysis (Fig. 1B).

To systematically identify genes involved in the pathophysiology of HF, the differential gene expression was analyzed in liver tissues of BDL and control rats using mRNA microarray. Two major clusters were identified using unsupervised hierarchical clustering analysis among the differentially expressed mRNAs, with one set closely

Table 1. Liver to body mass ratio and serum biochemical test results of rats in each group

Groups	Growth rate (daily %)	Liver/body weight [g/kg]	Serum ALT [U/L]	Serum AST [U/L]	Serum TBA [µmol/L]	Serum TBIL [U/L]
Sham group (n = 7)	0.39 ±0.05	25.53 ±1.37	45.13 ±1.7	134.03 ±9.53	5.39 ±3.54	10.65 ±1.22
BDL group (n = 7)	0.30 ±0.07	61.99 ±2.49	101.76 ±3.87	438.49 ±14.33	215.17 ±11.39	150.16 ±9.99
Lower limit of the 95% CI	0.0205	–38.3728	–59.5055	–317.2698	–218.8642	–146.4505
Upper limit of the 95% CI	0.1496	–34.2832	–53.2831	–292.6431	–201.0801	–131.8945

The t-test bootstrap was used to compare the difference between the sham group and the bile duct ligation (BDL) group. Each value represents the mean ± standard deviation (M ±SD) of 7 rats. ALT – alanine aminotransferase; AST – aspartate aminotransferase; TBA – total bile acid; TBIL – total bilirubin; 95% CI – 95% confidence interval.

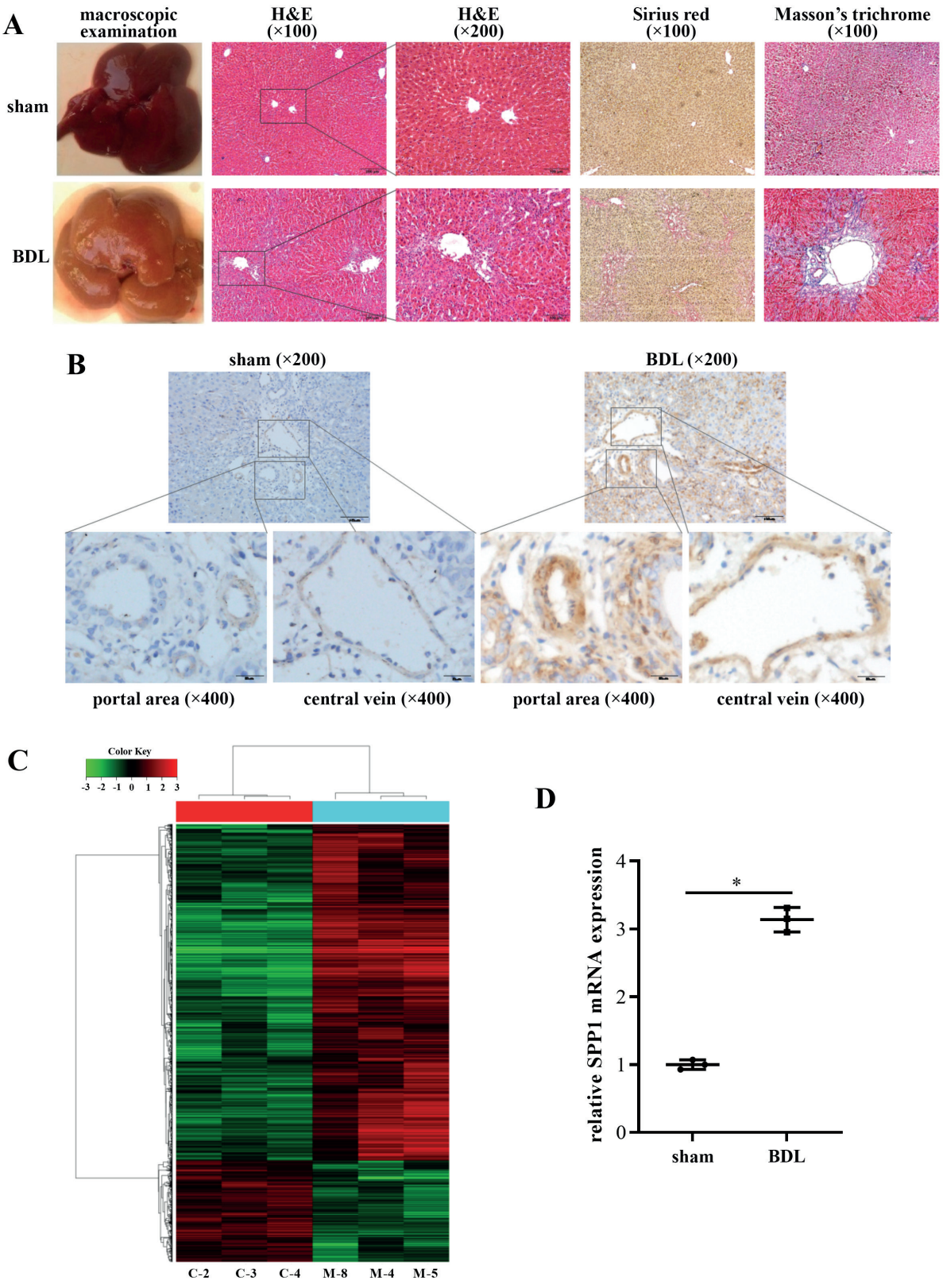

Fig. 1. Differential expression of genes in bile duct ligation (BDL) rat liver. A. Macroscopic examination of rat liver and representative images of hematoxylin and eosin (H&E) staining (×100 and ×200 magnification), Sirius Red staining (×100 magnification), Masson's trichrome staining (×100 magnification) of rat liver tissue sections, n = 7; B. Immunohistochemical staining for alpha smooth muscle actin (α-SMA) in the central vein and portal region of rat liver tissue sections (×200 and ×400 magnification), n = 7; C. Microarray analysis of liver tissue mRNAs in sham or BDL group. Hierarchical cluster analysis was performed for differential expression mRNAs; green – low expression; black – no difference; red – high expression; C-2, C-3 and C-4 are the sham group results; M-4, M-5 and M-8 are the BDL group results, n = 3; D. The expression of secreted phosphoprotein 1 (SPP1) in liver tissues was analyzed with quantitative real-time polymerase chain reaction (qRT-PCR) using samples from the sham and BDL groups. Data for SPP1 were normalized to mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the data for the sham group were standardized to 1. Results are shown as mean ± standard deviation (M ±SD), n = 3, *$p < 0.05$ compared to the control; the results were analyzed using the Student's t-test bootstrap

associated with the BDL group and the other with the sham group. Compared to the sham group, there were 1985 upregulated and 598 downregulated mRNAs in the BDL group (Fig. 1C), with SPP1 being one of the most upregulated mRNAs ($p < 0.05$, $n = 3$) (Fig. 1D). The SPP1 is related to the TGF-β1/Smads signaling pathway, as indicated by Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) analyses, which suggests that SPP1 is likely critical in HF.

SPP1 enhances the proliferation and activation of LX-2 cells through promotion of the TGF-β1/Smads signaling pathway

To determine the function and possible mechanism of SPP1 in HF, LX-2 cells and TGF-β1-activated LX-2 cells were used. It is reported that LX-2 cells can be further activated by TGF-β1.[18] The TGF-β1 interacts with receptor II (TGFBR2) on the cell surface, which leads to the phosphorylation of receptor I (TGFBR1). Smad2 and Smad3 are phosphorylated to become p-Smad2 and p-Smad3 by p-TGFBR1, where they form a cytoplasmic heteromeric complex that traffics to the nucleus to mediate HF.[20] This leads to the expression of SPP1 that were detected in activated LX-2 cells. The levels of SPP1 mRNA and protein were significantly enhanced in LX-2 cells treated with TGF-β1, as examined using qRT-PCR and western blot, respectively (all $p < 0.05$, Fig. 2A,B). Additional studies were performed in LX-2 cells by transfecting them with pcDNA3/SPP1, shR-SPP1 or NCs. The levels of SPP1 mRNA and protein in LX-2 cells transfected with pcDNA3/SPP1 or shR-SPP1 were increased or decreased relative to NCs, as detected using qRT-PCR or western blot, respectively (all $p < 0.05$, Fig. 2C,F). The proliferation of LX-2 cells transfected with pcDNA3/SPP1 or shR-SPP1 was significantly increased and decreased relative to controls, respectively, as determined with CCK-8 analysis (all $p < 0.05$, Fig. 2D). Additionally, the mRNA and protein levels of HF markers Collagen I and α-SMA in LX-2 cells transfected with pcDNA3/SPP1 or shR-SPP1 were significantly higher and lower than in their NCs, respectively (all $p < 0.05$, Fig. 2E,F). The protein expression of p-Smad2 and p-Smad3 in LX-2 cells transfected with pcDNA3/SPP1 were increased, while those transfected with shR-SPP1 were decreased when normalized to the total Smad2 or Smad3 protein (all $p < 0.05$, Fig. 2G). Thus, SPP1 might promote HSC proliferation and activation through the TGF-β1/Smads signaling pathway.

SPP1 is directly targeted by miR-340-5p

A growing number of studies have indicated that miRNAs directly interfere with the expression of their target genes, which likely happens in the occurrence and development of HF. Thus, the question of whether SPP1 is regulated by miRNAs was investigated. The results obtained using TargetScan and miRDB database analysis suggested that SPP1 can be directly targeted and regulated by miR-340-5p (Fig. 3A). The cells co-transfected with miR-340-5p and pcDNA3/EGFP-SPP1 3'UTR vector had a decreased fluorescence intensity ($p < 0.05$, Fig. 3B). However, no significant difference was seen in the co-transfection of miR-340-5p and the mutant pcDNA3/EGFP-SPP1 3'UTR-MUT ($p > 0.05$, Fig. 3B). Furthermore, SPP1 mRNA and protein levels in LX-2 cells transfected with pcDNA3/miR-340-5p were significantly decreased, while those with miR-340-5p inhibitor were increased (all $p < 0.05$, Fig. 3C,D). These results suggest that miR-340-5p directly suppresses SPP1 by interfering with its 3'UTR.

miR-340-5p inhibits the proliferation and activation of LX-2 cells by repressing the TGF-β1/Smads pathway

Next, LX-2 cells were used to systematically identify the effects of miR-340-5p in the pathophysiology of HF. The cells were transfected with pcDNA3/miR-340-5p, pcDNA3, miR-340-5p inhibitor or NC-inhibitor, and analyzed for cell proliferation, mRNA and protein expression levels of fibrosis markers Collagen I and α-SMA, as well as protein levels of p-Smad2 and p-Smad3. Compared with TGF-β1-untreated groups, the activated NFs and LX-2 cells had significantly reduced levels of miR-340-5p ($p < 0.05$, Fig. 4A). Also, the expression of miR-340-5p in LX-2 was dramatically enhanced and decreased by the transfection of its overexpression vectors and inhibitors, respectively (both $p < 0.05$, Fig. 4B). The proliferation of LX-2 cells was strikingly inhibited and promoted by pcDNA3/miR-340-5p and miR-340-5p inhibitor, respectively (all $p < 0.05$, Fig. 4C). Collagen I, α-SMA mRNA and protein levels were significantly downregulated by pcDNA3/miR-340-5p and significantly upregulated by miR-340-5p inhibitor (all $p < 0.05$, Fig. 4D,E). Additionally, the levels of p-Smad2 and p-Smad3 were reduced with pcDNA3/miR-340-5p or increased with miR-340-5p inhibitor (all $p < 0.05$, Fig. 4F), when normalized to total protein Smad2 or Smad3. These findings suggest that miR-340-5p inhibits the proliferation and activation of LX-2 cells by inhibiting the TGF-β1/Smads canonical pathway (Fig. 5).

Discussion

Factors such as viral infection, alcohol abuse and metabolic or genetic disorders cause HF, and its main characteristic is ECM protein accumulation, including Collagen I and α-SMA. Activated HSCs are responsible for the excessive ECM protein accumulation.[21] Hepatic stellate cells, which account for approx. 5% of all hepatocytes, were first described by Kupffer in 1876, and they exist in the space between parenchymal cells, hepatocytes and sinusoidal endothelial cells.[22] They participate in liver development,

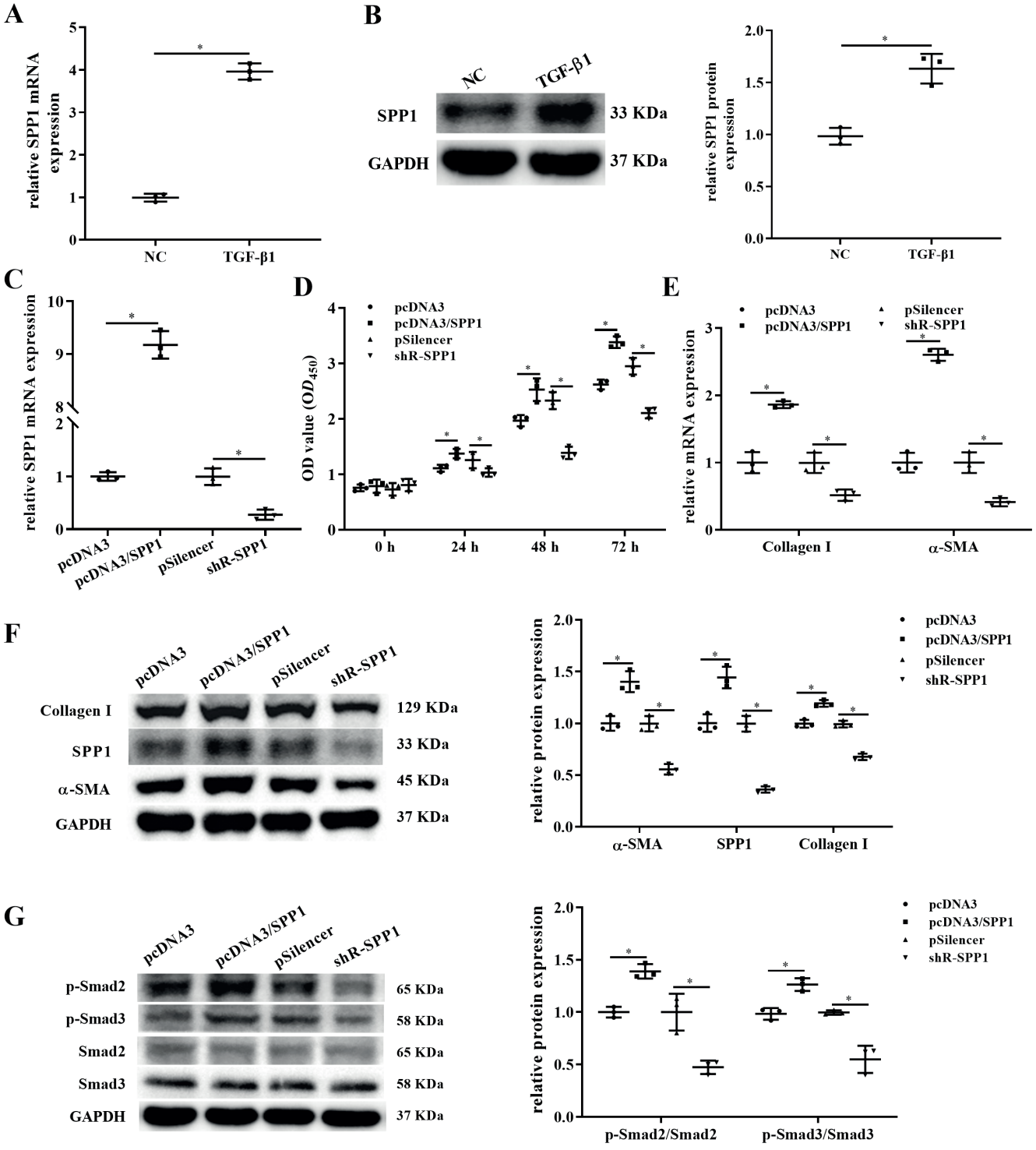

Fig. 2. Secreted phosphoprotein 1 (SPP1) promotes hepatic stellate cell (HSC) proliferation and activation. A. The SPP1 mRNA expression in activated LX-2 cells was examined using quantitative real-time polymerase chain reaction (qRT-PCR); B. The SPP1 protein expression in transforming growth factor beta 1 (TGF-β1)-activated LX-2 cells was examined with western blot; C. The SPP1 mRNA expression in LX-2 cells transfected with pcDNA3/SPP1 or shR-SPP1 was quantified using qRT-PCR; D. The proliferation of LX-2 cells transfected with pcDNA3/SPP1 or shR-SPP1 was determined using cell counting kit-8 (CCK-8) assay; E. The mRNA expression levels of Collagen I and alpha smooth muscle actin (α-SMA) in LX-2 cells transfected with pcDNA3/SPP1 or shR-SPP1 were quantified using qRT-PCR; F. The protein expression levels of Collagen I, α-SMA and SPP1 in LX-2 cells transfected with pcDNA3/SPP1 or shR-SPP1 were determined with western blot; G. Phosphorylated (p)-Smad2 and p-Smad3 expression levels were determined using western blot in LX-2 cells transfected with pcDNA3/SPP1 or shR-SPP1, which were normalized to the total Smad2 or Smad3, respectively. The gene expression levels in LX-2 cells transfected with pcDNA3 or pSilencer were normalized to 1; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for internal control. Results are shown as mean ± standard deviation (M ±SD), n = 3, *p < 0.05 compared to the control; the results were analyzed using the Student's t-test bootstrap or one-way analysis of variance (ANOVA) bootstrap followed by least significant difference (LSD) test or Dunnett T3 post hoc test

NC – negative control; TGF-β1 – transforming growth factor beta 1; OD – optical density.

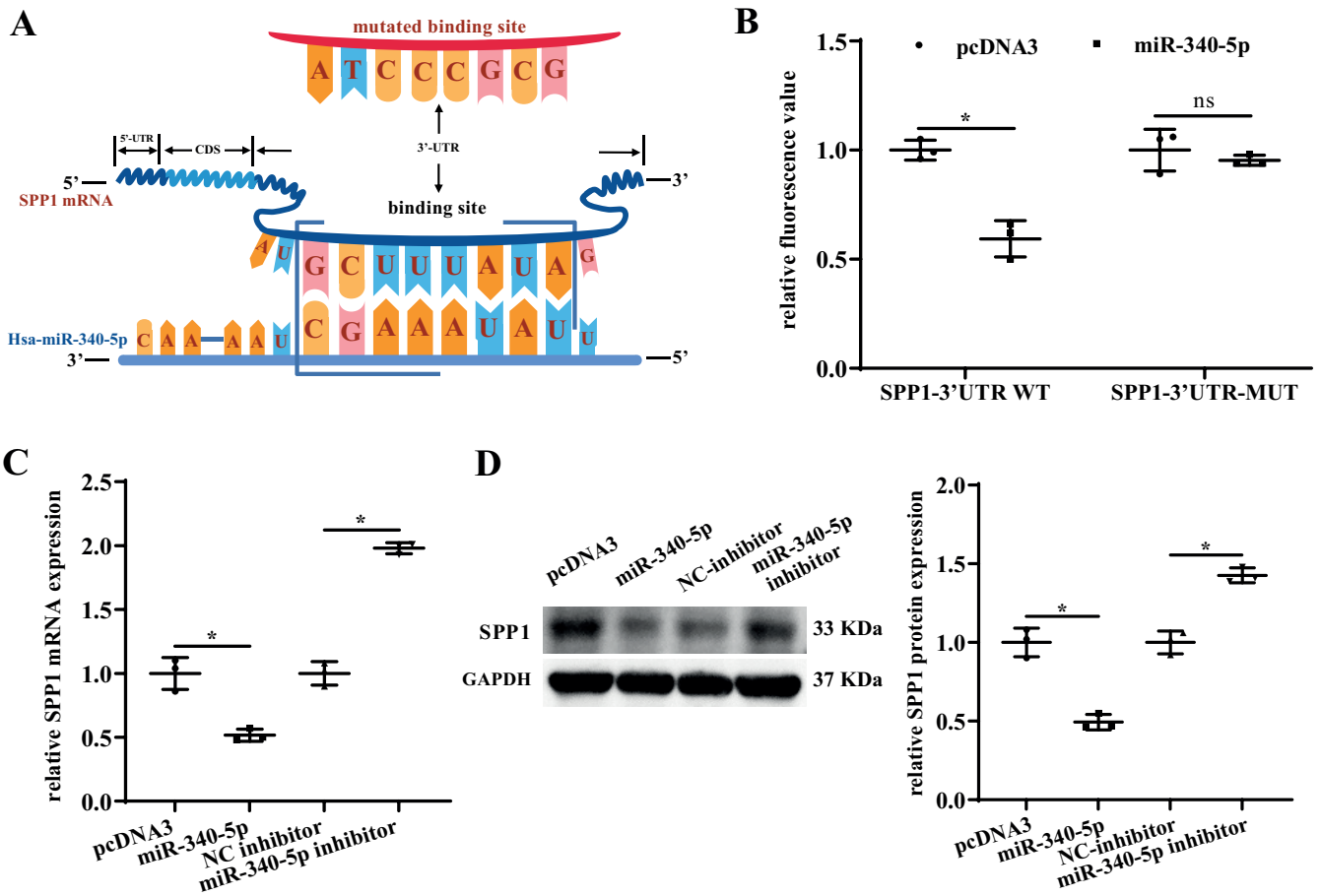

Fig. 3. Secreted phosphoprotein 1 (SPP1) is the target of miR-340-5p. A. miR-340-5p binding site and the mutated binding site in SPP1 3'UTR; B. LX-2 cells were transfected with pcDNA3/miR-340-5p and SPP1-3'UTR WT or MUT, and the enhanced green fluorescence protein (EGFP) intensity was determined and normalized to 1 in the control group; C,D. mRNA and protein expression levels of SPP1 in LX-2 cells transfected with pcDNA3/miR-340-5p or miR-340-5p inhibitor were detected using quantitative real-time polymerase chain reaction (qRT-PCR) and western blot. The mRNA levels in LX-2 cells transfected with pcDNA3 or NC-inhibitor were normalized to 1, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for internal control. Results are shown as mean ± standard deviation (M ±SD), n = 3, ns – not significant, *p < 0.05 compared to the control. The results were analyzed using one-way analysis of variance (ANOVA) bootstrap followed by least significant difference (LSD) post hoc test

differentiation, regeneration, immune regulation, inflammatory response, and liver blood flow control, as well as regulate the occurrence and development of some liver diseases. Once a liver injury occurs, quiescent HSCs are activated and transformed into contractile myofibroblasts, which induce the transcription of Collagen I and α-SMA, and lead to the formation of stress fibers and ECM deposition, which results in increased cell contact.[23] Since the complexity of HSC activation and the pathogenesis of HF are not fully understood, it is critical to clarify the regulatory mechanisms, so as to improve the treatment options for HF.

The *SPP1* gene is on chromosome 4 (4q13) and encodes a multifunctional matricellular protein that is abundantly expressed during inflammation and repair.[24] The *SPP1* also promotes inflammation and fibrosis of the prostate,[25] as well as aggravates the lungs[26] and promotes myocardial fibrosis[27] through different signaling pathways. It is known to regulate radiotherapy sensitivity in gastric adenocarcinoma through the Wnt/β-catenin pathway.[28] Similarly, our results revealed that a high expression of *SPP1* occurs in fibrotic liver tissue of BDL rats and activated LX-2 cells, and it is related to the TGF-β1/Smads signaling pathway, as indicated by KEGG and GO analysis.

Hepatic stellate cells gradually become activated during culturing in vitro, and LX-2 cells used in these experiments are an activated immortalized cell line.[29,30] Therefore, LX-2 cells were used to study SPP1 in order to avoid the influence of exogenous TGF-β1. These studies showed an increase in the levels of mRNA and protein for the markers of HF (Collagen I and α-SMA), as well as the proteins p-Smad2 and p-Smad3, with proliferation occurring when LX-2 cells overexpressed SPP1.

The results of the bioinformatics prediction combined with fluorescence reporter studies showed that SPP1 is the target of miR-340-5p. This is the first study to report on the effect of miR-340-5p in HF. Additional experiments showed that the expression of miR-340-5p was significantly downregulated both in the activated NFs and the activated LX-2 cells. Also, when pcDNA3/

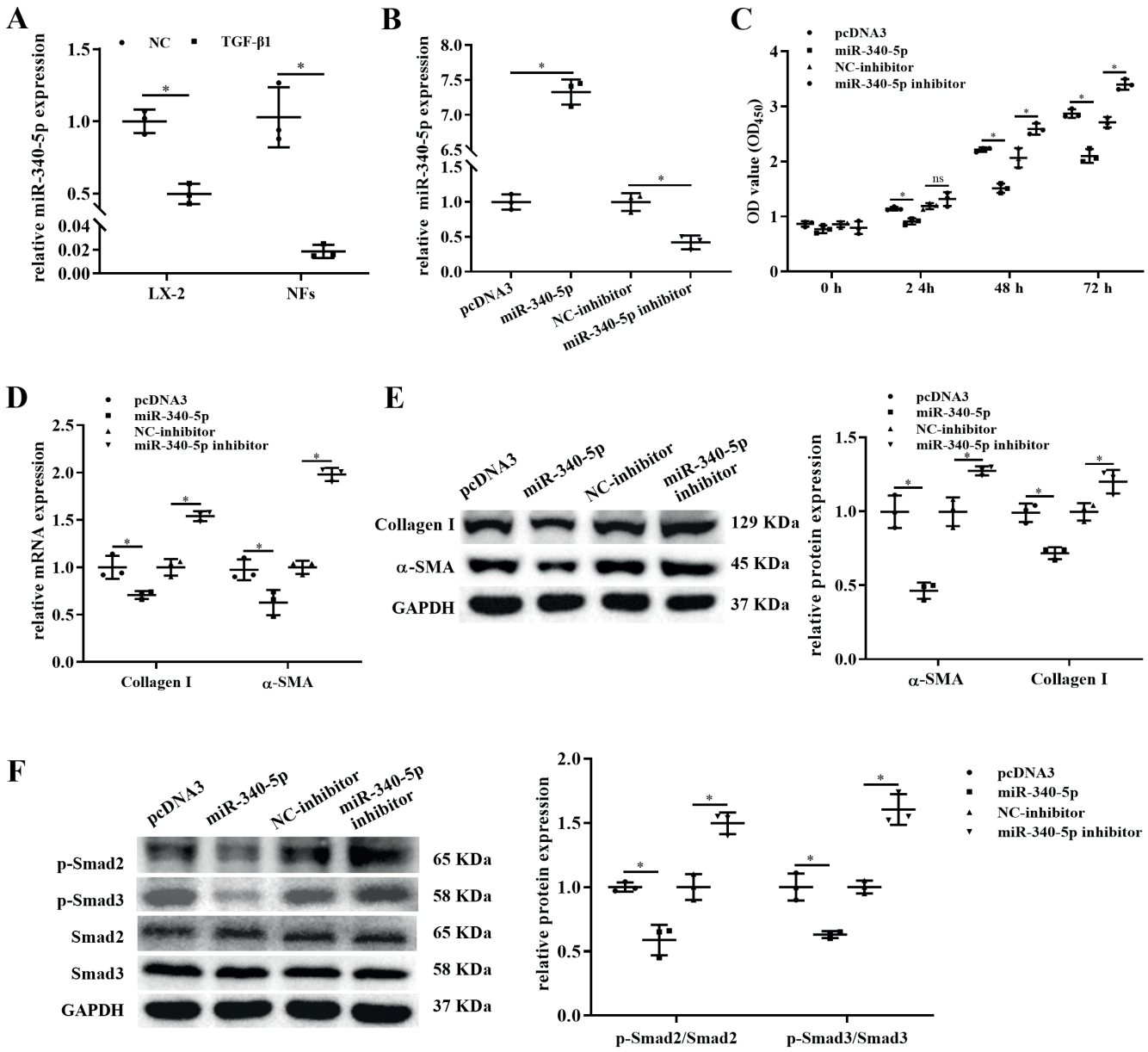

Fig. 4. The miR-340-5p inhibits hepatic stellate cell (HSC) proliferation and activation. A. Expression of miR-340-5p in activated normal fibroblasts (NFs) and activated LX-2 cells was detected using quantitative real-time polymerase chain reaction (qRT-PCR); B. The expression of miR-340-5p in LX-2 cells transfected with pcDNA3/miR-340-5p or miR-340-5p inhibitor was quantified using qRT-PCR; C. Cell proliferation was determined using cell counting kit-8 (CCK-8) assay in LX-2 cells transfected with either pcDNA3/miR-340-5p or miR-340-5p inhibitor; D,E. The mRNA and protein expression levels of Collagen I and alpha smooth muscle actin (α-SMA) in LX-2 cells transfected with pcDNA3/miR-340-5p or miR-340-5p inhibitor were detected using qRT-PCR and western blot; F. The levels of p-Smad2 and p-Smad3 were detected using western blot in LX-2 cells transfected with pcDNA3/miR-340-5p or miR-340-5p inhibitor, which were normalized to the total Smad2 or Smad3 protein. The gene expression in LX-2 cells transfected with pcDNA3 or NC-inhibitor were normalized to 1; U6 was used as the internal reference for miR-340-5p and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference for Collagen I, α-SMA, p-Smad2, and p-Smad3. Results are shown as mean ± standard deviation (M ±SD), n = 3, ns – not significant, *p < 0.05 compared to the control. The results were analyzed using one-way analysis of variance (ANOVA) bootstrap followed by least significant difference (LSD) or Dunnett T3 post hoc test

NC – negative control; OD – optical density; TGF-β1 – transforming growth factor beta 1.

miR-340-5p or miR-340-5p inhibitor was transfected into LX-2 cells to upregulate or knock down the expression of miR-340-5p, respectively, the proliferation of LX-2 cells was significantly downregulated with high levels of miR-340-5p, and upregulated with low levels of miR-340-5p in LX-2 cells. These results, along with the changes to HF markers Collagen I, α-SMA, p-Smad2, and p-Smad3, suggest altered levels of SPP1.

Limitations

In this study, we only carried out cellular experiments to investigate the effect of miR-340-5p on LX-2 cell proliferation and activation by targeting SPP1, there should be some focus on validating these findings in vivo. Therefore, animal experiments and clinical HF tissue samples are needed to confirm the effect of miR-340-5p on HF.

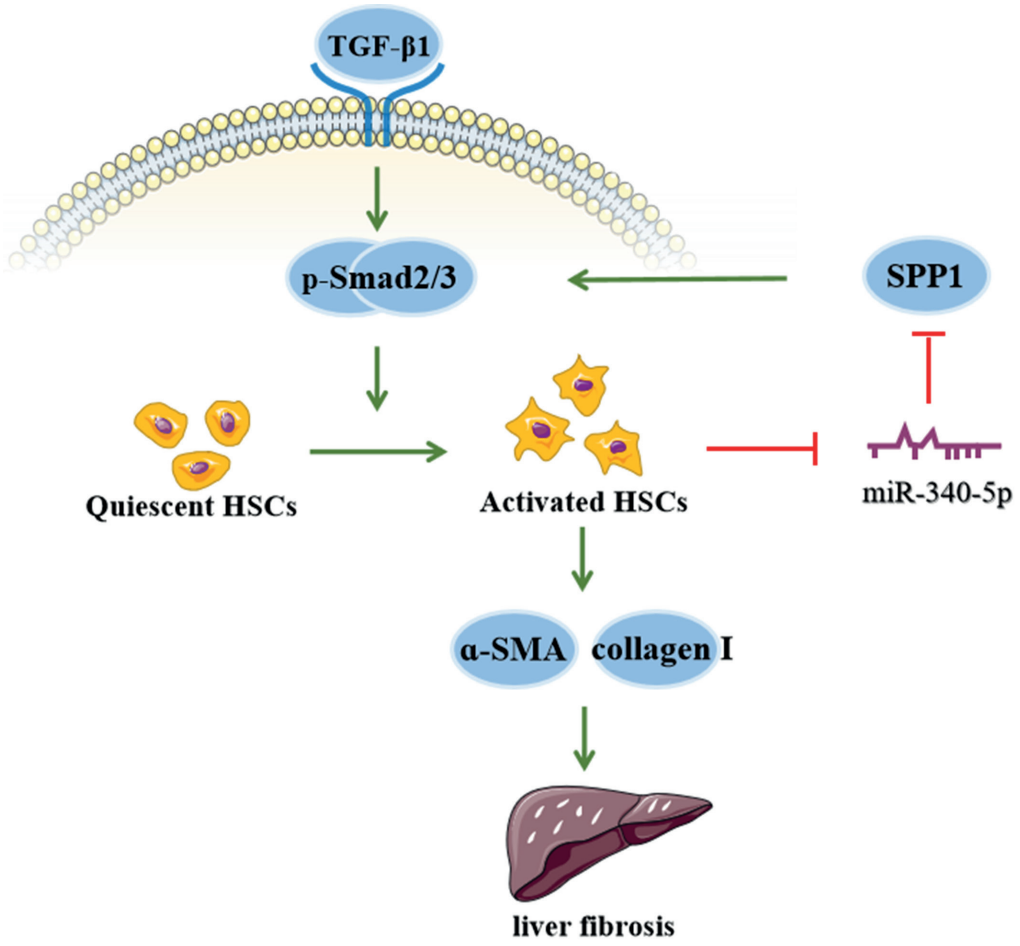

Fig. 5. Schematic representation of a working model of how miR-340-5p suppresses hepatic stellate cell (HSC) activation via the inhibition of the transforming growth factor beta 1 (TGF-β1)/Smads signaling pathway

SPP1 – secreted phosphoprotein 1; α-SMA – alpha smooth muscle actin.

Conclusions

Our findings provide evidence that SPP1 promotes LX-2 cell proliferation and activation through TGF-β1/Smads signaling and that it is the target of miR-340-5p. Therefore, miR-340-5p and SPP1 may be potential therapeutic targets for HF. However, in vivo studies are needed to evaluate the effect of miR-340-5p and SPP1 on HF.

Supplementary materials

The supplementary materials are available at https://doi.org/10.5281/zenodo.7115492.

Supplementary Table 1. Results of t-test bootstrap presented in Table 1.

Supplementary Table 2. Results of t-test bootstrap presented in Fig. 1D.

Supplementary Table 3. Results of t-test bootstrap presented in Fig. 2A,B.

Supplementary Table 4. Results of one-way ANOVA bootstrap followed by post hoc test presented in Fig. 2C–G

Supplementary Table 5. Results of one-way ANOVA bootstrap followed by post hoc test presented in Fig. 3B–D

Supplementary Table 6. Results of one-way ANOVA bootstrap followed by post hoc test presented in Fig. 4A–F.

ORCID iDs

Ronghua Zhang https://orcid.org/0000-0003-2433-1288
Meimei Wang https://orcid.org/0000-0001-9357-8466
Hongjian Lu https://orcid.org/0000-0002-0345-6500
Jingyao Wang https://orcid.org/0000-0003-3684-120X
Xiangyang Han https://orcid.org/0000-0001-7189-5700
Zhiyong Liu https://orcid.org/0000-0003-0900-2275
Lin Li https://orcid.org/0000-0002-1759-7582
Mingming Li https://orcid.org/0000-0001-9922-0940
Xiaoli Tian https://orcid.org/0000-0002-1609-131X
Shuang Chen https://orcid.org/0000-0001-5254-6353
Guangling Zhang https://orcid.org/0000-0002-9542-4986
Yanan Xiong https://orcid.org/0000-0002-5638-4889
Jingwu Li https://orcid.org/0000-0002-7851-5624

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Reduced sodium absorption in the colon under serotonin is a potential factor aggravating secretory diarrhea

Jarosław Koza[1,2,A–F], Ariel Liebert[1,2,C–F], Iga Hołyńska-Iwan[3,B–F], Elżbieta Piskorska[3,B–F], Piotr Kaczorowski[3,B–F]

[1] Department of Gastroenterology and Nutrition Disorders, Faculty of Health Sciences, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland
[2] Clinic of Gastroenterology, Jan Biziel University Hospital No. 2 in Bydgoszcz, Poland
[3] Department of Pathobiochemistry and Clinical Chemistry, Faculty of Pharmacy, Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Jarosław Koza
E-mail: jarosław.koza@cm.umk.pl

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Abstract

Background. Serotonin is a substance with a propulsive effect on the gastrointestinal tract. It stimulates the intestinal secretion of water and electrolytes, and plays an important role in the pathophysiology of secretory diarrhea. However, the influence of serotonin on intestinal absorption is very poorly understood.

Objectives. This study aimed to evaluate the serotonin and selected antagonists of serotonin receptors, i.e., ondansetron (5-HT3) and GR113808 (5-HT4), on electrogenic sodium ion absorption in the colon.

Materials and methods. The electrophysiologic method developed by Ussing and modified with a stimulating function on the mucosal side of the isolated colon wall was used. The influence of selected serotonergic compounds on the electrogenic transport of sodium ions under stationary conditions and mechanical stimulation was investigated. For this purpose, experiments were performed on specimens of isolated rabbit colon. Amiloride and bumetanide were used as reagents directly controlling individual ion transport. The data were analyzed using tests for paired samples (paired sample t-test, Wilcoxon signed-rank test and one-sided sign test).

Results. Serotonin reduced stationary and stimulated colonic sodium absorption. The 5-HT_3 receptor antagonist did not influence the studied phenomenon, while 5-HT_4 antagonists acted contrary to serotonin.

Conclusions. Serotonin reduces both stationary and stimulated sodium ion absorption, thus playing an important role in the pathophysiology of secretory diarrhea. The described phenomenon depends on serotonin's action on 5-HT_4 receptors.

Key words: colon, secretory diarrhea, serotonin, sodium absorption

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Background

Intestinal secretion and absorption are complex processes. Their disturbance can lead to constipation or diarrhea. There are many etiological factors causing both disorders, including diseases outside the digestive system. Generally, diarrhea is more dangerous to human life. Although usually a relatively common and mild symptom, it can in some cases be severe and fatal. Regardless of the cause, it is always associated with intestinal malabsorption. The expected amount of water content in the stool of healthy people is approx. 10 mL/kg/day in infants and young children and 200 g/day in adolescents and adults. Diarrhea is the excretion of stool with increased water content, as well as an increased volume, and is manifested by more frequent bowel movements, i.e., 3 or more loose or watery stools a day. Diarrhea creates a risk of both dehydration and dyselectrolytemia regardless of the mechanism.[1,2]

The evaluation in terms of duration distinguishes acute and chronic diarrhea. Acute diarrhea lasts up to 2 weeks and usually has an infectious cause or is associated with a dietary mistake or the use of laxatives. Chronic diarrhea lasts for more than 4 weeks and its etiology is diverse. Although diarrhea is a manifestation of intestinal dysfunction, its primary cause can be completely different and independent of digestive system disorders (e.g., hyperthyroidism, medullary carcinoma of the thyroid, diabetes, systemic mastocytosis, and vagotomy). The majority of diarrhea causes are almost completely understood, but the particular pathophysiological mechanisms involved in the pathogenesis of secretory diarrhea are still being studied.[2,3]

Generally speaking, diarrhea is the result of reduced water absorption or increased water secretion by the intestines. The amount of water in the stool and its appearance is primarily the result of these 2 opposing processes.[2,3] The dominance of intestinal secretion over absorption, which is the leading reason for diarrhea, is usually caused by one of 2 main pathophysiological mechanisms. These include secretory and osmotic factors, thus osmotic and secretory diarrhea can be distinguished from one another.[4,5] Osmotic diarhea occurs due to the presence of osmotically active substances in the intestines, making it difficult to absorb and retain water. Secretory diarrhea results from an increase in active secretion by intestinal epithelial cells.[6] Chloride secretion and related water transport are considered the leading causes of secretory diarrhea and this phenomenon is well known and described in other studies.[3,7–9] Increased chloride secretion is accompanied by a decrease in sodium ion absorption; however, these processes are much less understood. Chloride secretion is aggravated by serotonin (5-hydroxytryptamine (5-HT)). Thus, chloride secretion plays a pivotal role in the pathophysiology of diarrhea when 5-HT production is increased (e.g., in carcinoid tumor). In physiological conditions, 5-HT within the gastrointestinal tract is derived mainly from enterochromaffin cells, but small amounts of 5-HT also come from the neurons of the enteric nervous system.[3,10]

The 5-HT mediates intrinsic and extrinsic neuronal reflexes, stimulates motility, and increases intestinal secretion and vasodilation. There are many disorders causing 5-HT disturbances in the gastrointestinal tract. The most common causes include brain–gut axis disorders, inflammatory bowel diseases and carcinoid syndrome. The clinical symptoms of carcinoid syndrome are mainly caused by its biologically active substance production, the most important being 5-HT. The physiological role of 5-HT in the gastrointestinal tract is disturbed by its overproduction. Normally, the desired 5-HT concentration is sufficient to regulate basic physiological intestine functions, e.g., secretion and motility. Increased 5-HT content intensifies the basic functions of this hormone, causing secretory diarrhea. Despite the well-known influence of 5-HT on secretion in the gastrointestinal tract, its effects on absorption are poorly understood.[3,11–14] Unraveling the pathophysiology of diarrhea allows to search for the most optimal treatment. Thus, our study was designed to discover new mechanisms involved in secretory diarrhea.

Objectives

This study aimed to determine whether 5-HT can affect sodium absorption in the gastrointestinal tract or not. For this purpose, the effects of 5-HT on electrogenic sodium absorption in the isolated distal colon were evaluated. The additional tested substances were 5-HT_3 antagonist (ondansetron) and 5-HT_4 antagonist (GR113808).

Materials and methods

Study design and pharmacological agents

For our study, the basic experimental environment was assured by the transepithelial chloride transport inhibitor (bumetanide). Thus, the measured electrical properties (potential difference and electric resistance) were based on sodium ion transport.[15–18]

The solutions used during experiments (concentration in mmol/L) were:

RF – Ringer fluid (Na 147.2, K 4.0, Ca 2.2, Cl 155.6, Hepes 10.0) buffered to pH 7.4;

AMI – amiloride (0.1) dissolved and diluted with RF;

BUME – bumetanide (0.1) dissolved in dimethyl sulfoxide (0.1%) and diluted with RF;

5-HT – serotonin (0.005) dissolved with BUME;

GR – GR113808 (0.005) dissolved with BUME;

ON – ondansetron (0.005) dissolved with BUME.

Ondansetron, the 5-HT_3 receptor antagonist, was supplied by GlaxoSmithKline (London, UK), while GR113808, the 5-HT_4 receptor antagonist, and other drugs used in this study were supplied by Sigma-Aldrich Ltd., Poznań, Poland.

Animals

The experiments were performed on the distal colon wall isolated from New Zealand white male healthy rabbits weighing 2.5–4.0 kg. There were 10 animals used in the study (3 in the 5-HT group, 3 in the ondansetron group and 4 in the GR113808 group). Before the experiments, the rabbits had unlimited access to water and food. The experiments were approved by Local Committee for Ethical Animal Experiments of the Universities of Bydgoszcz, Poland (approval No. 23/2009).

The preparatory stage

The rabbits were euthanized with isoflurane (4%) dispersed in carbon dioxide. Next, the colonic wall was excised by laparotomy and a 10-cm-long piece was excised from the border of the mesocolon and divided into 4–5 pieces (each about 2.5 cm^2). The pieces were subsequently incubated in BUME and aerated at room temperature for 60 min.

Ussing chamber

For experimental purposes, each colonic specimen was mounted in a Ussing chamber[16–18] filled with a fluid having the same composition as used during the incubation (that is, BUME). The stimulation consisted of gently rinsing the mucosal surface of the examined colonic wall with the experimental fluid through a nozzle connected to a peristaltic pump. The washing nozzle was set at a distance of 2 mm from the mucosa and the rinse was applied using pressure of approx. 6 Pa. Each stimulus lasted 15 s. The experimental procedure was conducted at a temperature of 37°C.

A pair of Ag/AgCl electrodes was used to measure the value of the electric potential difference (PD) between the mucosal and serous surfaces of the isolated colonic wall. The electrodes were connected to an EVC 4000 (World Precision Instruments, Sarasota, USA) amplifier and the data acquisition system MP 100 (Biopack System, Goleta, USA). The system was operated using AcqKnowledge software v. 3.8.1 (Biopack System). The other ends of the electrodes were connected to the half of the Ussing chamber with the agar bridges.

The experiments were always carried out in the same order. The 1st stimulus was BUME (control), while the 2nd were the serotonergic agents dissolved in BUME (the essential part of the experiment). Then, stimulation with BUME was repeated. Next, to inhibit sodium ion transport, AMI was added to the experimental environment alone, and finally AMI with BUME were inserted to obtain a condition with sodium and chloride inhibition. The electrical resistance of the tissue under study was always measured between stimuli.

Statistical analyses

The data were analyzed using tests for paired samples. The Shapiro–Wilk testing allowed to choose the appropriate statistical tool for further evaluation. When the distribution of differences between evaluated couples was normal, a paired sample t-test was used. When the distribution of differences between couples was not normal, non-parametric tests were applied. The Cabilio–Masaro test was used to check if the data were symmetric around the median.[19] When they were symmetric, the Wilcoxon signed rank test was applied. If they were not symmetric, the one-sided sign test was used.

A p-value <0.05 was considered statistically significant. The Cabilio–Masaro formula was calculated using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, USA), while the other experimental data were evaluated using Statistica v. 12 (StatSoft Poland, Kraków, Poland).

Results

There were no excluded animals or experiments. All obtained data were evaluated. The results of the preliminary experiment using GR113808 are shown in Fig. 1. The protocol of test solutions containing different serotonergic agents used in the studies was the same (infusion of drugs was administered in the same order). The stationary transepithelial PD was measured constantly during the experimental period and was negative in relation to the basolateral side. Moreover, it was found that the reaction of isolated colonic wall in a Ussing chamber when gently stimulated with fluid from a peristaltic pump was hyperpolarization (dPD). After the stimulation stopped, the transepithelial PD returned to the baseline conditions, in which the value of the PD was dependent on the use of a serotonergic agent. The results of our statistical analysis are presented in Table 1. Most of the studied results were normally distributed. The statistical analysis of these data was performed using paired t-tests. The distribution of differences between pairs for the electric resistance of bumetanide and 5-HT was symmetric, while the distribution of the potential differences between bumetanide and GR113808 was asymmetric. Therefore, the Wilcoxon test for the 1st analysis and the sign test for the 2nd analysis were used.

Addition of 5-HT (Table 2, Fig. 2) to the fluid washing the mucosal surface of the studied colon caused a reduction in PD and dPD absolute values. The inhibition of the 5-HT_4 receptor (by GR113808) caused opposite effects on 5-HT (Table 3, Fig. 3). Ondansetron, the 5-HT_3

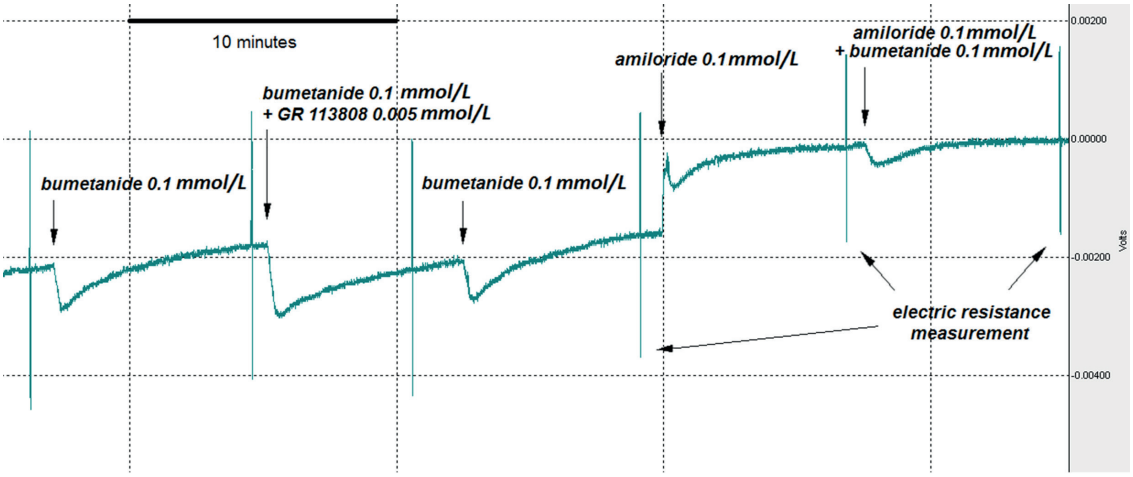

Fig. 1. The course of experimental procedure. Presented example is performed with GR113808 as the tested agent. The vertical arrows indicate the origin of mechanical stimulus. Electrophysiological reaction (dPD) of an isolated colonic wall caused by its mucosal surface stimulation is aggravated by the tested substance (5-HT4 antagonist)

Table 1. Verification of statistical tests assumptions

Variable	n	W_{Sh-W}	p-value	$\|Sk_0\|$	Statistically significant α = 0.05
PD_{B-S}	12	0.967	0.8800	0.56	no
dPD_{B-S}		0.944	0.5573	0.43	no
R_{B-S}		0.6612	0.0004	0.50	no
PD_{B-O}	14	0.943	0.4624	0.25	no
dPD_{B-O}		0.964	0.7937	0.19	no
R_{B-O}		0.891	0.0821	0.08	no
PD_{B-G}	16	0.820	0.0051	1.50	yes
dPD_{B-G}		0.893	0.0630	0.94	no
R_{B-G}		0.901	0.0986	0.56	no

Variables are evaluated differences between means (control compare to the study group). n – number of samples tested consecutively according to the scheme: control compared to the tested substance; W_{Sh-W} and p-value – results of the Shapiro–Wilk test allowing to evaluate data distribution (when $p < 0.05$, then the data distribution is not normal); $|Sk_0|$ – results of the Cabilio–Masaro test allowing to evaluate the symmetry around the median (statistically significant difference for α = 0.05 means asymmetric data distribution); $PD_{B-S, B-O, B-G}$ – differences between paired measurements of transepithelial potential difference for 3 tested groups: bumetanide compared to serotonin, bumetanide compared to ondansetron, bumetanide compared to GR113808, respectively; $dPD_{B-S, B-O, B-G}$ – differences between paired measurements of stimulated transepithelial potential difference for 3 tested groups: bumetanide compared to serotonin, bumetanide compared to ondansetron, bumetanide compared to GR113808, respectively; $R_{B-S, B-O, B-G}$ – differences between paired measurements of electric resistance for 3 tested groups: bumetanide compared to serotonin, bumetanide compared to ondansetron, bumetanide compared to GR113808, respectively.

Table 2. The influence of serotonin on the basic electrical properties of epithelium in the distal colon wall (n = 12)

Variable	Bumetanide (0.1 mmol/L) – control			Serotonin (0.005 mmol/L)		
	PD [mV]	dPD [mV]	R [Ω x cm^2]	PD [mV]	dPD [mV]	R [Ω x cm^2]
Min	–11.63	–1.83	232	–10.13	–1.23	238
Median	–8.93	–1.37	346	–7.51	–0.82	324
Max	–4.67	–0.73	615	–4.06	–0.34	756
IQR	4.57	0.66	227	3.95	0.48	194
Mean	–7.92	–1.34	382	–7.00	–0.75	385
SE	0.74	0.11	39	0.63	0.08	45
n.d.d.b.m.	–	–	–	yes	yes	no
p-values						
t-test	–	–	–	<0.001	<0.001	–
Wilcoxon test	–	–	–	–	–	0.327

Serotonin (0.005) – dissolved with bumetanide (0.1 mmol/L); Min – minimal value in the data; Max – maximal value in the data; IQR – interquartile range; SE – standard error of the mean; n – number of samples tested consecutively according to the scheme: control compared to the tested substance; PD – transepithelial potential difference; dPD – stimulated transepithelial potential difference; R – electric resistance; n.d.d.b.m. – normal data distribution of the differences between paired measurements (control compared to the study group). The Wilcoxon test was used only when t-test could not been considered (not normal data distribution of the differences) and at the same time data were symmetric around the median (the Cabilio–Masaro test).

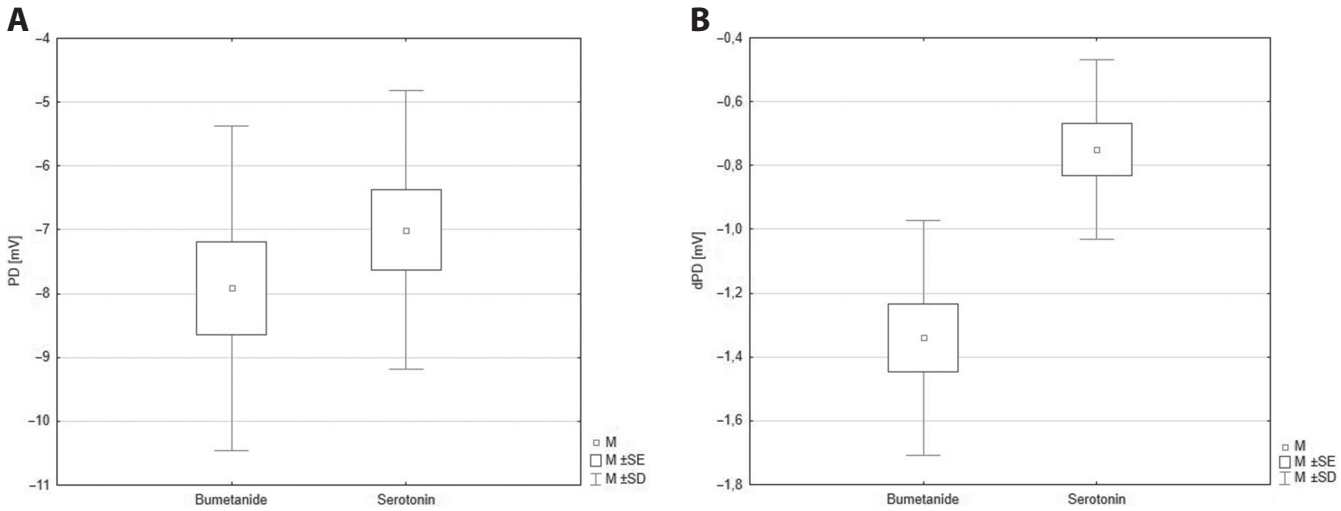

Fig. 2. A. The influence of serotonin on transepithelial electric potential difference (PD) in colon; B. The influence of serotonin on stimulated transepithelial electric potential difference (dPD) in colon

M – mean; SE – standard error; SD – standard deviation.

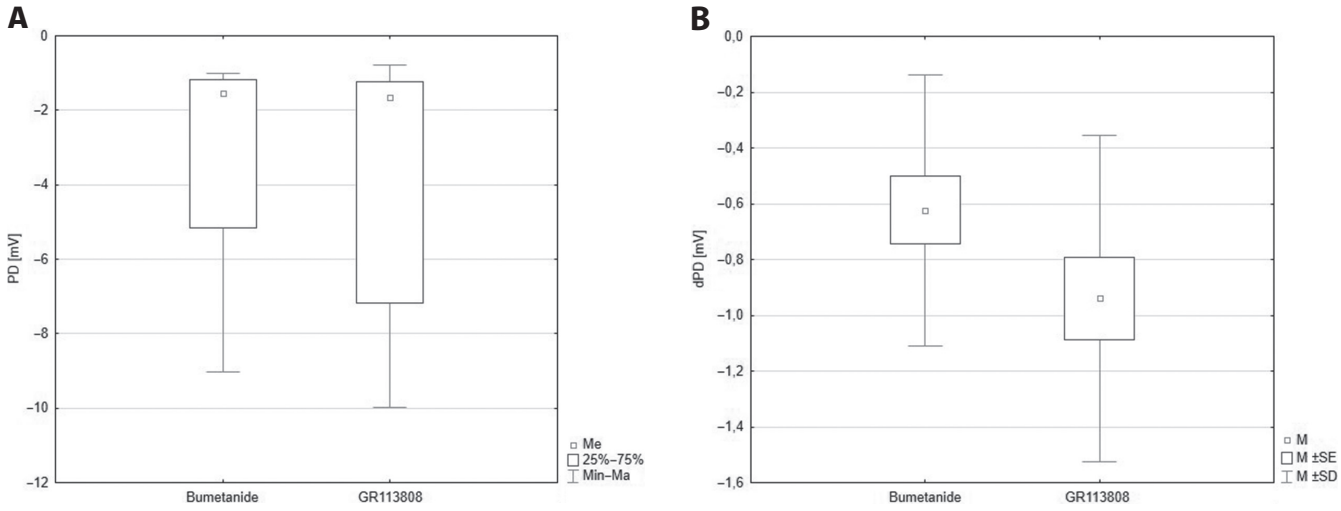

Fig. 3. A. The influence of 5-HT_4 receptor antagonist (GR113808) on transepithelial electric potential difference (PD) in colon; B. The influence of 5-HT_4 receptor antagonist (GR113808) on stimulated transepithelial electric potential difference (dPD) in colon

Me – median; 25%–75% – the range of results in indicated area; Min–Max – the range of minimal and maximal results; M – mean; SE – standard error; SD – standard deviation.

receptor antagonist, did not significantly change any electric properties of the tested specimens (Table 4). The electric resistance was not modified by any of the tested serotonergic drugs.

Discussion

All presented experiments were conducted in an environment with the inhibition of the transepithelial chloride transport; thus, the electric transepithelial PD and its changes were mediated by sodium ion transepithelial flux. Bumetanide used in experiments allowed us to focus on the evaluation of sodium electrogenic transport. The final sequence, with the addition of amiloride to the experimental chamber, confirmed the expected rapid disappearance of the transepithelial electric potential difference.[16]

The 5-HT reduced both the stationary and stimulated absolute values of PD. Thus, 5-HT reduces colonic sodium absorption. The increase in intestinal secretion caused by 5-HT is a well-known phenomenon[3,20–22] and has been confirmed by electrophysiological methods, including the Ussing chamber.[23,24] Studies have reported a reduction in sodium absorption associated with serotonin.[23–25] In the current study, the effects of 5-HT on the reduction of sodium absorption in the large intestine are shown. Subsequently, attempts were made to identify the type of receptor through which the observed reduction of sodium ion transport could be realized. For this study, the antagonists of two 5-HT receptors were also evaluated.

Table 3. The influence of GR113808 (5-HT_4 antagonist) on the basic electrical properties of epithelium in the distal colon wall (n = 16)

Variable	Bumetanide (0.1 mmol/L) – control			GR113808 (0.005 mmol/L)		
	PD [mV]	dPD [mV]	R [Ω x cm^2]	PD [mV]	dPD [mV]	R [Ω x cm^2]
Min	–9.04	–1.67	178	–9.97	–1.92	159
Median	–1.54	–0.40	334	–1.66	–0.86	295
Max	–1.01	–0.23	517	–0.78	–0.27	571
IQR	–3.97	0.63	211	5.95	0.98	298
Mean	–3.17	–0.62	333	–3.87	0.94	341
SE	–0.75	0.12	29	0.89	0.15	39
n.d.d.b.m.	–	–	–	no	yes	yes
p-values						
t-test	–	–	–	–	<0.001	0.775
Wilcoxon test	–	–	–	–	–	–
sign test	–	–	–	0.040	–	–

GR113808 (0.005 mmol/L) – dissolved with bumetanide (0.1 mmol/L); Min – minimal value in the data; Max – maximal value in the data; IQR – interquartile range; SE – standard error of the mean; n – number of samples tested consecutively according to the scheme: control compared to the tested substance; PD – transepithelial potential difference; dPD – stimulated transepithelial potential difference; R – electric resistance; n.d.d.b.m. – normal data distribution of the differences between paired measurements (control compared to the study group). The Wilcoxon test was used only when t-test must not been considered (not normal data distribution of the differences) and data are symmetric around the median (the Cabilio–Masaro test). The one-sided sign test was used for asymmetric data distribution.

Table 4. The influence of ondansetron (5-HT_3 antagonist) on the basic electrical properties of epithelium in the distal colon wall (n = 14)

Variable	Bumetanide (0.1 mmol/L) – control			Ondansetron (0.005 mmol/L)		
	PD [mV]	dPD (mV)	R [Ω x cm^2]	PD [mV]	dPD [mV]	R [Ω x cm^2]
Min	–5.24	–1.94	204	–5.84	–1.92	209
Median	–2.25	–0.75	268	–1.63	–0.72	294
Max	–0.57	–0.23	486	–0.44	–0.29	498
IQR	3.29	0.69	190	3.98	0.83	170
Mean	–2.67	–0.83	321	–2.43	–0.90	335
SE	0.47	0.14	30	0.54	0.15	28
n.d.d.b.m.	–	–	–	yes	yes	yes
p-values (t-test)	–	–	–	0.110	0.233	0.187

Ondansetron (0.005 mmol/L) – dissolved with bumetanide (0.1 mmol/L); Min – minimal value in the data; Max – maximal value in the data; IQR – interquartile range; SE – standard error of the mean; n – number of samples tested consecutively according to the scheme: control compared to the tested substance; PD – transepithelial potential difference; dPD – stimulated transepithelial potential difference; R – electric resistance; n.d.d.b.m. – normal data distribution of the differences between paired measurements (control compared to the study group).

Of all the recognized 5-HT receptors, 5-HT_3 and 5-HT_4 dominate in the intestines and their importance in this section of the gastrointestinal tract is well-known.[26] Thus, antagonists of their activity were tested to determine their 5-HT effects on sodium ion transport.

The primary use of ondansetron and other 5-HT_3 receptor antagonists is for their antiemetic effect.[27] Nevertheless, this group of drugs has been effectively shown to reduce the severity of diarrhea. In clinical trials, 5-HT_3 receptor antagonists reduced diarrhea intensification by influencing intestinal peristalsis and ion transport processes.[28,29] In vitro, studies did not confirm the influence of 5-HT_3 receptor antagonists on intestinal ion transport.[30] The changes caused by 5-HT in the present study were independent also of these receptors.

Experiments using GR113808 confirmed the complete inhibition of 5-HT-induced ion transport under 5-HT_4 receptor blockade.[23] In turn, the stimulation of the 5-HT_4 receptor induces secretion. This phenomenon has been shown with the use of mechanical stimulation on the intestinal mucosa.[31] The participation of 5-HT_4 receptors located on epithelial cells in secretory processes has also been proven.[32]

Limitations

The present study was carried out in vitro and concerned only electrogenic ion transport. Thus, the performed experiments do not reflect all aspects of colonic physiology. However, observed differences should be considered representative of in vivo conditions. Efforts were made to recreate the physiological state as much as possible. For this purpose, several important procedures were

performed. In order to increase the credibility of the results, the experiments were performed at the same time of the day. Since starvation causes disturbances in the transepithelial ion transport and additionally increases the permeability of the intestinal barrier, animals had constant access to water and food until the commencement of euthanasia using isoflurane.[33,34] This gas does not induce permanent changes in transepithelial ion transport and thus does not influence the experimental results.[35,36] It is well known that the mechanisms of ion transport in the rabbit colon and humans are extremely similar; thus, the observed differences are fully representative of humans.[37] In the available literature, numerous publications describe the use of the voltage clamp technique.[9,23,25,30] This form of measurement applies an external electric source to the colon and it is not physiological. In order to achieve a state close to the physiologic environment, a transepithelial electric PD measurement was used.

Conclusions

Mechanical stimulation of colonic mucosa under chloride ion transport inhibition causes an increase in sodium ion absorption processes, manifested by a transient enhancement in the electrical polarity of epithelial tissue. The 5-HT reduces the absorption of sodium in the colon during both mechanical stimulation (dPD) and stationary conditions (PD). Thus, this is one of the implicit mechanisms enhancing 5-HT-dependent secretory diarrhea in vivo. The role of the 5-HT_4 receptor in the described phenomenon concerning sodium absorption has been demonstrated in our study.

ORCID iDs

Jarosław Koza https://orcid.org/0000-0001-7965-5154
Ariel Liebert https://orcid.org/0000-0002-1020-4707
Iga Hołyńska-Iwan https://orcid.org/0000-0002-0986-5604
Elżbieta Piskorska https://orcid.org/0000-0003-4622-4993
Piotr Kaczorowski https://orcid.org/0000-0002-2970-5528

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Awareness and practice of preventive measures among healthcare workers in medical institutions in Beijing during influenza season on the eve of COVID-19 epidemic: A cross-sectional survey

Mei-Lian Xie[1,A,C,F], Jun-Xia Zhang[1,B,F], Zhi-Yun Zhang[1,D,F], Zhao-Xia Lin[2,A,F]

[1] Department of Nursing, Beijing Ditan Hospital, China
[2] Infection Center, Beijing Ditan Hospital, China

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Zhi-yun Zhang
E-mail: zhiyunzhang40@163.com

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Abstract

Background. Influenza is an acute respiratory infectious disease caused by the influenza virus, which poses a certain threat to humans due to its short incubation period, fast transmission and strong infectivity.

Objectives. To evaluate the awareness and prevention behavior against influenza among healthcare workers on the eve of the coronavirus disease 2019 (COVID-19) epidemic in Beijing, China.

Materials and methods. Using the cross-sectional research design based on the principle of convenience sampling, an online questionnaire survey on the knowledge of flu, vaccination, medical protection behavior, and flu medication was conducted between January and February 2020. Healthcare workers from different healthcare facilities and different job positions in Beijing participated in this survey.

Results. A total of 1910 healthcare workers from different medical institutions and jobs were included in the study. The mean age of the participants was 32.69 ±8.72 years (range: 18–64 years). There were significant differences in knowledge about clinical signs about flu and prevention approaches among different age groups, individuals with different work experience and job titles ($\chi^2 = 8.903–32.839$; $p < 0.05$). Personnel with different job positions and education levels differed only in the knowledge about clinical signs of flu and identification of high-risk populations. A multivariate logistic regression analysis revealed that age (odds ratio (OR) = 0.979, 95% confidence interval (95% CI): 0.966–0.992) and education level (OR = 0.736, 95% CI: 0.588–0.921) were risk factors for hand hygiene practices, whereas job position (OR = 1.757, 95% CI: 1.146–2.695) and awareness of high-risk populations (OR = 1.405, 95% CI: 1.096–1.800) were protective factors influencing hand hygiene practices ($p < 0.05$). The only factor influencing mask wearing was the education level (OR = 0.610, 95% CI: 0.450–0.828).

Conclusions. The knowledge level and preventive behavior of healthcare workers before the outbreak of COVID-19 has been insufficient.

Key words: influenza, awareness, healthcare workers, preventive measure

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Introduction

Influenza (flu) is an acute respiratory infectious disease caused by the influenza virus, which poses a certain threat to humans due to its short incubation period, fast transmission and strong infectivity.[1] At present, non-pharmaceutical interventions (NPIs) are an effective influenza prevention and control measure because they are easy to implement and can be used to prevent the spread of flu.[2] The World Health Organization (WHO) released the Global Influenza Strategy 2019–2030, integrating NPIs into prevention and control programs as expanding policy and planning of seasonal influenza prevention and control to protect the vulnerable groups.[3] Similarly, over the last decade, scholars in China have considered the necessity of carrying out health education, timely vaccinations and relevant healthcare measures within the high-risk group to reduce the mortality risk from influenza-related diseases.[4]

Many countries and health institutions have been struggling to raise public awareness of and ability for influenza prevention and control vigorously. However, previous studies have shown that the public implementation of influenza prevention is still insufficient.[5,6] Due to the character of their work, healthcare workers have a high probability of contact with influenza patients compared with other professions. Healthcare workers' knowledge and protective ability are related to their safety and the health or awareness of every patient they contact. Understanding the current situation in medical personnel's prevention can provide important directions and ideas for medical institutions and the public to improve preventive measures in the future.

Objectives

This study was aimed to evaluate awareness and preventive behavior against influenza among healthcare workers on the eve of the coronavirus disease 2019 (COVID-19) epidemic in Beijing, China.

Materials and methods

Participants

Using the cross-sectional research design based on the principle of convenience sampling, an online questionnaire survey was conducted between January and February 2020. Healthcare workers from different healthcare facilities and different job positions in Beijing were included in this survey, including clinicians, nursing staff, medical technicians, and administrative or auxiliary staff. Subjects who studied or pursued further education in a healthcare facility in Beijing, and those who could not complete the survey due to technical difficulties, were excluded.

The study was approved by the Ethics Committee of Beijing Ditan Hospital, Capital Medical University, China (Approval No. 2020-046-02-A issued on February 21, 2021). Informed consent was obtained from all the subjects before participation in this study.

Survey tools

This survey was designed according to the results of on-site interviews with 10 frontline healthcare workers in healthcare facilities, relevant literature, and the content of related domestic and foreign flu awareness questionnaires. The questionnaire included questions concerning basic characteristics of the studied healthcare workers (job position, length of work experience in years, and living environment), their knowledge about flu (clinical signs of flu, high-risk populations, prevention, and treatment), flu vaccination and its reasons, their medical protection behavior (hand hygiene and wearing masks) during the flu season, and willingness to take flu medication.

The questionnaire contained 27 items, and all multiple-choice items were to be answered using a binary response (yes or no). Questions regarding basic characteristics of the healthcare workers were provided in a text form.

The reliability of the survey was evaluated and the questionnaire was revised through a pre-survey of a large sample which included 323 healthcare workers employed in the Beijing Ditan Hospital. The content of the survey was also validated by 5 experts with senior titles and more than 10 years of work experience in a related field (infectious diseases, pediatrics, nursing, and nosocomial infection). The final reliability and retest reliability of the questionnaire were indicated by Cronbach's $\alpha = 0.872$ and $r = 0.956$, respectively; the mean content validity index (CVI) of all items was 1 and the sampling validity was 90.8%. These scores indicated good reliability and validity for this study.

Survey methods

We used a convenience sampling method to explain the purpose and meaning of the study to the managers of healthcare facilities in Beijing. The managers were invited to send the online questionnaires to their employees who met the inclusion criteria. The survey was conducted anonymously.

Statistical analyses

The general data of the survey respondents were subjected to descriptive statistical analysis. The χ^2 test was applied to compare the differences between categorical variables. Binary logistic regression was employed to analyze the factors influencing the implementation of personal protective measures among healthcare workers. Hand hygiene and mask wearing are 2 very important such measures. Considering that there would be different influencing

factors which restrict the implementation rate, we have performed univariable logistic and backward-stepwise logistic regression for these 2 dependent variables. Microsoft Excel 2016 (Microsoft Corporation, Redmond, USA) was used to record the data from the online questionnaire responses. This study did not employ interactive analysis, which is indeed a disadvantage, but we also believe that it should have no impact on the overall outcome. The likelihood ratio test was used for nested models to evaluate the global null hypothesis that 1 or more of the regression coefficients were equal to 0. The Hosmer–Lemeshow test was used to evaluate goodness-of-fit measures. The IBM SPSS v. 20.0 software (IBM Corp., Armonk, USA) was used to import the data and perform the statistical analysis, with $p < 0.05$ indicating statistical significance.

Results

Basic characteristics

Data were initially collected from 2002 healthcare workers. After excluding 8 nonlocal training subjects, 1994 healthcare workers from healthcare facilities in Beijing remained. As this study investigated and analyzed the situation in tertiary hospitals, 84 workers employed in primary and secondary healthcare facilities were excluded, leaving 1910 participants in the final analysis. The mean age of the participants was 32.69 ±8.72 years (range: 18–64 years). The general characterists of the participants including age, sex, job position, work experience in years, job title, education level, and type of hospital in which they work are shown in Table 1.

Table 1. Participant demographics

Index		Number	Percentage (%)
Age [years]	<30	823	43.1
	30–39	708	37.1
	40–49	274	14.3
	≥50	105	5.5
Sex	male	150	7.9
	female	1760	92.1
Job position	medical technician	169	8.8
	nursing staff	1741	91.2
Work experience [years]	<5	611	32
	5–10	517	27.1
	10–20	437	22.9
	>20	345	18.1
Job title	junior	1426	74.7
	intermediate	392	20.5
	senior	92	4.8
Education level	junior college and below	720	37.7
	undergraduate	1055	55.2
	graduate and above	135	7.1
Type of hospital	general	1711	89.6
	specialized	199	10.4

Awareness of flu preparedness in healthcare workers

The knowledge about flu among healthcare workers was assessed using 3 knowledge dimensions. It was measured using multiple-choice questions, and the selection was considered correct only if all options were selected. Answers were compared according to age, sex, job position, work experience (in years), job title, education level, and type of employing insitution. There were significant differences in the knowledge on clinical signs of flu and prevention approaches among different age groups, individuals with different work experience (in years), and persons with different job titles ($\chi^2 = 8.903–32.839$; $p < 0.05$). There were significant differences in the knowledge about high-risk populations of flu only among workers from different types of hospitals and holding different job titles.

The incidence of errors was high in those aged <30 years, whereas the correct answer rates in the older age groups were high ($\chi^2 = 12.877$; $p = 0.005$). The cognitive error rate was higher in the group with <5 years of work experience compared with other groups with more years of work experience ($\chi^2 = 10.113$; $p = 0.018$). The level about knowledge about the clinical signs of flu varied between workers with different job positions ($\chi^2 = 22.422$; $p < 0.001$). The correct answer rate of participants with higher education levels was higher compared with those with an education level of college or below ($\chi^2 = 29.296$; $p < 0.001$). There were significant differences in awareness of high-risk groups among participants from different levels of healthcare. ($\chi^2 = 6.976$; $p = 0.008$). Personnel with different job positions and education levels differed only in the knowledge about the clinical signs of flu and the identification of high-risk populations (Table 2).

Factors influencing flu preparedness in healthcare workers

The dependent variables included the practice of hand hygiene and mask wearing, while the independent variables included: age, sex, job position, work experience (in years), job title, education level, type of employing insitution, and awareness regarding the clinical signs of flu, high-risk populations and prevention approaches. Table 3 shows the assignment of each variable. The development of a multivariate logistic regression model was a two-step process. First, a univariate analysis was carried out to screen for independent variables. Multivariate logistic regression was then performed on statistically significant variables using a backward-stepwise method.

Table 2. Analysis of differences in answers to questions assessing knowledge about protective measures among 1910 medical workers

Index		Clinical manifestations, n (%)		High-risk population, n (%)		Prevention method, n (%)	
		correct	wrong	correct	wrong	correct	wrong
Age [years]	<30	384 (46.7)	439 (53.3)	581 (70.6)	242 (29.4)	574 (69.7)	249 (30.3)
	30–39	390 (55.1)	318 (44.9)	519 (73.4)	189 (26.6)	490 (69.3)	218 (30.7)
	40–49	145 (52.9)	129 (47.1)	194 (70.8)	80 (29.2)	154 (56.2)	120 (43.8)
	≥50	60 (57.1)	45 (42.9)	80 (76.2)	25 (23.8)	52 (49.5)	53 (50.5)
	χ^2	12.877		2.506		32.839	
	p-value	0.005*		0.474		<0.001*	
Sex	male	80 (53.3)	70 (46.7)	98 (65.3)	52 (34.7)	102 (68.0)	48 (32.0)
	female	899 (51.1)	861 (48.9)	1276 (72.5)	484 (27.5)	1168 (66.4)	592 (33.6)
	χ^2	0.281		3.517		0.166	
	p-value	0.596		0.061		0.684	
Job position	medical technician	116 (68.6)	53 (31.4)	120 (71.0)	49 (29.0)	113 (66.9)	56 (33.1)
	nursing staff	863 (49.6)	878 (50.4)	1254 (72.0)	487 (28.0)	1157 (66.5)	584 (33.5)
	χ^2	22.422		0.08		0.012	
	p-value	<0.001*		0.778		0.915	
Work experience [years]	<5	281 (46.0)	330 (54.0)	420 (68.7)	191 (31.3)	415 (67.9)	196 (32.1)
	5–10	276 (53.4)	241 (46.6)	384 (74.3)	133 (25.7)	369 (71.4)	148 (28.6)
	10–20	238 (54.5)	199 (45.5)	317 (72.5)	120 (27.5)	298 (68.2)	139 (31.8)
	>20	184 (53.3)	161 (46.7)	253 (73.3)	92 (26.7)	188 (54.5)	157 (45.5)
	χ^2	10.113		4.905		28.951	
	p-value	0.018*		0.179		<0.001*	
Job title	junior	711 (49.9)	715 (50.1)	1004 (70.4)	422 (29.6)	974 (68.3)	452 (31.7)
	intermediate	203 (51.8)	189 (48.2)	304 (77.6)	88 (22.4)	242 (61.7)	150 (38.3)
	senior	65 (70.7)	27 (29.3)	66 (71.7)	26 (28.3)	54 (58.7)	38 (41.3)
	χ^2	15.169		8.984		8.903	
	p-value	0.002*		0.03*		0.031*	
Education level	junior college and below	340 (47.2)	380 (52.8)	502 (69.7)	218 (30.3)	464 (64.4)	256 (35.6)
	undergraduate	541 (51.3)	514 (48.7)	775 (73.5)	280 (26.5)	718 (68.1)	337 (31.9)
	graduate and above	98 (72.6)	37 (27.4)	97 (71.9)	38 (28.1)	88 (65.2)	47 (34.8)
	χ^2	29.296		3.004		5.346	
	p-value	<0.001*		0.391		0.148	
Type of hospital	general	867 (50.7)	844 (49.3)	1215 (71.0)	496 (29.0)	1127 (65.9)	584 (34.1)
	specialized	112 (56.3)	87 (43.7)	159 (79.9)	40 (20.1)	143 (71.9)	56 (28.1)
	χ^2	2.245		6.976		2.872	
	p-value	0.134		0.008*		0.090	

* $p < 0.05$

The univariable logistic regression model showed that the risk factors associated with hand hygiene were: age, job position, work experience, job title, education level, and awareness of high-risk populations (Table 4). In univariable logistic regression model of mask wearing, the risk factors were: age, job position, work experience, job title, education level, type of employing insitution, awareness of high-risk populations, and awareness of prevention approaches (Table 5).

In the multivariable logistic regression model of hand hygiene practices, age (odds ratio (OR) = 0.979, 95% confidence interval (95% CI): 0.966–0.992) and education level (OR = 0.736, 95% CI: 0.588–0.921) were risk factors, whereas job position (OR = 1.757, 95% CI: 1.146–2.695) and awareness of high-risk populations (OR = 1.405, 95% CI: 1.096–1.800) were preventive factors ($p < 0.05$, R^2 Nagelkerke: 0.049) (Table 6). Testing the global null hypothesis for regression coefficients equal to 0 indicated that these factors could predict the outcome. According to the Hosmer–Lemeshow test, the model is well-fitted ($p = 0.856$).

In the multivariable logistic regression model of mask wearing, age (OR = 0.95; 95% CI: 0.935–0.966) and education level (OR = 0.57; 95% CI: 0.452–0.720) were risk factors, and the awareness of high-risk populations (OR = 1.469;

Table 3. Variables and assignments for multivariate logistic regression

Variable	Assignment
Hand hygiene practices	0 – no, 1 – yes
Wearing masks	0 – no, 1 – yes
Age [years]	1 – <30 2 – 30–39 3 – 40–49 4 – ≥50
Sex	1 – male, 2 – female
Job position	1 – medical technician, 2 – nursing staff
Job title	1 – primary, 2 – secondary, 3 – tertiary
Education level	1 – junior college and below, 2 – undergraduate, 3 – graduate and above
Type of hospital	1 – general, 2 – specialized
Awareness of clinical signs of flu	0 – wrong, 1 – correct
Awareness of high-risk populations	0 – wrong, 1 – correct
Awareness of prevention approaches	0 – wrong, 1 – correct

95% CI: 1.076–2.006) was a preventive factor for mask wearing practices ($p < 0.05$, R^2 Nagelkerke: 0.075) (Table 7). The result of likelihood ratio test was $p < 0.001$; therefore, the null hypothesis of regression coefficient equaling 0 was rejected. However, according to the Hosmer–Lemeshow test, the overall model fit was poor ($p = 0.046$).

Discussion

During an epidemic of infectious respiratory diseases, prevention is crucial for healthcare workers, not only to protect their health but also to ensure the safety of patients and related populations. Due to their daily interaction with sick people in general, and especially those with influenza, healthcare workers are at a higher risk of infection,[8] and are also more likely to transmit influenza virus, especially as they can be asymptomatic carriers.[9–13]

In this survey, 3 main knowledge dimensions of flu were examined, namely clinical signs, high-risk populations

Table 4. Univariate logistic regression results of factors influencing preparedness capabilities of hand hygiene

Variables	B	SE	Wald	p-value	OR	EXP(B) 95% CI		R^2 Nagelkerke	p-value of likelihood-ratio test
						lower limit	upper limit		
Age	–0.026	0.006	–4.140	<0.001	0.974	0.962	0.986	0.014	<0.001
Sex	0.146	0.206	0.707	0.480	1.157	0.773	1.732	<0.001	0.487
Job position	1.008	0.173	5.827	<0.001	2.741	1.953	3.847	0.027	<0.001
Work experience [years]	–0.147	0.052	–2.820	0.005	0.863	0.779	0.956	0.007	0.005
Job title	–0.417	0.083	–5.015	<0.001	0.659	0.560	0.776	0.020	<0.001
Education level	–0.503	0.094	–5.328	<0.001	0.605	0.502	0.728	0.024	<0.001
Type of hospital	–0.316	0.176	–1.802	0.072	0.729	0.517	1.028	0.003	0.078
Awareness of clinical signs of influenza	0.142	0.115	1.235	0.217	1.152	0.920	1.444	0.001	0.217
Awareness of high-risk populations	0.344	0.123	2.794	0.005	1.411	1.108	1.795	0.006	0.006
Awareness of prevention approaches	0.078	0.121	0.650	0.516	1.082	0.854	1.370	<0.001	0.517

95% CI – 95% confidence interval; OR – odds ratio; SE – standard error; EXP(B) – exponential function of B.

Table 5. Univariate logistic regression results of factors influencing preparedness capabilities of mask wearing

Variables	B	SE	Wald	p-value	OR	EXP(B) 95% CI		R^2 Nagelkerke	p-value of likelihood-ratio test
						lower limit	upper limit		
Age	–0.051	0.008	–6.656	<0.001	0.950	0.936	0.965	0.044	<0.001
Sex	0.164	0.257	0.636	0.525	1.178	0.711	1.949	<0.001	0.532
Job position	0.929	0.205	4.535	<0.001	2.532	1.695	3.784	0.019	<0.001
Work experience [years]	–0.416	0.067	–6.179	<0.001	0.660	0.578	0.753	0.041	<0.001
Job title	–0.615	0.094	–6.518	<0.001	0.541	0.450	0.651	0.039	<0.001
Education level	–0.627	0.120	–5.214	<0.001	0.534	0.422	0.676	0.029	<0.001
Type of hospital	–0.532	0.206	–2.576	0.010	0.588	0.392	0.881	0.006	0.014
Awareness of clinical signs of influenza	0.142	0.146	0.971	0.332	1.152	0.866	1.533	0.001	0.332
Awareness of high-risk populations	0.361	0.154	2.343	0.019	1.435	1.061	1.940	0.006	0.021
Awareness of prevention approaches	0.321	0.149	2.148	0.032	1.378	1.028	1.847	0.005	0.033

95% CI – 95% confidence interval; OR – odds ratio; SE – standard error; EXP(B) – exponential function of B.

Table 6. Multivariate logistic regression results of factors influencing preparedness capabilities of hand hygiene

Variables in the equation in step 1	B	SE	Wald	df	p-value	OR	EXP(B) 95% CI	
							lower limit	upper limit
Age	–0.021	0.007	9.604	1	0.002	0.979	0.966	0.992
Job position	0.564	0.218	6.675	1	0.010	1.757	1.146	2.695
Education level	–0.307	0.115	7.159	1	0.007	0.736	0.588	0.921
Awareness of clinical signs of influenza	0.221	0.120	3.420	1	0.064	1.247	0.987	1.577
Awareness of high-risk populations	0.340	0.126	7.223	1	0.007	1.405	1.096	1.800
Constant	1.509	0.719	4.407	1	0.036	4.524	–	–

df – degrees of freedom; 95% CI – 95% confidence interval; OR – odds ratio; SE – standard error; EXP(B) – exponential function of B.

Table 7. Multivariate logistic regression results of factors influencing preparedness capabilities of mask wearing

Variables in the equation in step 1	B	SE	Wald	df	p-value	OR	EXP(B) 95% CI	
							lower limit	upper limit
Age	–0.051	0.008	–6.274	1	0.000	0.950	0.935	0.966
Education level	–0.562	0.119	–4.715	1	0.000	0.570	0.452	0.720
Awareness of clinical signs of influenza	0.238	0.152	1.571	1	0.116	1.269	0.943	1.708
Awareness of high-risk populations	0.385	0.159	2.420	1	0.016	1.469	1.076	2.006
Constant	4.992	0.445	11.207	1	0.000	147.229	61.496	352.483

df – degrees of freedom; 95% CI – 95% confidence interval; OR – odds ratio; SE – standard error; EXP(B) – exponential function of B.

and prevention approaches. The awareness rates for high-risk populations and prevention approaches were relatively high. However, in terms of the awareness of clinical signs, only 16.65% of the participants answered correctly for the item "do not have symptoms of a common cold", 72.61% of the participants answered correctly for "also have gastrointestinal symptoms such as vomiting, abdominal pain and diarrhea", and the rate was from 92.48% to 97.22% for other items. This indicated that there was a confusion about the clinical signs of flu even among healthcare workers. Although the National Health Commission of the People's Republic of China issued the Guidelines for the Diagnosis and Treatment of Flu in 2011 and updated it in 2018,[14] after nearly a decade, the level of awareness of flu among healthcare workers remains low and needs to be improved through organizational training.

In the analysis of demographic factors, there were statistical differences in the perception of clinical representations of influenza by age, job position, work experience, job title, education level, and type of employing insitution. The cognitive error rate was higher in the group with <5 years of work experience compared to other groups with more years of work experience. It was because healthcare workers with longer work experience had more training opportunities during their clinical practice. The level of knowledge about the clinical signs of flu varied between job positions, with the higher correct answer rates among clinicians and medical technicians compared to the nursing staff, which could be attributed to their participation in the diagnosis, treatment and administration of medication, as these require a higher level of knowledge. The healthcare workers with education levels of college or below were mainly nursing staff (90.69%), suggesting that the nursing staff needed to improve their knowledge on the clinical signs of flu. Regarding the question on who was at a high risk of flu, there were differences in the responses based on the type of employing insitution. Respondents from specialized hospitals had a higher correct answer rate than healthcare workers from general hospitals. This may be because the specialized hospital in question is predominantly an infectious disease hospital, where the healthcare workers are more knowledgeable about flu. Finally, the differences in responses to prevention approaches were mainly related to age and job title. The results indicated that healthcare workers <30 years of age and junior staff were more likely to be correct regarding this item than other groups, as the concept of "post-exposure prophylaxis" was more accepted among young population because it received more emphasis during their education.

In this study, all healthcare workers believed that they practiced hand hygiene properly, indicating that the overall hand hygiene and mask wearing practices among the healthcare workers were satisfactory. The "Five Moments for Hand Hygiene" advocated by the World Health Organization (WHO) were developed for the hospital setting and may require some adaptation before the implementation in the primary care context,[15] considering that hand hygiene, if done properly, can reduce the transmission of influenza.[16] However, the survey results showed that 8 staff members did not wear masks during the flu season. All of them were women aged 22–54 years. The 8 interviewed staff members (1 nurse and 7 doctors) worked

in different types of hospitals (general hospitals or specialized hospitals). Although these 8 people accounted for a small proportion of the 1910 respondents, it indicates some inadequacies in the infection prevention in healthcare facilities and implies that there are still problems in the preparedness of healthcare workers in general hospitals that need attention and better management. The factors influencing hand hygiene practices included age, education level, job position, and the number of correct answers about high-risk populations; older age and higher education were related to less meticulous hand hygiene practices. This may be attributed to the low compliance with hand hygiene practices in older people, while habitual thinking patterns and years of taking potluck lead to poor hand hygiene practices in more educated teams of doctors. The factors influencing mask wearing were age, education level and awareness of high-risk populations. Higher educated individuals showed a poorer rate of the mask wearing. This may also be related to the lack of awareness regarding the importance of wearing masks among the higher-educated staff. Therefore, it is crucial to enhance the education and supervision of healthcare workers, especially physicians, during the implementation of protective measures. However, this result differs from previous findings in different knowledge levels, where the high level of awareness shown by the physician community did not guide the clinical practice of self-protection, which suggests that there might be a need to explore the relationships among knowledge, beliefs and actions.

Therefore, while supervising healthcare workers and guiding them in self-protection and management of facilities, training should be provided and emphasis should be put on finding strategies to enhance their knowledge to promote the standardized implementation of protective measures. Moreover, it is recommended to use distance learning techniques to provide layered training tailored to the healthcare workers' needs in order to minimize knowledge gaps, reduce misunderstandings, improve knowledge, and ultimately achieve the goal of improving the overall protection capabilities.

Limitations

There were also some limitations to this study. Our study used convenience sampling, which inevitably resulted in bias. Moreover, this survey did not include other levels of healthcare facilities, resulting in incomplete information on the overall awareness and implementation of prevention among healthcare workers in the region. However, the results of the survey conducted among healthcare workers in tertiary healthcare facilities may also provide insights and reflections for relevant departments, which may be of a great practical significance. Future research should explore strategies for improvements in a larger population. In the results of logistic regression, we found that R^2 Nagelkerke was very low ($p < 0.01$), indicating that the variation in the outcome variable cannot be explained based on the variables selected from the survey of this study. Therefore, there is still a need to explore more desirable variables in further studies.

Conclusions

Influenza prevention education in various regions of China has been effective among the public during the influenza epidemic. Although these educational programs were effective, our study showed that healthcare workers still performed poorly at the implementation of prevention strategies. In the future, continuous attention should be paid to improving the preparedness ability and awareness of healthcare workers. Especially under the impact of the COVID-19 pandemic, substantial changes will be possible.

Data availability statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

ORCID iDs

Mei-Lian Xie https://orcid.org/0000-0002-1531-7698
Jun-Xia Zhang https://orcid.org/0000-0002-6029-2485
Zhi-Yun Zhang https://orcid.org/0000-0003-2301-9102
Zhao-Xia Lin https://orcid.org/0000-0002-0398-7985

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Nursing interventions reduce postoperative urinary retention in fast-track total hip arthroplasty: A pilot study

Łukasz Kołodziej[1,2,A–D], Alina Jurewicz[1,B], Magdalena Gębska[2,D–F]

[1] Department of Orthopedic Traumatology and Musculoskeletal Oncology, Pomeranian Medical University in Szczecin, Poland
[2] Department of Movement Rehabilitation, Pomeranian Medical University in Szczecin, Poland

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;
D – writing the article; E – critical revision of the article; F – final approval of the article

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Address for correspondence
Łukasz Kołodziej
E-mail: Lukas@hot.pl

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Abstract

Background. Postoperative urinary retention (POUR) is a common complication of spinal anesthesia that occurs in 10–80% of patients after total hip replacement (THR). Bladder catheterization carries risks for urinary tract infections, mechanical urethral trauma, urethral inflammation and subsequent strictures, pain, discomfort, an increased length of hospital stay, and a loss of patient dignity.

Objectives. We investigated whether simple postoperative nurse-driven intervention protocols, including the sound of running tap water, followed by caffeinated hot beverages (tea or coffee) and pouring warm saline on the perineal area, could reduce POUR and the need for bladder catheterization.

Materials and methods. This pilot study included 60 patients undergoing elective fast-track THR with spinal anesthesia and early patient ambulation. Patients with postoperative voiding difficulties received nursing interventions, including hearing running tap water, ingesting caffeinated beverages (tea and coffee), and warm saline poured over the perineal area. If voiding difficulties continued, bladder distention was examined by ultrasound. Catheterization was performed if the volume exceeded 500 mL or if distension caused discomfort or pain.

Results. Seven patients (11%) were excluded from the study due to prophylactic preoperative catheterization. Among the 53 included patients, 27 (51%) experienced spontaneous voiding difficulties and received nursing interventions, which induced voiding in 24 patients (45%, $p = 0.0027$), while 3 (6%) required catheterization.

Conclusions. Simple nursing interventions reduced the need for bladder catheterization after fast-track THR.

Key words: total hip replacement, urinary retention, fast track, nursing intervention, urinary catheter

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Introduction

Acute postoperative urinary retention (POUR) occurs as a complication of surgical anesthesia in 10–80% of patients.[1–3] Intermittent or indwelling bladder catheterization reduces the risks of POUR, bladder overdistention and possible renal impairment. However, catheterization carries increased risks of hospital-acquired urinary tract infections, mechanical urethral trauma, urethral inflammation and subsequent strictures, pain, discomfort, an increased length of hospital stay, and a loss of patient dignity. Moreover, both POUR and catheterization can lead to hematogenous bacteremia, seeding of the implant and subsequent periprosthetic infection.[4–7]

The classic criteria for urinary catheterization, published in 1999 by Saint and Lipsky, do not recognize elective total hip replacement (THR) as an appropriate indication for indwelling bladder catheter use.[8] Compared to general anesthesia, spinal anesthesia with the routine addition of intrathecal morphine is superior in fast-track total hip arthroplasty, but may increase the risk of POUR.[5,9] Although there are currently no standard protocols for urinary catheter use in elective total hip arthroplasty, many institutions routinely use urinary catheters in patients who undergo spinal anesthesia.

Bladder function and micturition are complex physiological processes. However, bladder emptying can sometimes be encouraged using simple procedures described in every classic handbook for nurses and doctors, including the sound of tap running water, ingesting caffeinated beverages, or pouring warm water on the perineal area. The effectiveness of these techniques for reducing POUR and the need for bladder catheterization in patients undergoing fast-track total hip arthroplasty remains unknown.

Objectives

The present study aimed to investigate the effectiveness of classic nursing interventions for reducing the incidence of POUR and the need for bladder catheterization among patients undergoing fast-track THR.

Materials and methods

This prospective observational pilot study included 60 consecutive patients undergoing fast-track primary THR. No exclusion criteria were applied. All participants signed written consent forms, and the study protocol was approved by the bioethical committee at the Pomeranian Medical University in Szczecin, Poland (approval No. KB-0012/71/11). The average participant age was 64 ±11.12 years, and the average participant body mass index (BMI) was 26 ±4 kg/m^2. Table 1 shows the patients' preoperative characteristics and comorbidities.

Table 1. Patients' preoperative characteristics and comorbidities

Patients' characteristics and comorbidities		n (%)
Gender	male	28 (53.0)
	female	25 (47.0)
Diabetes type I		2 (3.5)
Diabetes type II		5 (9.5)
Hypo-/hyperthyroidism		7 (13.0)
Gout		3 (5.7)
Prostate cancer		1 (1.9)
Coronary artery disease		10 (18.9)
Hypertension		22 (41.5)

A modified fast-track protocol was utilized in all patients. Thirty minutes before surgery, the patients received intravenous cefazolin (1.0 g). Spinal anesthesia was performed using Marcaine Spinal 0.5% Heavy (Aspen Pharmacare, Durban, South Africa; dose depending on the patient's height (3.5–4 mL), planned level of anesthesia was Th10). The surgery was carried out using an anterolateral approach to the hip joint, and all patients received a cementless hip implant. Several surgical parameters were recorded, including the duration of surgery, blood loss and the need for blood transfusion.

After the operation, the patients received multimodal, opioid-sparing analgesia, including intravenous administration of ketoprofen (100 mg) every 12 h (changing to oral administration on the 1st postoperative day), oral paracetamol (100 mg) every 6 h and oral tramadol (50–100 mg) every 8 h. For breakthrough pain, patients received intravenous morphine (1–2 mg). At 3–4 h after surgery, once motor blockade resolved, physical therapy and ambulation with weight-bearing as tolerated were initiated.

In every patient who experienced postoperative difficulties in voiding after ambulation, a nurse-driven intervention protocol was introduced. First, the sound of running tap water was initiated, followed by the ingestion of caffeinated hot beverages (tea or coffee) and the pouring of warm saline on the perineal area. If the patient was still unable to void, bladder distention was examined by ultrasound. If the volume exceeded 500 mL, or if bladder distension caused the patient discomfort and pain, a Foley catheter was inserted.

Statistical analyses

Dichotomous data are presented as percentages, while continuous variables are presented as medians. Dichotomous data were compared using the Fisher's exact test. A value of $p < 0.05$ was considered statistically significant. Statistical analyses were performed using Statistica v. 10 (StatSoft Inc., Tulsa, USA).

Results

Seven patients (11%) were prophylactically catheterized after surgery without clear indication (due to anesthesiologist preferences) and were therefore excluded from the analysis. Among the 53 patients available for further assessment, 26 (49%) spontaneously emptied their bladder after early ambulation. The remaining 27 patients (51%) experienced voiding difficulty and urine retention, which the nursing staff recognized and responded to with micturition-supporting interventions. The sound of running tap water was successful in 14 patients (26%), caffeinated beverages (tea/coffee) in 7 patients (13%), and pouring warm saline on the perineal area in 3 patients (6%). Only 3 patients (6%) required subsequent catheterization. Overall, using all methods together one after the other appeared very effective in promoting bladder emptying in 24 patients (45%). The methods were ineffective in only 3 patients (6%) who required subsequent catheterization ($p = 0.0004$; Fisher's exact test).

Discussion

Postoperative urinary retention can prolong hospitalization, increase hospital readmission rates and negate the benefits of THR. However, bladder catheterization and related complications can lead to the same negative consequences. There are presently no clear guidelines for orthopedic surgeons and nurses regarding POUR prevention and treatment, and only limited evidence is available indicating whether intermittent or indwelling catheterization is preferable after THR.[4–6]

The use of the International Prostate Symptoms Score (IPSS) revealed that older patients with obstructive symptoms are at an elevated risk of developing POUR. Weekes et al. created a urinary symptoms questionnaire aiming to identify patients at high risk for POUR before surgery.[3] However, the use of that questionnaire does not help avoid the complications of bladder catheterization itself or reduce the number of unnecessary elective catheterizations.[1,10,11] Bjerregaard et al. identified other modifiable risk factors for POUR, including anesthesia technique, opioid use (intrathecal and parenteral), postoperative pain management, and fluid therapy.[7] Griesdale et al. found that POUR commonly occurs after total knee replacement in patients receiving spinal anesthesia with morphine.[2] In contrast, Miller et al. found that spinal anesthesia is associated with a low POUR risk in THR patients.[5] Advances in fast-track total hip arthroplasty have led to a number of positive modifications in perioperative bladder management.[7] Balderi et al. confirmed the usefulness of an ultrasonographic nurse-driven protocol for avoiding elective and unnecessary catheterization.[12] Leach et al. suggested that caffeine ingestion increases voiding volume and helps to avoid subsequent catheterization after the removal of the indwelling catheter following joint arthroplasty.[13]

The current pilot study was designed to measure the clinical effectiveness of simple and well-known nursing techniques for avoiding elective and unnecessary catheterizations. To our knowledge, no similar study has been performed before. The results showed that the nursing protocol was successful in the selected group of patients. The present study has several limitations, including a small number of participants and the lack of a control group. Moreover, discriminant analyses do not identify any factors related to urine retention or to the effectiveness of micturition-supporting methods in the examined group. Rather, the current results indicate that the tested methods were effective, with no relation to epidemiological differences.

Conclusions

Overall, the present results suggest that simple nursing techniques can successfully reduce POUR in patients undergoing fast-track THR, and encourage further studies on this subject.

ORCID iDs

Łukasz Kołodziej https://orcid.org/0000-0002-0400-7918
Alina Jurewicz https://orcid.org/0000-0002-2822-858X
Magdalena Gębska https://orcid.org/0000-0002-1477-8302

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