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MINIREVIEWS

Diagnosis and treatment of chronic osteomyelitis based on nanomaterials

Ming Zeng, Zheng Xu, Zhen-Qi Song, Jie-Xiao Li, Zhong-Wen Tang, Sheng Xiao, Jie Wen

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Abstract

Chronic osteomyelitis is a painful and serious disease caused by infected surgical prostheses or infected fractures. Traditional treatment includes surgical debridement followed by prolonged systemic antibiotics. However, excessive antibiotic use has been inducing rapid emergence of antibiotic-resistant bacteria worldwide. Additionally, it is difficult for antibiotics to penetrate internal sites of infection such as bone, thus limiting their efficacy. New approaches to treat chronic osteomyelitis remain a major challenge for orthopedic surgeons. Luckily, the development of nanotechnology has brought new antimicrobial options with high specificity to infection sites, offering a possible way to address these challenges. Substantial progress has been made in constructing antibacterial nanomaterials for treatment of chronic osteomyelitis. Here, we review some current strategies for treatment of chronic osteomyelitis and their underlying mechanisms.

Key Words: Osteomyelitis; Nanomaterials; Infectious disease; Drug delivery

Core Tip: Chronic osteomyelitis is a painful and serious disease caused by infected surgical prostheses or infected fractures. Traditional treatment includes surgical debridement followed by prolonged systemic antibiotics treatment. But as antibiotics is difficult to penetrate into the internal infection areas of bone, thus limiting the efficacy of systemic antibiotic therapy, new therapeutic approach to treat this disease remains a major challenge for orthopedic surgeons. Substantial progress has been made in constructing antibacterial nanomaterials for treatment of chronic osteomyelitis. We review some current strategies for treatment of chronic osteomyelitis and their underlying mechanisms.

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INTRODUCTION

Pyogenic osteomyelitis is the inflammation of bone tissue caused by pyogenic bacterial infection, including bone marrow, bone cortex, periosteum, and surrounding soft tissue infections, confined to one site or distributed throughout the body. The infection types are as follows: (1) Blood-borne infection: Pathogenic bacteria are transferred from distant infection foci to the bone tissue through the blood circulation, which is termed as bloodborne osteomyelitis; (2) Post-traumatic infection, also known as post-traumatic osteomyelitis, includes direct contamination of open fractures or bone infection after fracture surgery, especially after internal fixation or prosthesis implantation; and (3) Adjacent infection: Foreign body infection, pressure ulcers, and other adjacent soft tissue infections spread to the bone tissue, ulcers caused by diabetes and arteriosclerosis, and osteomyelitis caused by tissue necrosis. The most common site in children is the metaphysis of the long bones (distal femur and proximal tibia), or penetrating bone injury due to trauma[1-4]. Pyogenic osteomyelitis can be divided into acute and chronic types according to disease progression. It is speculated that the formation of dead bone is asign of chronic osteomyelitis as it appears 6 wk after disease onset[5,6].

OSTEOMYELITIS

Epidemiology of osteomyelitis

The incidence of osteomyelitis has increased with the upgrade of diagnostic technology, increase in prosthetic implants in orthopedic surgery, and increase in diabetes. For example, German researchers conducted statistical analysis of patients with osteomyelitis and found that, compared with 10 years ago, the overall incidence of osteomyelitis rose from 15.5/100000 people/year to 16.7/100000 people/year, an increase of 10.44%; however, this number was higher in developing countries and lower in undeveloped countries[1,7,8]. Kremers *et al*[9] assessed osteomyelitis from January 1969 to December 2009 and found that the total annual incidence of osteomyelitis was 21.8/100000 people/year. Nonetheless, the annual incidence of osteomyelitis was lower in women than in men and the infection rate increased with age. The incidence increased significantly from 11.4/100000 person-years in 1969-1979 to 24.4/100000 person-years in 2000-2009. The rates were stable in children and young adults but almost three times higher in those aged > 60 years, which could be attributed to a large increase in cases of diabetes-related osteomyelitis[10], of which, 44% had *Staphylococcus aureus* (*S. aureus*) infection. Lindbloom *et al*[11] investigated diabetic-foot-related osteomyelitis and found that diabetic foot infection was 36.5 per 1000 people/year, and the incidence of diabetic foot ulcers was 25%. About 20%-68% of diabetic foot ulcers are potentially associated with osteomyelitis. The amputation rate after osteomyelitis in diabetic foot infection is 66%. In one study, diabetes was associated with a 1.6% in-hospital mortality rate for osteomyelitis[12].

Common pathogenic bacteria of osteomyelitis

Among many pathogenic bacteria, *S. aureus* is the most common cause of chronic osteomyelitis[13,14]. However, specific bacterial distribution and drug resistance may vary across some regions due to patterns of use of antibacterial drugs in different areas. Data from southwest China were as follows: 467 cases (92.8%) had antibiotic treatment before admission, 324 cases (64.4%) were positive for culture, and 377 strains of microorganisms were cultivated. *S. aureus* (159 strains) accounted for 42.2%, and 38 strains were methicillin-resistant *S. aureus* (MRSA). There were 49 strains of *Pseudomonas aeruginosa* (*P. aeruginosa*) (13.0%), 35 of *Enterobacter cloacae* (*E. cloacae*) (9.2%), 33 of *Escherichia coli* (*E. coli*) (8.8%), seven of fungi (1.9%), 17 of *Acinetobacter baumannii* (4.5%), and 77 other microorganisms (20.4%)[15]. Jiang *et al*

[16] retrospectively analyzed 394 patients with chronic osteomyelitis of limbs treated in the Nan Fang Hospital from January 2010 to April 2015. The study cohort comprised 307 men and 87 women at a sex ratio of 3.53. The mean age at initial visit of all patients was 42 years. The positive rate of intraoperative culture was 70.63% (214/303), of which a single-strain infection accounted for 78.97% (169/214). Most of the single-strain infections were *S. aureus* (59 cases), while a few were *P. aeruginosa* (29 cases) or *E. coli* (11 cases). From January 1, 2012 to December 31, 2015, 5268 patients with limb fractures were admitted to this hospital, and 108 cases were diagnosed as post-traumatic osteomyelitis[16]. The bacterial cultures were positive in 77.8% (84/108) of patients. Among them, 104 microbial strains were detected, including 56 Gram-positive bacteria (53.9%), 39 *S. aureus* (37.5%), and six *Staphylococcus epidermidis* (*S. epidermidis*) (5.8%). There were 48 Gram-negative strains (46.1%), including 16 (15.4%) *E.coli* and 11 (10.6%) *E. cloacae* [17]. Another study showed that most bacteria, such as *S. aureus*, *P. aeruginosa*, *S. epidermidis*, and *Streptococcus pneumoniae*, adhere to the contact surface after invading the host[18]. There are even rarer cases of osteomyelitis being caused by *Salmonella*[19,20]. Surface anchoring proteins play a critical role in host cell adhesion and invasion, biofilm formation, and secretion of polysaccharide matrix, fibrin, and lipoprotein[21], which form an organized microbial aggregate biofilm[22]. This makes the bacteria resistant to the immune system[23]. Also, it is difficult to remove the biofilm completely with antibiotics, but the antibiotics kill the free bacteria in the blood that cause outbreaks of infection. When the body's resistance is reduced, the bacteria living in the biofilm are released, causing repeat infection. Biofilms are like shelters for microorganisms, leading to repeated outbreaks of chronic infection, which are prolonged and unhealed[24,25].

The interaction between osteoblasts and osteoclasts tightly regulates bone remodeling. The osteoblasts and osteoclasts communicate through direct contact between the cells or by secreting proteins that regulate their behavior, survival and differentiation[26]. *In vitro* studies have shown that bone tissue in an inflammatory and infective environment increases osteoclast activity and decreases osteoblast activity[27]. Biofilm-derived factors can decrease osteoblast activity by activating apoptosis under biofilm formation[28]. SPA mainly upregulates the expression of NFATc1 and C-FOS by activating the mitogen-activated protein kinase pathway, thus promoting the formation of osteoclasts [29]. It also binds to tumor necrosis factor receptor 1, which is highly expressed in osteoblasts[30], and then activates the downstream nuclear factor-kB pathway, leading to interleukin (IL)-6 release[31].

Therefore, we must focus on the research and development of new technologies and materials for the bacteria that cannot be controlled by antibiotics to achieve precise delivery of the drugs into the capsule. A specific drug can directly destroy the capsule of the bacteria to overcome the current disadvantages (Figure 1).

Pathophysiological mechanism of osteomyelitis

Before the onset of acute bloodborne disease, acute osteomyelitis often appears before the onset of disease in parts of the body with different degrees of infection foci[32], such as the upper respiratory tract and open fractures. With improper handling or immunodeficiency, the infection can spread throughout the body *via* the blood circulation and bacteria with the oven, and the bloodstream of children's long bone epiphysis nourishing artery is slow. The blood vessels are dense, retaining the bacteria and allowing their multiplication[33]. Bacteria multiply in cancellous bone and cause a local acute inflammatory reaction, such as hyperemia, edema, and leukocyte infiltration. Subsequently, local intraosseous pressure increases, causing severe pain, and then leukocyte necrosis releases lysozyme to destroy trabecular bone matrix abscess as it expands in the direction of low pressure. The local foci of infection can spread to the surrounding articular structures through the Havelian and Volkmann canals, and vascular occlusion exacerbates osteonecrosis. The necrotic bone is surrounded by granulation and fibrous tissues and is retained for a long time, resulting in the formation of dead bone, where a dead cavity is formed, termed a bone defect[34]. Often the course of osteomyelitis changes from acute to chronic. If the periosteum is not destroyed by infection, inflammation stimulates the formation of new bone beneath the periosteum, which can wrap around the dead bone and its upper and lower living segments. The dead bone and pericarp can cause the infected lesions to persist. Chronic osteomyelitis is likely to result from acute childhood osteomyelitis, followed by post-traumatic osteomyelitis, which causes bacterial colonization of bone tissue during open injury (Figure 2). In patients with reduced immunity, diabetes, atherosclerosis, and other conditions, the incidence of the disease is significantly increased.

In case of poor control of the lesion, the infection is surrounded by dead bone with no vascular supply, thickened periosteum, and fibrous tissue, eventually forming a zone of isolation. This isolation band can prevent the antibiotics from reaching this avascular region to kill the bacteria. Since the body's immune system cannot work correctly, it can easily lead to failure of drug treatment of osteomyelitis [35]. The lesions can exist for a long time and have intermittent episodes. If these persist for an extended period, they may become resistant to antibiotics[36]. In the face of intelligent bacteria, we must immediately find new materials that can fill bone defects, accurately deliver antibiotics to lesions, and assess auxiliary bactericidal and antibacterial effects.

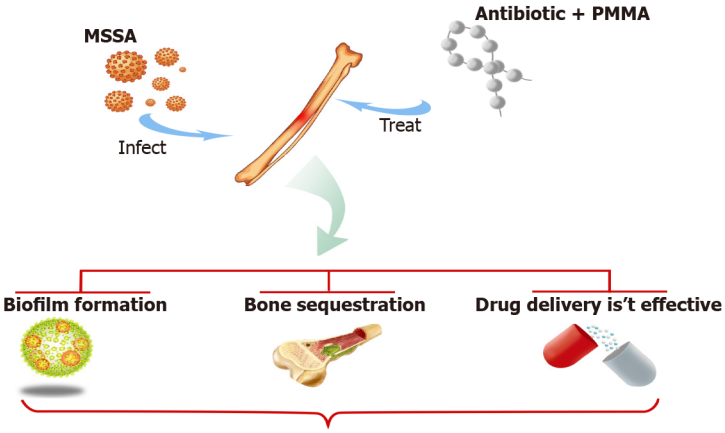

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Figure 1 A schematic of major challenges in the treatment of osteomyelitis. MSSA: Methicillin-susceptible *Staphylococcus aureus*; PMMA: Polymethyl methacrylate.

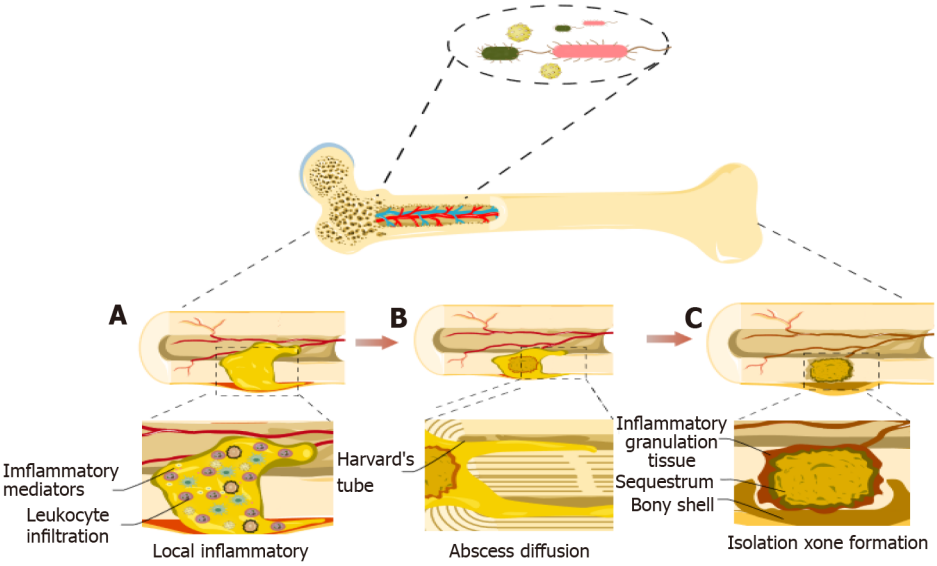

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Figure 2 Pathophysiological mechanism of osteomyelitis. A: Leukocyte necrosis releases inflammatory mediators to destroy bone matrix and bone trabeculae, forming abscesses; B: The infected lesions spread to the adjacent bone structures through Harvard's tube, and the intraosseous pressure is increased; C: Bone destruction and vascular obstruction resulted in different degrees of osteonecrosis and encapsulation.

NANOMATERIALS FOR DIAGNOSIS AND TREATMENT OF OSTEOMYELITIS

Nanotechnology is defined as the ability to translate the theory of nanoscience into practical applications by observing, measuring, manipulating, assembling, controlling, and manufacturing matter at the nanoscale (1-100 nm)[37]. After decades of rapid development, nanotechnology has achieved excellent results in other industries, and human life has become more convenient[38]. During this period, scientists have shown significant interest in the medical application of nanomaterials. Since nanomaterials are of similar size as biological molecules, all kinds of materials can be designed as carriers with a variety of functions that allow nanomaterials to pass through the capillaries in the human body or through the cells to regulate cell behavior and genes[39]. Therefore, nanotechnology has potential medical applications[40]. In recent years, many researchers have proposed nanomaterials for treating osteomyelitis to overcome a series of previously encountered problems (Table 1). It has been found that nanomaterials have many advantages in the treatment of osteomyelitis[41].

Nanomaterials for the diagnosis of osteomyelitis

Osteomyelitis has an insidious onset. Current imaging techniques make it challenging to make an accurate and specific diagnosis of osteomyelitis, which needs to be differentiated from other diseases, such as bone metastases, nonspecific inflammation, and Charcot arthropathy. Early osteomyelitis

Table 1 Application of novel nanomaterials in the treatment of osteomyelitis

Ref.	Material	Conclusion	Application
Bruna *et al* [46], 2021	Silver nanoparticles	With antibacterial agents as organic compounds or antibiotics it has shown synergistic effect against pathogens bacteria such as *Escherichia coli* and *Staphylococcus aureus*	Treat infections or prevent them efficiently
Zheng *et al* [57], 2018	Gold nanoclusters	Exhibit excellent treatment effects in both macrophages and animal infection models induced by MRSA as representative	Inhibition of MRSA biofilm formation. The induction of intracellular ROS production in bacterial cells
Gowri *et al* [68], 2021	Ca-Alg nanoparticle	Clindamycin loaded Ca-Alg/PPAA system showed sustained Clindamycin release from the carrier. Exhibited better cell viability of synthesized materials against MG63 cells	Sustained drug release. Promotes bone regeneration
Krishnan *et al*[69], 2020	Silica coated nanohydroxyapatite-gelatin reinforced with poly-l-lactic acid yarns	The nanocomposite fibrous scaffold containing vancomycin can be proposed as a bifunctional graft that can reduce bacterial infection, while subsequently engineer new bone in osteomyelitis	Reduce bacterial growth engineer. New bone in osteomyelitis
Hassani Besheli *et al* [71], 2017	Silk fibroin nanoparticles	The VANCO-loaded silk fibroin nanoparticles entrapped in scaffolds reduced bone infections at the defected site with better outcomes than the other treatment groups	With good biocompatibility and sustained release properties
Mahon *et al* [75], 2020	BMnP	In-house generated BMnP preferentially polarize human macrophages towards an M2 phenotype, activate the transcription factor cMaf and specifically enhance production of the anti-inflammatory cytokine, IL-10	Driving pro-angiogenic responses in human macrophages and HUVECs. Promoted M2 macrophage polarization, tissue vascularization and increased bone volume
Chen *et al* [58], 2021	Aptamer-functionalized platinum nanozymes	The activity switching and enhanced antibacterial effect of the nanocapsule were verified *in vitro* and in diabetic wounds	Catalyzing H_2O_2 into OH. Chemodynamic sterilization

Ca-Alg: Calcium-Alginate; BMnP: Bone mimetic nano hydroxyapatite particles; IL: Interleukin; HUVECs: Human umbilical vein endothelial cells; ROS: Reactive oxygen species; PPAA: Phosphorylating polyallylamine; MRSA: Methicillin-resistant *Staphylococcus aureus*.

patients are not identified by X-ray lesions as symptoms usually appear 10-14 d after infection. Without timely treatment for osteomyelitis, most cases progress to chronic osteomyelitis, making diagnosis and treatment complex and painful. Therefore, improving the accuracy of early imaging of osteomyelitis has become essential to managing this disease.

Superparamagnetic iron oxide nanoparticles (SPIONs)-ferumoxytol have been approved by the United States Food and Drug Administration for clinical application, and they can be absorbed in lymphatic tissue and bone marrow with < 20-nm lesions. In preliminary animal studies, Tsuda *et al*[42] and Fukuda *et al*[43] found that ferucarbotran was able to identify and diagnose bone metastases. Hence, they performed controlled clinical trials, in which patients with injection of SPIONs had significantly lowered signaling in bone metastases (-12.2%), osteomyelitis (-35%) or normal bone marrow (-46.6%). This indicates that SPIONs have the potential to differentiate bone metastases from osteomyelitis. Xiao *et al*[44] prepared uniform and bio-efficient IL-13-TAMRA-Gd3N@C80(OH)30-(CH2CH2-COOH)20 nanoparticles, which are a novel gadolinium cluster-coated metal-fullerenes (Gd3N@C80) obtained by coupling with IL-13 fragments. In a mouse model of chronic post-traumatic osteomyelitis, this novel nano-targeted probe specifically bound lipopolysaccharide to stimulate macrophages, which showed a high signal on the T1-weighted sequence of infection foci. This suggested that this novel targeted magnetic resonance imaging probe detected and distinguished CPO from sterile inflammation. Quantum dots (QDs) comprise a type of low-dimensional semiconductor material with photostability. They are a new fluorescent probe that can be used for biomolecular and cell imaging. Yousefi *et al*[45] used intermittent fluorescence emission (optical scintillation), electron density, and biocompatibility of QD nanoparticles. The combination of two different color QDs (red and green) can be used to distinguish osteomyelitis from Charcot neuroarthropathy.

Nanomaterials for sterilization

We have found that nanomaterials have many antibacterial properties and specific characteristics, and silver and silver nanoparticles (Ag-NPs) have been used as antibacterial agents. These nanoparticles exhibited antibacterial properties against various microorganisms (*P. aeruginosa*, *S. aureus* and *Vibrio cholerae*)[46]. The prevailing view is that, based on the microscopic properties of Ag-NPs, Ag^+ infiltrates the bacteria through the cell wall, causing the cell wall to rupture and increase cell permeability[47].

The respiratory chain in the intima is modified to produce reactive oxygen species (ROS) and free radicals that cause protein denaturation. The positively charged Ag^+ can bind to the negatively charged cell membrane[48]. Aurore *et al*[49] found that Ag-NPs enhanced the cytotoxicity of osteoclasts to MRSA, *P. aeruginosa*, and other microorganisms, and increased the reaction of free radicals to pathogenic microorganisms. Nandi *et al*[50] demonstrated that high-dose Ag-NPs had a good effect on the treatment of infection in an animal model of osteomyelitis but caused no toxicity to other significant organs. Wang *et al*[51] found that Ag-NPs inhibited biofilm formation of pathogenic microorganisms and reduced bacterial adhesion by regulating expression of bacterial genes (*ICAR* and *ICAA* of *S. epidermidis* and *fnbA* and *fnbB* of *S. aureus*). Secinti *et al*[52] confirmed that nanosilver ion coating inhibits biofilm formation on implants. Afzal *et al*[53] showed that addition of hydroxyapatite (HA) and carbon nanotubes (CNTs) to 5% Ag-NPs enhanced the bactericidal performance of these composites.

However, in mammalian cell models, the antimicrobial efficacy of Ag-NPs is largely due to the production of Ag ions, which are potentially cytotoxic to both target bacterial cells and normal host cells [54], posing a serious safety threat to practical clinical applications. We are also looking for other unique materials that do not attack indiscriminately. Unlike silver, gold is inert and does not readily decompose into ions to produce cytotoxicity, but it has been observed in mammalian models with high biocompatibility and low biotoxicity. The biological activity of Au-NPs has been studied for a long time. Due to the characteristics of stability and high biocompatibility, Au-NPs have become the optimal choice for nanocarriers[55]. When Au-NPs attach to the surface of the microbial cell wall, due to their inherent antibacterial properties, both bacteria and nanoparticles combine to produce physical and chemical surface modifications and produce ROS, which cause bacterial protein degeneration, DNA damage, mitochondrial dysfunction, and ultimately cell death[56]. Zheng *et al*[57] evaluated the effects of gold-4-amino-6-hydroxy-2-mercaptopyrimidine (Au-DAMP) on biofilm formation and maturation in mice infected with MRSA. The results showed that Au-NPs had high anti-biofilm activity, and the formation of MRSA biofilm was inhibited significantly even at a low concentration of Au-NPs. Unlike other precious metal nanomaterials, Au-DAMP can eliminate MRSA mature biofilms at low concentrations; a property not possessed by the most common antibacterial agents. Chen *et al*[58] prepared an activated nanocase with chemodynamic therapy. This new platinum nanocase and glucose oxidase were encapsulated in an HA shell and termed APGH nanocapsules (Figure 3). *In vitro* experiments demonstrated that the novel nanocapsule reduced the pH and H_2O_2 constraints. The nanocapsule retained peroxidase-like activity, making it suitable for antibacterial treatment and accelerating healing in diabetic wound models.

In addition to chemodynamic therapy, photodynamic therapy (PDT) nanomaterials have been widely used in diagnosing and treating tumors, but relevant studies have assessed the antibacterial effect of PDT. Kuo *et al*[59] performed water-soluble C60 (OH) 30 on Gram-positive MRSA to prove that nanomaterial photochemokinetics also effectuate sterilization. In this study, ROS were produced using water-soluble fullerenol, and PDT killed most Gram-positive bacteria within a short period. Faced with the challenge of the increase in drug-resistant bacteria worldwide, scientists are looking for ways to replace or enhance the sterilization effect of antibiotics. Some scientists have started innovative research on the mechanism of action of magnetic nanomaterials on bacteria. The hyperthermic effect induced by low-intensity magnetic fields destroyed the bacterial biofilm and promoted absorption of antibiotics [60]. Hyperthermia has been evaluated in clinical trials for prostate cancer and glioma with remarkable results and no severe side effects[61,62]. By embedding the heating source into the tissue, magnetic particle hyperthermia uses an external alternating magnetic field to increase the accuracy and reduce the occurrence of damage to the surrounding normal cells. Magnetic particle hyperthermia improves the accuracy of heating. Fang *et al*[60] used magnetic nanoprecision-induced hyperthermia to destroy the bacterial biofilm of infected lesions, thus improving the sterilization effect of antibiotics. Fe_3O_4 nanoparticles implanted into a rat model of osteomyelitis were heated to 75 °C by magnetic heating, which did not cause tissue loss but could destroy the biofilm polysaccharide matrix to enhance the permeability of the antibiotics.

A previous study discovered carbon-based nanomaterials, such as graphene and CNTs[63]. Because of its unique structural characteristics and physical and chemical properties, carbon-based nanomaterials have high antibacterial activity. Embedded in the phospholipid bilayer, the phospholipid membrane structure and phospholipid molecular configuration are disturbed, which directly or indirectly achieve a bactericidal effect or oxidize bacterial molecules, such as lipids and proteins, through the generation of oxidative stress *via* ROS[64].

Nanomaterials for drug delivery

The main goal of our nanotechnology application is to load the treatment unit into the nanocarrier and transport the therapeutic drugs to a lesion without leakage, which increases the local concentration of the drug. Eventually, the disease-causing organism is destroyed because the carrier acts like a precision-guided missile to deliver a precise strike to the target. The standard treatment for osteomyelitis consists of thorough debridement of the infected bone-removal of dead bone and elimination of dead cavities-followed by long-term systemic antibiotic therapy based on bacterial culture results[65]. The major challenge to this method is how to keep the normal bone tissue while maintaining complete debridement to avoid nonunion, limb dysfunction, and pathological fracture. Furthermore, long-term

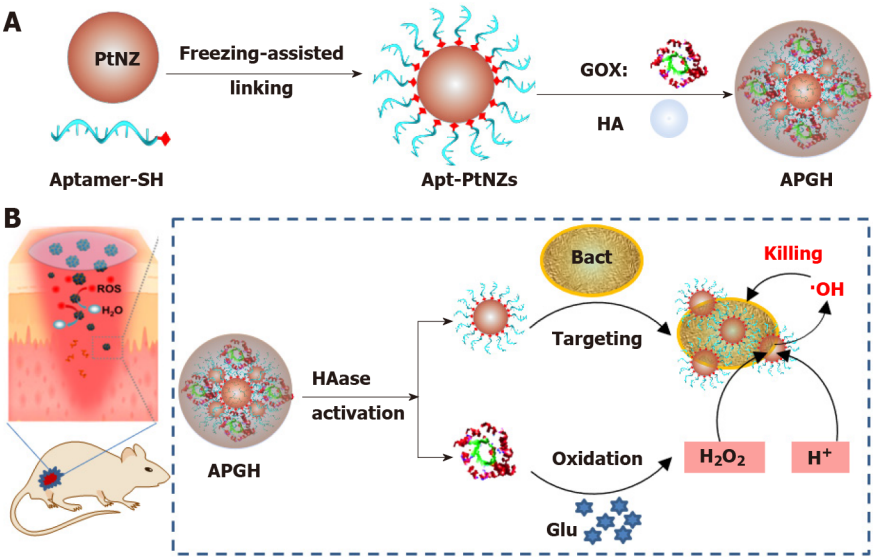

Figure 3 The illustration of APGH released the Aptamer-functionalized platinum nanozymes and glucose oxidase and its application for chemodynamic sterilization. A: The preparation route for the nanozyme capsule (APGH) with aptamer-functionalized platinum nanozymes, glucose oxidase and hyaluronic acid; B: Schematic illustration of APGH activation, activity switching in the infected wound, and its application for chemodynamic sterilization through *in situ* generation of COH on bacteria surface. Apt-PtNZ: Aptamer-functionalized platinum nanozymes; HA: Hyaluronic acid; GOX: Glucose oxidase. Citation: Chen L, Xing S, Lei Y, Chen Q, Zou Z, Quan K, Qing Z, Liu J, Yang R. A Glucose-Powered Activatable Nanozyme Breaking pH and H_2O_2 Limitations for Treating Diabetic Infections. *Angew Chem Int Ed Engl* 2021; 60: 23534-23539. Copyright ©The Author(s) 2021. Published by John Wiley and Sons. The authors have obtained the permission for figure using from the John Wiley and Sons and Copyright Clearance Center (Supplementary material).

systemic use of large doses of antibiotics can easily lead to antibiotic resistance and side effects, such as rashes, abdominal pain, and other gastrointestinal reactions. However, it is difficult for antibiotics to reach the osteomyelitic focus, resulting in insufficient local drug concentration and delayed healing.

Hence, we altered the treatment approach by filling the locally infected site with polymethyl methacrylate (PMMA) bone cement and microbeads loaded with antibiotics. The advantage of this approach is that it facilitates the delivery of a high concentration of the drug to the local infection site while reducing systemic toxicity. However, the PMMA bone cement delivery system also has some deficiencies in the treatment of osteomyelitis: (1) Heat generated during the preparation of PMMA implants affects the activity of loaded drugs; (2) PMMA is expensive; (3) PMMA bone cement cannot be absorbed in the body, requiring a second operation forits removal; (4) PMMA stents cannot achieve sustained drug release, and the drug release kinetics are poor; and (5) After the release of the loaded antibiotics, the remaining PMMA scaffold may become a site for bacterial determination[66]. Recent animal experiments and clinical applications show that nanoparticles have potential compared with existing drugs because of the size of nanoparticles that can enter pathogenic microorganisms or bone cells. Currently, we are exploring ways to target the drugs into specific bone sites, reducing costs, and improving compliance by maximizing controlled drug release such that the required drug concentration can be maintained over an extended period without exceeding toxicity levels or dropping below the minimum effective dose. Nanoparticles can deliver drugs through two mechanisms: (1) Nanoparticles bind to the microbial cell wall/membrane or osteoblast cell wall/membrane to release drugs into the cytoplasm; and (2) Nanoparticles can attach to the cell walls and act as drug repositories to continuously release the drug, which can then spread into the cell[67].

Currently, a variety of nanomaterials suitable for drug delivery for the precise treatment of osteomyelitis are being developed. Gowri *et al*[68] prepared calcium alginate (Ca-Alg) nanoparticles by salting out and loading clindamycin after crosslinking and phosphorylating polyallylamine (PPAA). The *in vitro* drug release experiments showed that the Ca-Alg/PPAA system had a sustained-release effect. The results of trypan blue colorimetry and MTT cytotoxic colorimetry showed that the system had good biocompatibility with osteoblasts (MG63). The Ca-Alg/PPAA/clindamycin system can control the synthesis of microbial flagella, affect the growth rate of cells, and reduce the viability of cell colonies by inhibiting viability. Krishnan *et al*[69] produced vancomycin-supported nanocomposite fiber Scaffidi (silica-coated nano-HA gelatin matrix). The porous structure (average pore size 150-350 μm) provides a large surface area and absorbs a large volume of antibiotics. In male Wistar rats (250-350 g), vancomycin release of all composite scaffolds was 10-20-fold higher than the minimum inhibitory concentration within 30 d. Anagar diffusion test, turbidity test, and bacterial adhesion test showed an excellent antibacterial effect, which could remove bacteria and promote the formation of new bone in 3 mo after implantation in a bone marrow disease model. Zhou *et al*[70] showed that mesoporous silica nanoparticle gelatin matrix composite fiber scaffold loaded with vancomycin promoted bone healing and significantly reduced bacterial contamination. Hassani Besheli *et al*[71] demonstrated that a silk

fibroin nanoparticle delivery system had strong antibacterial effect, targeted delivery, and continuous release of drug activity in a model of severe osteomyelitis induced by MRSA injection into the tibia of male Wistar rats (260-330 g). Wan *et al*[72] prepared polyvinyl alcohol/polycaprolactone (PCL) nanocomposite film and combined it with cefuroxime sodium. They measured the drug release from the nanocomposite material by spectrophotometry and concluded that the system was simple to prepare and had good biocompatibility. The stable and slow release of the drug may cause reversible absorption of the drug in PCL and forma hydrophobic layer.

Nanomaterials for treatment of chronic osteomyelitis

According to the clinicopathological mechanism of osteomyelitis, we considered the selection of nanomaterials for treatment after the formation of dead bone. The severe infection environment reduces the self-repair ability of bone tissue and delays bone regeneration[73]. A large area of the bone defect is often formed in the bone marrow cavity. There may be nonunion, pathological fracture, and other severe complications in the absence of intervention. Presently, autologous bone transplantation or artificial bone is used for clinical intervention; however, this scheme cannot inhibit bacterial growth and has a prolonged prognosis. Therefore, it is crucial to find materials that can promote proliferation of osteoblasts, inhibit osteoclast activity, and serve as vectors for antibiotics. This approach ensures antimicrobial efficacy, thereby minimizing the risk of antimicrobial resistance and infection recurrence, and stimulates osteoblast differentiation and proliferation, thereby promoting the formation of healthy bone tissue.

Jiang *et al*[74] divided 45 rabbits with chronic osteomyelitis into experimental, control, and blank groups, and evaluated them by X-ray, biopsy, and microbial culture. Vancomycin-loaded nano-HA pellets controlled the infection and repaired bone defects caused by MRSA-associated osteomyelitis. No significant or isolated nano-HA was observed in the experimental group 3 wk after implantation. The particles were involved in the formation of bone trabeculae or were replaced by medullary luminal tissue, and new bone was formed around the implanted particles. After 6 wk, the bone in the experimental group returned to normal and the periosteal reaction was weakened. Mahon *et al*[75] studied how nano-hydroxyphospholime promoted bone regeneration. They demonstrated that bone mimetic nano hydroxyapatite particles (BMnPs) preferentially polarized the M2 phenotype of human macrophages and specifically enhanced the production of the anti-inflammatory cytokine IL-10. The secretion of BMnP-treated macrophages promoted mesenchymal stem cell osteogenesis in an IL-10-dependent manner, suggesting that BMnPs directly promoted the osteogenic effect. In addition, BMnP-treated rats had significantly increased bone volume and stimulated expression of osteogenic genes, bone morphogenetic protein 2 (BMP2) and alkaline phosphatase (ALP), suggesting that BMnPs promote bone regeneration. IL-10 promotes chondrocyte differentiation and proliferation through the BMP pathway, and recombinant IL-10 promotes the expression of BMP-2, alkaline phosphatase, and osteopontin. Westhauser *et al*[76] observed that zinc-loaded mesoporous bioactive glass nanoparticles (5Zn-MBGNs) promoted osteogenic differentiation and expression of genes related to the extracellular matrix (ECM), and significantly promoted the formation and calcification of ECM, suggesting an excellent osteogenic effect. These genes also increased ALP activity, promoted DNA synthesis, and significantly increased calcium deposition. 5Zn-MBGNs IDPS upregulated expression of *OCN* and *COL1A1* genes, which significantly promoted ECM formation and calcification. The novel chitosan nanohybrid hydrogel and scaffold materials prepared by Mahanta *et al*[77] were highly porous, open, and three-dimensionally interconnected. In a rat femoral defect model, the bone healing rate of the nanohybrid scaffold was faster compared with that of the pure chitosan scaffold, while the cell growth rate of the nanohybrid scaffold was faster, and the cell proliferation was rapid.

BMP-2 is the most widely studied BMP, with the strongest activity in inducing endogenous bone formation, and it exists as a 30-kDa molecule in the form of a dimer[78]. Recombinant human (rh)BMP-2 has been expressed by gene recombination technology; however, the osteogenic activity of rhBMP-2 is lower than that of natural BMP-2, and the ideal vector has not been identified. Therefore, the search for a new nanomaterial containing BMP-2 has become a new direction for treatment of bone defect caused by osteomyelitis. Qiu *et al*[79] preloaded BMP-2 onto mesoporous HANPs and synthesized it into silk fibroin/chitosan composite. The results showed that scaffolds loaded with BMP-2 had better cell adhesion, provided an ideal microenvironment for cell proliferation, and significantly increased bone formation. SCH scaffolds containing BMP-2 had more material absorption and bone trabecular deposition than non-BMP-2 scaffolds had. The mesoporous HANPs maintained continuous release of BMP-2. This mode of delivery preserves the bioactivity of BMP-2. Therefore, the osteogenic effect of the scaffold can be improved. Qiao *et al*[80] reported a microwave-excited antibacterial nanocapturer system for treating deep tissue infections that consisted of microwave-responsive Fe_3O_4/CNT and gentamicin (Figure 4). They suggested that Fe_3O_4/CNT/gentamicin efficiently targeted and eradicated MRSA-infected rabbit tibia osteomyelitis.

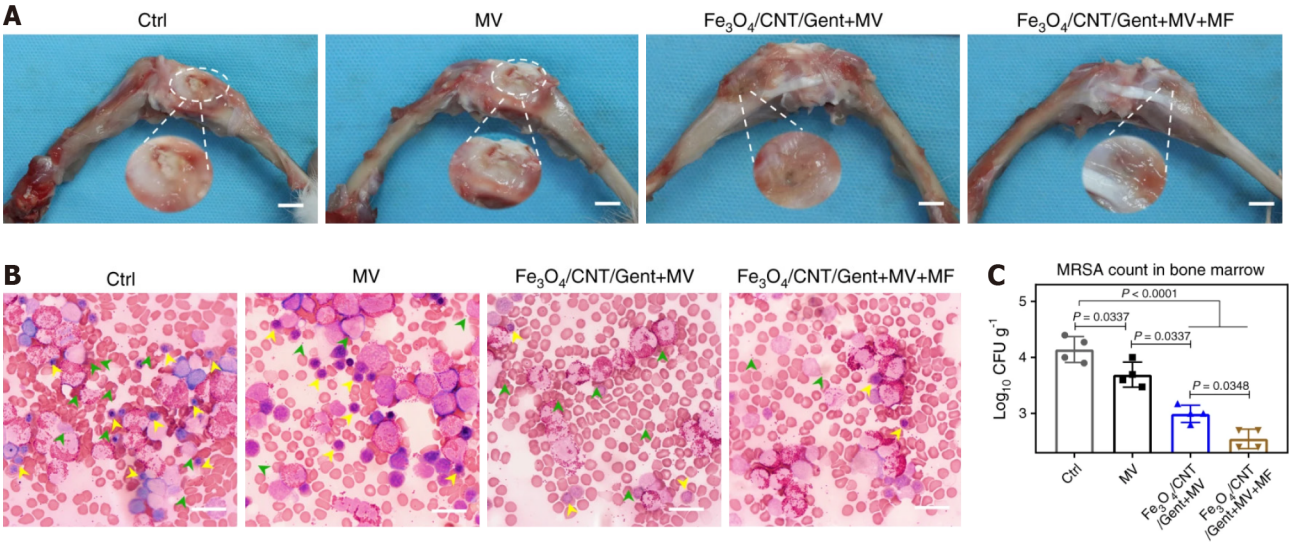

Figure 4 Antibacterial effects of Fe_3O_4/carbon nanotube/Gent on methicillin-resistant *Staphylococcus aureus*-infected osteomyelitis *in vivo*. A: Macro images of femur and tibia in each group of animal models 14 d after treatment. Scale bars = 1 cm; B: Wet-stained images, scale bars = 20 μm, 14 d after treatment; C: Methicillin-resistant *Staphylococcus aureus* count in bone marrow of each group after 2 d of antibacterial treatment. CNT: Carbon nanotubes. Citation: Qiao Y, Liu X, Li B, Han Y, Zheng Y, Yeung KWK, Li C, Cui Z, Liang Y, Li Z, Zhu S, Wang X, Wu S. Treatment of MRSA-infected osteomyelitis using bacterial capturing, magnetically targeted composites with microwave-assisted bacterial killing. *Nat Commun* 2020; 11: 4446. MRSA: Methicillin-resistant *Staphylococcus aureus*.

CONCLUSION

Continuous research and development of nanomaterial preparations have enhanced the therapeutic effect of nanomaterials on infection, including improved treatment of osteomyelitis. Our goal is to perfect the treatment of chronic osteomyelitis by using a variety of new nanomaterials, which cannot be reached by either common antibiotics or sustained-releasing antibiotics. Because of the material properties, nanomaterials have antibacterial activity with as low-releasing and bone regeneration promoting ability. Recent research has been limited to animal experiments and has not been applied to clinical treatment. Thus, we need to understand the toxic effects and metabolism of nanoparticles, their properties, and the cost of preparing these materials to consider whether nanomaterials are suitable for treating chronic osteomyelitis. These tasks require a multidisciplinary collaboration.

FOOTNOTES

Author contributions: Zeng M and Xu Z contribute equally to this study, they share co-first author; Xu Z wrote the paper; Zeng M and Li J did the literature review; Tang ZW and Song ZQ did the data analysis; Xiao S conceived and coordinated the study; Wen J revised the paper; and all authors reviewed the results and approved the final version of the manuscript.

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MINIREVIEWS

Fungal arthritis: A challenging clinical entity

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Abstract

There has been an increasing incidence of fungal infections in recent years. Rarely joints are also affected by fungal infections. Mainly, these infections develop in prosthetic joints, but sometimes native joints are also involved. *Candida* infections are mostly reported, but patients may also develop infections secondary to non-*Candida* fungi, especially *Aspergillus*. Diagnosis and management of these infections is challenging and may involve multiple surgical interventions and prolonged antifungal therapy. Despite this, these infections are associated with high morbidity and mortality. This review described the clinical features, risk factors, and therapeutic interventions required to manage fungal arthritis.

Key Words: *Aspergillus*; *Candida*; Fungal arthritis; Invasive fungal infections; Osteomyelitis

Core Tip: Fungal arthritis and osteomyelitis are rare diseases, but their incidence is increasing with the rising prevalence of predisposing factors. Most infections are secondary to *Candida spp.*, especially *Candida albicans*, but patients rarely develop infections secondary to other fungi, including *Aspergillus*, *Histoplasma*, *Cryptococcus* and *Coccidioides*. Fungal biomarkers may aid in rapid diagnosis in high-risk patients, but definitive diagnosis requires bone or synovial culture or biopsy. Surgical intervention and prolonged antifungal therapy form the mainstay of therapy, with azoles and echinocandins providing a safe and effective therapeutic option.

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INTRODUCTION

Fungal septic arthritis is a rare but severe and sometimes even life-threatening infection. It requires long-term medical and, in most cases, surgical management. During the past few years, there has been a dramatic surge in invasive fungal infections (IFIs). This is majorly attributable to the rise in the number of immunocompromised patients, including those on immunosuppression or broad-spectrum antibiotics, neutropenia, indwelling prosthesis, Human immunodeficiency virus (HIV), diabetes mellitus, burns, and long-term parenteral alimentation. A marked improvement in diagnostic techniques, including molecular methods, has also contributed to the early and rapid detection of these infections[1-3]. The course of bone and joint fungal infection can vary from indolent to highly aggressive. Spread is usually hematogenous (due to the high vascularity of synovial tissue), direct extension from a nearby infective focus or direct inoculation[4]. An indolent infection may be challenging to diagnose due to a lack of systemic inflammatory response and the absence of typical imaging features. However, the absence of a periosteal reaction and new bone formation at the site of osteomyelitis may indicate fungal etiology[5]. Therefore, diagnosing such cases may be highly dependent on a thorough history and physical examination. The clinical course and outcomes vary depending on the specific fungal species and the host factors.

PATHOPHYSIOLOGY

Hematogenous dissemination is the most frequent route of spread for IFIs. The other common route is direct inoculation from an exogenous source, such as surgery, prosthetic implantation, intra-articular corticosteroid injection, arthrocentesis, trauma, and open fractures[4,6]. The most common presenting complaint is localized pain followed by signs of local inflammation (swelling, erythema, and effusion), fever, and decreased range of motion. The large weight-bearing joints such as knees are most frequently affected[7]. However, there are no distinct clinical clues that may help differentiate fungal from bacterial arthritis. Hence, a detailed histopathological diagnosis is needed to elucidate a definitive pathogen in cases of high suspicion. In some cases, lytic lesions, cortical erosions, or adjacent osteoporosis and osteomyelitis may be seen on imaging scans. Findings of necrotizing granulomas on pathological examination make tubercular arthritis a close differential diagnosis (Table 1)[5,7].

DIAGNOSIS

Diagnosis of fungal arthritis can be quite challenging because of insidious disease onset with slow progression and lack of characteristic findings. The typical stains and smears used for fungal identification, such as potassium hydroxide or gram stain, may fail to identify the organism, and routine cultures are often non-diagnostic. The routine biochemistry and synovial fluid leukocyte count resemble a picture suggestive of non-infectious arthritis[7]. The demonstration of fungi on synovial, bone, or tissue culture and biopsy can be more indicative of an actual infection. However, the biggest limitation is the time taken to grow, especially in filamentous fungi cases, which potentially delays the treatment [3,8]. The fungal cultures are plated on Sabouraud dextrose agar at 24 °C-25 °C and should not be reported negative for growth until 4 wk after incubation[8].

As a consequence of difficult diagnosis and rising uncertainty of the classical phenotypic methods of fungal identification, there is now an increased focus on the use of molecular methods and antigen detection methods as surrogates for histopathology and cultures. The non-culture-based serological testing techniques that detect antibodies include enzyme immunoassay, immunodiffusion, and complement fixation. Among the molecular diagnostic tests, only a few tests, such as Film Array Blood Culture Identification (BioFire Diagnostics, Inc.), which is a PCR-based test, has been approved by the Food and Drug Administration. The matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) assay is a new test rapidly gaining popularity. It is a non-nucleic acid sequence-based molecular diagnostic assay for fungi, especially filamentous fungi such as *Aspergillus spp.*, and its strength lies in speed and accuracy[9].

There is an increasing interest in the use of biomarkers for diagnosing IFIs. β-D-glucan (BDG) is a non-specific fungal marker that may be positive in many fungal infections like *Candida*, *Aspergillus*, *Pneumocystis jirovecii*, and *Fusarium*. However, BDG may be negative in patients with *Cryptococcus*, *Blastomyces* (yeast form), or Zygomycetes (*Absidia*, *Mucor*, or *Rhizopus*) infection. Galactomannan is more

Table 1 Differential diagnosis for fungal arthritis

No.	Diagnosis
1	Bacterial arthritis
2	Tubercular arthritis
3	Sarcoidosis
4	Ewing's sarcoma
5	Osteogenic sarcoma
6	Langerhans cell histiocytosis
7	Malignant metastasis

specific to *Aspergillus* infections. These biomarkers have the advantage of rapid turn-around time and hence, if applied in high-risk patients with a moderate to high chance of IFI, may enable an early diagnosis. However, these biomarkers may also be falsely elevated in many patients, especially those on intravenous beta-lactam antibiotics (amoxicillin-clavulanate and piperacillin-tazobactam) and those on hemodialysis.

MANAGEMENT

Surgical

In cases of fungal native joint arthritis, a combined approach using medical and surgical therapy is typically desired for optimal results. Most cases of fungal osteomyelitis and septic arthritis need irrigation and surgical debridement. At the same time, tissue or fluid specimens can be collected for histopathological examination. Drainage and debridement are often unnecessary in the established diagnosis of cryptococcal infections.

The surgical process involves debridement of bony and soft tissues, removal of sinus tracts, and insertion of antifungal beads, if required[4]. Many cases of fungal vertebral osteomyelitis are associated with spinal instability and nerve root compression; hence spinal stabilization and arthrodesis are typically required. Early surgical interventions have been shown to prevent neurological injury in such patients[10].

Pharmacological management

Antifungal therapy, along with surgical intervention, forms the mainstay of therapy for fungal arthritis. Historically, the primary clinical experience treating invasive fungal arthritis has been with amphotericin B (Amp B), with or without flucytosine. However, recently azoles (fluconazole and extended-spectrum triazoles such as voriconazole, posaconazole, and isavuconazole) and echinocandins (caspofungin, micafungin, and anidulafungin) have been added to the list of available therapeutic options[11]. The recent clinical practice guideline for the management of osteoarticular candidiasis updated by the Infectious Diseases Society of America (IDSA) in 2016 recommends fluconazole and echinocandins as the first drugs of choice for *Candida* infections, given good efficacy and a better safety profile. Liposomal Amp B has been recommended as an alternative[12].

Azoles

The azole group blocks ergosterol synthesis in the cell membrane by inhibiting the enzyme lanosterol 14A demethylase and alters the functions of the membrane enzymes resulting in its dysfunction[13]. Fluconazole has good bone and tissue penetration, as indicated by the synovial fluid levels. Except for *Candida (C.) krusei*, *C. glabrata*, and some strains of *C. auris*, the majority of the *Candida* species are susceptible to fluconazole[14]. However, it has poor activity against *Aspergillus*. Voriconazole is the drug of choice for *Aspergillus* infections and has good bone penetration.

Polyenes

Amp B belongs to the polyene class of antifungal drugs that binds to ergosterol and results in the formation of pores, leakage of ions, and cell death. Additionally, Amp B also causes lipid peroxidation and oxidation reaction. The drug's newer lipid formulations are less nephrotoxic than conventional Amp B deoxycholate and have a better tolerability and toxicity profile. However, because of the better safety profile of azoles and echinocandins, it is typically reserved for invasive and resistant fungal infections. The toxicity profile of Amp B is directly related to its affinity and interaction with cholesterol. The most common adverse effects include infusion-related reactions (fever, chills, malaise, and hypotension), hypokalemia, hypomagnesemia, thrombocytopenia, leucopenia, normochromic, and

normocytic anemia with long-term use. Renal toxicity is dose-related, and renal failure is generally reversible after discontinuation of the drug. Adequate hydration and avoiding concomitant use of other nephrotoxic drugs may decrease the incidence of nephrotoxicity. The infusion-related toxicity can be reduced by administering a slow infusion over an extended period[4,15].

Echinocandins

Echinocandins inhibit the synthesis of 1,3 β-glucan, a major polysaccharide component of the cell wall and cause osmotic instability. This class of antifungals has a better tolerance profile with fewer adverse effects and ease of dosing in renal and liver dysfunctions. Echinocandins are active against most triazole-resistant pathogens and demonstrate fungicidal activity towards *Candida* species and fungistatic against mold (*Aspergillus* species)[16]. They are relatively less active against *C. guilliermondii* and *C. parapsilosis* and lack any activity against *Cryptococcus neoformans*, Zygomycetes and *Trichosporon* species due to the absence of β-glucan in the cell walls of these organisms[17]. Echinocandins are most effective against the biofilm formed by certain fungal species like *C. albicans* on retention hardware and medical devices. Studies have demonstrated the effectiveness of caspofungin and micafungin in killing preformed *Candida*-related biofilms at concentrations achievable *in vivo*[18].

Pyrimidine analogue

Flucytosine is an antimetabolite agent, approved by the Food and Drug Administration in 1971, for treating invasive cryptococcal and *Candida* infections. The drug carries a high risk of resistance to its use and is almost always used in combination with other antifungals, such as Amp B, for systemic and IFIs. It can cause acute hepatitis and severe hematological adverse effects, including pancytopenia, agranulocytosis, aplastic anemia, and bone marrow suppression[19].

COMMON PATHOGENS

Several different fungi may cause arthritis with varying clinical presentations. It is pertinent to make a pathological diagnosis as therapeutic options, the duration of therapy, and patient prognosis vary. Many risk factors have been identified for infection with different fungi, which may aid in raising suspicion and enabling early diagnosis (Table 2)[20,21].

Candida species

Candida species are the most common cause of fungal arthritis and osteomyelitis, especially in immunocompromised patients. An episode of candidemia, despite adequate antifungal treatment, may cause invasive joint and bone disease even after a long latent period of several months and sometimes even years[22]. *C. albicans* is the most commonly isolated species. *Candida* osteomyelitis often involves vertebrae, sternum, femur, humerus, and tibia. It usually starts as synovitis, eventually involving the adjacent bone causing osteomyelitis[4,5,23]. *Candida* arthritis, however, targets large-weight joints such as the knee, hip, shoulder, and ankle[20]. Joints affected by rheumatoid arthritis are predisposed to *Candida* infection. Infection can occur during aspiration, intra-articular steroid injection, or arthrotomy [11].

Surgical findings are cartilage erosion, thickening and hyperemia of the synovium with fibrosis, scarring, and purulence. Radiographically, *Candida* osteomyelitis of the spine shares certain findings with bacterial infections such as disc space narrowing, end plate destruction of adjacent vertebral bodies, and demineralization and mottled trabecular pattern in cases of long bone infections[5,24].

Treatment of *Candida* joint disease includes antifungal therapy and surgical debridement. Fluconazole remains the mainstay of medical management as most *C. albicans* are still susceptible. For septic arthritis, it is a 6-wk course of treatment with fluconazole or an echinocandin for 2 wk, followed by oral fluconazole for at least 4 wk. Lipid formulation of Amp B is another alternative, with therapy for 2 wk before transitioning to fluconazole. For osteomyelitis, a longer course of 6 mo of fluconazole is generally required. Alternatively, an echinocandin or liposomal Amp B may be used for 2 wk, followed by 6-12 mo of fluconazole, depending upon the clinical and radiographical improvement (Table 3). Surgical drainage is indicated in all cases of septic arthritis. Removal is recommended in cases of infected prosthetic joints[12].

Aspergillus species

Aspergillus spp. are ubiquitous saprophytes, and the pathogenic species are *Aspergillus (A.) fumigatus*, *A. niger*, *A. flavus*, and *A. terreus*. Infection most commonly occurs through inhalation of spores or hematogenous spread from a primary pulmonary focus. Extension of infection may involve maxillofacial structures, mastoids, sphenoid bones, or basilar skull, but most cases of *Aspergillus* osteomyelitis involve the vertebrae[25]. The lumbar spine is the most frequently involved site in cases of hematogenous spread[5].

The most common presentation is fever, pain, tenderness, and swelling of the joint. Cases of head and neck infections may present with headache, conjunctivitis, periorbital cellulitis, proptosis, and epistaxis. Radiologically, vertebral aspergillosis may be challenging to distinguish from tuberculosis. Cultures of

Table 2 Risk factors for specific fungal infections

Fungal infections	Risk factors
Candida spp.	Immunosuppression
	Chemotherapy
	Recent surgery
	Uncontrolled diabetes
	Corticosteroids
	Broad spectrum antibiotics
	Intravenous drug abuse
	Indwelling central venous catheters
	Hemodialysis
	Multiple site colonization
Aspergillus spp.	Neutropenia
	Chronic granulomatous disease
	Post-organ transplant
Coccidioides immitis	Uncontrolled diabetes
	Immunosuppression
	Advanced age
	HIV
Histoplasma capsulatum	HIV
	Advanced age
	Post-organ transplant
	Corticosteroids
	Immunosuppression

HIV: Human immunodeficiency virus.

biopsy specimens or synovial fluid growing the characteristic acutely branching hyphae are usually diagnostic of *Aspergillus*[26]. Identification tests for *Aspergillus* also include serum beta-d-glucan, galactomannan antigen test, and PCR testing. Serum assays for BDG are not specific to *Aspergillus*. Galactomannan antigen test has prognostic value with a serial decline in the serum levels indicating an effective antifungal treatment[27].

Triazoles and Amp B have long been used to treat invasive aspergillosis. Voriconazole is recommended as the drug of choice in managing invasive aspergillosis as it has a broad antifungal spectrum of activity and lesser nephrotoxicity than amphotericin[28]. Antifungal treatment is recommended for 6 wk to 8 wk, along with surgical debridement. Surgical debridement of the infected material helps with reducing the infective burden and allowing better drug penetration[13].

Coccidioides species

Coccidioides species are dimorphic fungi found predominantly in regions of Mexico and central, southern, and southwestern regions of the United States. Extrapulmonary dissemination of the disease is rare, but polyarticular arthritis may occur during primary infection with rash, fever, eosinophilia, and bilateral hilar lymphadenopathy as hypersensitivity syndrome[29]. Although rare, hematogenous dissemination may result in septic arthritis, and weight-bearing joints, like the knee, are at greater risk of involvement. On standard laboratory tests, raised erythrocyte sedimentation rate, C-reactive protein, peripheral eosinophilia, and raised *Coccidioides* complement fixation titer may be seen[4].

Radiological findings include joint space narrowing with effusion, osteopenia, and lytic lesions with bone destruction. Histopathology may show villonodular synovitis, pannus and sinus tract formation, non-necrotizing granulomas, and spherules containing endospores. It is a septic arthritis demonstrating synovial proliferation rather than synovial fluid accumulation[30]. The main antifungal drugs are oral azoles (fluconazole and itraconazole), which have largely replaced the role of Amp B, except in immunocompromised patients and disseminated coccidioidomycosis[31].

Table 3 Therapeutic options for fungal arthritis

Antifungal drugs	Indication/pathogen	Dosage
Fluconazole	Candidiasis	400 mg (6 mg/kg) daily (IV/PO)
	Histoplasmosis	
	Blastomycosis	
	Coccidioides	
	Cryptococcus	
Voriconazole	*Aspergillus*	4-6 mg/kg BD; 200 mg BD (IV/PO)
	Candidiasis	
	Coccidioides	
	Blastomycosis	
	Cryptococcus	
	Histoplasmosis	
Itraconazole	*Aspergillus*	100-400 mg/d (PO)
	Sporothrix	
	Candidiasis	
	Coccidioides	
	Histoplasmosis	
	Cryptococcus	
	Blastomycosis	
Ketoconazole	Blastomycosis (mild to moderate cases)	200-400 mg OD (PO)
	Coccidioides	
	Cryptococcus	
	Histoplasmosis	
Liposomal amphotericin B	*Candida species* (except *C. lusitaniae*)	3-5 mg/kg/d (IV)
	Cryptococcus	
	Aspergillus	
	Coccidioides	
	Histoplasmosis	
	Sporothrix	
Echinocandins: Caspofungin, anidulafungin, micafungin	Candidiasis (fungicidal)	Caspofungin: 70 mg on day 1 followed by 50 mg OD (IV); anidulafungin: 200 mg on day 1 followed by 100 mg OD (IV); micafungin: 100 mg OD (IV)
	Aspergillus (fungistatic)	
5-Flucytosine	*Cryptococcus*	100 mg/kg/d divided q 6 h (PO)
	Candida	

IV: Intravenous; PO: Per os (oral administration).

Cryptococcus species

Cryptococcus is a basidiomycete that primarily involves the lungs and central nervous system. The route of infection could be inhalational, hematogenous from infected lungs in cases of disseminated disease, or rarely a direct trauma causing arthritis. Septic arthritis usually results from the contiguous spread of infection from adjacent osteomyelitis[32]. *Cryptococcus neoformans* has a unique polysaccharide capsule that protects it against phagocytosis and opsonization in the host[4].

The stains that aid in diagnosing cryptococcosis include periodic acid Schiff stains and methenamine silver nitrate. The diagnostic and prognostic value of serum cryptococcal antigen in osteoarticular cases needs to be better defined[33]. Histopathologic examination of tissue or bone specimens may demonstrate granulomatous changes with extensive fibrosis and giant cells. Radiological findings elicit

discrete, well-defined lytic lesions, usually without sclerotic or periosteal change. In cases of vertebral osteomyelitis, the intervertebral space is spared despite contiguous vertebral body involvement[26]. In non-complicated single-site infections and the absence of any immunosuppressive risk factors, fluconazole 400 mg (6 mg/kg) OD for 6-12 mo is the antifungal treatment of choice. In case of disseminated disease (at least 2 non-contiguous sites involved or high levels of serum cryptococcal antigen titer ≥ 1:512), a combination of liposomal Amp B and flucytosine for 2 wk followed by fluconazole as maintenance therapy for 6-12 mo is recommended by IDSA 2010 guidelines[34].

Histoplasma species

Histoplasma (*H.*) *capsulatum* usually causes a self-limited respiratory flu-like illness. The spores of this fungus have a predilection for the reticuloendothelial system and can spread to regional lymph nodes. Patients with impaired cellular immunity, such as HIV AIDS infection, are predisposed to *Histoplasma* primary infection or reactivation disease. It usually causes aseptic arthritis as a spectrum of immunologically mediated diseases. Synovial fluid analysis is inflammatory and demonstrates mononuclear cell predominance. The joint involvement is symmetric but may be migratory, and the most common sites of involvement include the knee, ankle, and small joints of the hands and wrist. Other than osteoarticular disease, *H. capsulatum* may also cause tenosynovitis and carpal tunnel syndrome[4,26,35]. *H. capsulatum*, along with *Coccidioides immitis*, are the two fungi most implicated in immune complex arthritis. This leads to joint swelling secondary to synovial proliferation rather than fluid accumulation, often manifested by symmetrical joint involvement[7]. The treatment recommendation is liposomal Amp B for 12 wk before transitioning to oral itraconazole. For mild cases, oral itraconazole is recommended[35].

PROSTHETIC JOINT INFECTION

Prosthetic joint infection (PJI) is a known complication of joint arthroplasty procedures and occurs in 1%-2% of cases of prosthetic joint implantations[36]. Overall, *Staphylococcus* is the most common organism isolated in 50% of cases, and *C. albicans* is the most common cause of fungal PJI. Most cases are encountered after revision arthroplasty. The risk factors include diabetes, immunosuppressive therapy, malignancy, obesity, prior antibiotic use, multiple revision surgeries, and preceding bacterial PJI. The clinical presentation is usually similar to septic arthritis as pain, effusion, erythema, joint tenderness with raised levels of erythrocyte sedimentation rate, C-reactive protein, and possibly a positive aspirate culture in cases of deep PJI[4,36,37].

The management consists of two-stage revision arthroplasty, separated by 3-6 mo with a prolonged systemic antifungal treatment[37]. Even after two-stage revision arthroplasty there may be a significant recurrence rate of 20%[38]. Fungal infections are relatively more difficult to treat and require longer time intervals for reimplantation in order to reduce the risk of infection recurrence. The reimplantation is based on the patient's stability and surgical wound status. Some surgical centers also practice joint re-aspiration before proceeding with reimplantation[3,8]. As updated by IDSA in 2016, drug therapy for *Candida* PJI includes fluconazole, liposomal Amp B and echinocandins, and prosthetic device removal [12]. Echinocandins are a lucrative alternative as they have the potential to penetrate the biofilm with the advantage of better tolerance and safety profile and fewer drug interactions than most antifungals [39].

CURRENT CHALLENGES AND FUTURE DIRECTION

In the present era of evolving anti-fungal resistance, treating invasive fungal diseases poses an immense challenge. Due to limited bone penetration for most of the anti-fungal drugs and high relapse and recurrence rates, there is a need for prolonged duration of therapy resulting in increased financial burden and high drop-out rates. Experiments are now being conducted to search for newer potential targets and generate new antifungal combinations to overcome drug resistance. An example of such a new target is heat shock protein 90, which modulates fungal virulence and drug resistance through certain downstream effectors and proteins such as calcineurin. The calcineurin inhibitor analogues have been studied to possess antifungal properties and may hold a promising role in abrogating drug resistance. Similarly, pharmacological inhibition of heat shock protein 90 expression with geldanamycin has been shown to reduce resistance to azoles and echinocandins, including resistance that has already evolved in humans treated with these antifungal drugs[40]. Development of newer drugs like ibrexafungerp, a triterpenoid anti-fungal agent, which has advantages of having oral formulation, broad-spectrum activity, and efficacy against newer strains of *Candida* like *C. auris*, may help in managing difficult and resistant infections[41].

CONCLUSION

Fungal arthritis and osteomyelitis are rare but difficult to treat conditions. The incidence of fungal disease is increasing as predisposing factors are more prevalent in the general population. The treatment for fungal arthritis requires surgical intervention and a prolonged course of antifungal agents. The treatment targets preservation of joints, eradication of the infection, and protection from future recurrence.

FOOTNOTES

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Mechanism of spinal cord injury regeneration and the effect of human neural stem cells-secretome treatment in rat model

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Abstract

BACKGROUND

Globally, complete neurological recovery of spinal cord injury (SCI) is still less than 1%, and 90% experience permanent disability. The key issue is that a pharmacological neuroprotective-neuroregenerative agent and SCI regeneration mechanism have not been found. The secretomes of stem cell are an emerging neurotrophic agent, but the effect of human neural stem cells (HNSCs) secretome on SCI is still unclear.

AIM

To investigate the regeneration mechanism of SCI and neuroprotective-neuroregenerative effects of HNSCs-secretome on subacute SCI post-laminectomy in rats.

METHODS

An experimental study was conducted with 45 Rattus norvegicus, divided into 15 normal, 15 control (10 mL physiologic saline), and 15 treatment (30 µL HNSCs-secretome, intrathecal T10, three days post-traumatic). Locomotor function was evaluated weekly by blinded evaluators. Fifty-six days post-injury, specimens were collected, and spinal cord lesion, free radical oxidative stress (F2-Isoprostanes), nuclear factor-kappa B (NF-κB), matrix metallopeptidase 9 (MMP9), tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), B cell lymphoma-2 (Bcl-2), nestin, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF) were analyzed. The SCI regeneration mechanism was analyzed using partial least squares structural equation modeling (PLS SEM).

RESULTS

HNSCs-secretome significantly improved locomotor recovery according to Basso, Beattie, Bresnahan (BBB) scores and increased neurogenesis (nestin, BDNF, and GDNF), neuroangiogenesis (VEGF), anti-apoptotic (Bcl-2), anti-inflammatory (IL-10 and TGF-β), but decreased pro-inflammatory (NF-κB, MMP9, TNF-α), F2-Isoprostanes, and spinal cord lesion size. The SCI regeneration mechanism is valid by analyzed outer model, inner model, and hypothesis testing in PLS SEM, started with pro-inflammation followed by anti-inflammation, anti-apoptotic, neuroangiogenesis, neurogenesis, and locomotor function.

CONCLUSION

HNSCs-secretome as a potential neuroprotective-neuroregenerative agent for the treatment of SCI and uncover the SCI regeneration mechanism.

Key Words: Secretome; Regeneration mechanism; Spinal cord injury; Locomotor; Biomarkers

Core Tip: The human neural stem cell secretomes is effective in spinal cord injury (SCI) treatment, based on locomotor function improvement, decreased size of spinal cord lesions, and biomarkers expression. Based on partial least squares structural equation modeling analysis, the regeneration mechanism of SCI started with pro-inflammation, anti-inflammation, anti-apoptotic, neuroangiogenesis, neurogenesis, finally, locomotor improvement.

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INTRODUCTION

Spinal cord injury (SCI) can result in permanent neurologic deficits; complete SCI neurological recovery is still less than 1%, and 90% experience permanent disability[1]. Secondary damage is caused by oxidative stress, inflammation, ischemia, apoptosis, and glial scar formation[2]. It can result in axon regeneration failure, leading to neurological deterioration[2]. The SCI regeneration mechanism is still uncertain[3]. Pajer *et al*[4] stipulate that SCI pathophysiology can be divided into three overlapping stages: Acute, subacute, and chronic. The injury begins with trauma that results in microvascular damage in the form of bleeding, thrombosis, and vasospasm[5]. This microvascular damage causes the spinal cord to undergo hypoperfusion, hypoxia, and ischemia[6]. Ischemia in the spinal cord affects cellular and molecular inflammation processes, neuron and neuroglia cell apoptosis, and glial scar formation, which mechanically and chemically inhibit SCI regeneration[5,6].

SCI management is still controversial, as there is no global consensus guideline and no effective pharmacological neuroprotective-neuroregenerative agent[7,8]. Current SCI management is focused on treating the secondary injury[2]. The secretomes of stem cell help mitigate the risk of immune rejection, reduce the risk of tumorigenesis, and cryopreserve treatments while avoiding the issues of maintaining cell viability[9]. The secretomes of stem cell are more economical and readily available in emergency cases as they can be mass-produced[10].

The effect of human neural stem cells (HNSCs) secretome on SCI is still unclear. Consequently, this study aimed to investigate the SCI regeneration mechanism and HNSCs-secretome treatment effects on subacute SCI post-laminectomy by analyzing free radical oxidative stress (F2-Isoprostanes), nuclear factor-kappa B (NF-κB), matrix metalloproteinase (MMP)-9, tumor necrosis factor (TNF)-α, interleukin-10 (IL-10), transforming growth factor (TGF)-β, vascular endothelial growth factor (VEGF), B cell lymphoma (Bcl)-2, nestin, brain-derived neurotrophic factor (BDNF), gleal cell line neurotrophic factor (GDNF), spinal cord lesion, and locomotor function. For this purpose, we used a well-established Rattus norvegicus model of SCI contusion-compression.

MATERIALS AND METHODS

Ethics statement

The study protocol was reviewed and approved by the Faculty Dentistry, University of Jember (REC.1112/UN25.8/KEPK/DL/2021). All rats were approved by the animal health office (No.503/A.1/0005. B/35.09.325/2020).

Study design

The research was a proper experimental study. The Lemeshow formula counted the sample size (n = 15 rats), with correction factors of 20%. The rats were randomly grouped into the following three groups: Normal (15 experimental rats did not have SCI and did not get HNSCs-secretome), control (15 experimental rats did have SCI with physiologic saline), and treatment (15 experimental rats did have SCI with HNSCs-secretome) (Figure 1). The treatment group received a 30 μL HNSCs-secretome intrathecal injection in T10 three days after the SCI and laminectomy. Treatment and control groups were replicated 15 times, and we observed the study over 56 d. The study's independent variable was HNSCs-secretome treatment, whereas the dependent variables were GDNF, BDNF, nestin, Bcl-2, VEGF, TGF-β, IL-10, MMP9, F2-Isoprostanes, TNF-α, NF-κB, locomotor function, and spinal cord lesion size.

Preparation of the HNSCs-secretome

HNSCs-secretome is characterized by the presence of nerve cells as well as the nestin, BDNF, and growth associated protein-43. NSCs were derived from 2 × 5 × 10^6 adipose-mesenchymal stem cells (MSC) with fresh frozen nerve scaffolds under 5% hypoxic conditions. Secretom does not provide an immune compatibility effect, therefore in this study secretoms were used from humans, not from rats[3, 11]. HNSCs-secretome 50 cc produced on June 21, 2021 at the Stem Cell Installation and Network Bank of RS Dr. Soetomo No. 301/VAL/FORM/BJRS/10/2021, with ethical clearance No. 0059/KEPK/IX/2020.

Rats and SCI models

The adult male Rattus norvegicus pure strain Sprague Dawley rats were three to four months old and weighed 300-350 g. Inclusion criteria were male, age 3-4 mo, weight 300-350 g, pure line, and healthy with a healthy statement from a veterinary polyclinic. The exclusion criteria were experimental rats that had received immunomodulatory therapy and were fatally ill. Acclimatization was carried out for seven days by one laboratory assistant and two veterinarians. The rats were kept in separate cages, consisting of a plastic box with woven wire as a cover, with each cage (45 cm × 30 cm × 15 cm) containing one rat. The floor mat was covered with wood shavings and a pad underneath to absorb urine and retain moisture. Air conditioning provided comfort and maintained a room temperature of 20-24 ˚C and humidity of 50%-70%. An exhaust fan was used to remove the ammonia smell, and the environment was a quiet room with 12-h light and dark cycles. The light sources were 300 Lux electric lamps positioned 1 m from the floor. The cage was cleaned every three days with soap and running water. Feed comprised 30-35 g of pellets (10% of bodyweight) and 30-35 mL of mineral water (10% of bodyweight).

Contusion-compression of the spinal cord was performed with the commercially available spinal cord impactor aneurysm Yasargil clip, with a length of 7 mm and a 65 g load (equivalent to 150 k Dyne). The rats were anesthetized using ketamine (75 mg/kg) and acepromazine 3 mg/kg intraperitoneal. The rats were placed on a fixation board in a prone position, and the back fur was shaved to approximately 2 mm. The operating area was disinfected with 10% betadine and 75% alcohol. The surgical level was marked by tracing the level of the T12 rib to the T12 spinous process using a 2 cm skin incision. A T10-T11 partial laminectomy was conducted to expose the spinal cord. The tip of the titanium aneurysm Yasargil clip was placed at a 1-mm distance from the anterior and posterior of the spinal cord, and the spinal cord was impacted suddenly for 60 s by retracting the tip using an applicator. This retraction induced an SCI contusion-compression model with the dura appearing flat and cloudy white. The operating field was cleaned using saline, and the muscle and skin were sutured together in layers.

Three days post-injury, the treatment and control group rats were completely paraplegic. The control group was administered an intrathecal injection of 10 mL physiologic saline. The treatment group was administered an intrathecal injection of 30 μL HNSCs-secretome under general anesthesia, which was centered at the injury site and 1.5-2 mm deep from the dura to the subarachnoid space, with a tilt angle of 30°–40° using a 50 μL Hamilton Syringe. The rats received normal saline, tolfenamic acid 4 mg/kg, and enrofloxacin 10 mg/kg subcutane, were placed under a 5 W heating lamp. Manual bladder drainage was conducted twice daily until micturition was normal.

Locomotor assessment

The Basso, Beattie, Bresnahan (BBB) open-field test was performed on days one, three, seven, 14, 21, 28, 35, 42, 49, and 56 after injury to assess locomotor expression. The BBB measures the tail, body, legs, trunk stability, limb movement, and toe clearance, all of which are examined to measure locomotor abilities. The score shows a range of numbers between 0 and 21. A score of 0 indicates no movement,

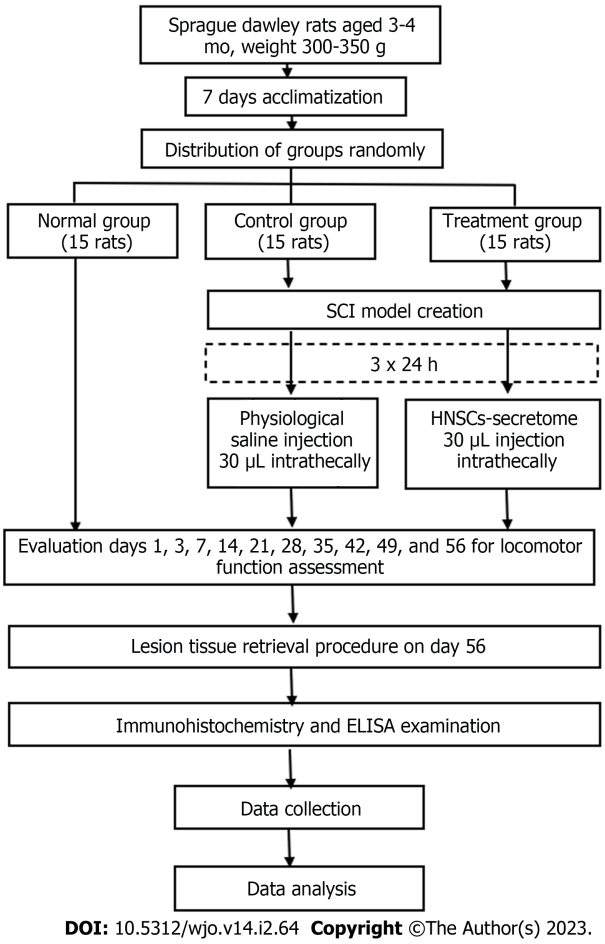

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Figure 1 Diagram of the animal model and grouping. SCI: Spinal cord injury; HNSCs: Human neural stem cells; ELISA: Enzyme-linked immunosorbent assay.

whereas a score of 21 represents normal movement without a locomotor disorder. The data collector and outcome adjudicator/data analyst were blinded.

Preparation of the tissue for the immunohistochemical and enzyme-linked immunosorbent assay assessment

The rats' termination was carried out on day 56 through the induction of inhalation anesthetics. The 5-cm SCI was separated from the vertebral column and marked at the cranial end. The SCI materials were put in a pot and fixed in a 10% formalin buffer. All SCI specimens were sent to the Anatomy and Pathophysiology Laboratory for enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry examination, tabulation of results, and analysis were conducted according to the blinding principle. The data collector and outcome adjudicator/data analyst were blinded.

Spinal cord lesion assessment

With hematoxylin and eosin staining, measurements of the spinal cord lesions were carried out in both the control and treatment groups, then analyzed with Statistical Package for Social Science (SPSS) software (Version 25, IBM) was used to analyze the differences between groups by non-parametric test, followed by Mann Whitney test. A *P*-value of more than 0.05 was considered statistically significant.

Immunohistochemical assessment

GDNF, BDNF, nestin, Bcl-2, VEGF, TGF-β, IL-10, MMP9, and NF-κB were evaluated using indirect immunohistochemical quantitative measurements. The data collector and outcome adjudicator/data analyst were blinded. Fifteen specimens of spinal cord tissue taken from animals in each group. In each group, we observed an average value of 10 fields of view, and every field of view had 625 μ^2 with 400 × magnification.

Immunohistochemical operational procedures were as follows: The specimens were immersed in the xylol solution for 3-5 min, then in absolute ethanol for 1-3 min, and finally in 70% ethanol for 1-3 min. They were then washed 3 times with Aquabidest, and the edge of the slide was cleaned with a tissue. They were then dropped with H_2O_2 3%, incubated at room temperature for 10 min, washed 3 times in

phosphate-buffered saline (PBS), and the edge of the slide was again cleaned with a tissue. They were then dropped with Trypsin 0.025%, and incubated at 37 °C for 6 min, washed 3 times in PBS, and the edge of the slide again cleaned with a tissue. Specimens were then dropped with Ultra V Block and incubated at room temperature for 5 min, with the edge of the slide cleaned again (no need to wash). They were then dropped with monoclonal antibody which has been diluted (1:100) and incubated at room temperature for 25-30 min, washed with PBS 3 times, and the edge of the slide cleaned with a tissue. Drops of biotin, incubation at room temperature for 10 min, washed with PBS 3 times, and the edge of the slide cleaned with a tissue. Specimens were then dropped with horseradish peroxidase polymer (streptavidin peroxidase conjugate), incubated at room temperature for 10 min, washed with PBS 3 times, and the edge of the slide cleaned with a tissue. They were then dropped with diaminobenzidine chromogen (20 mL/1 mL substrate), incubated at room temperature for 5-15 min in a dark room, washed Aquabidest 3 times, cleaned, painted with Meyer Hematoxylin at room temperature, then incubated for 6-15 min, washed in running water 3 times, and finally soaked in water for 10 min, drained, mounting, and microscopic readings were taken.

ELISA

The specimen for ELISA was collected from cardiac blood. The TNF-α analysis used serum, and F2-Isoprostanes used plasma for the analysis. The ELISA kit for the TNF-α analysis used the Sandwich-ELISA principle, while the ELISA kit for the F2-Isoprostanes analysis used the Competitive-ELISA principle. TNF-α and F2-Isoprostanes were evaluated using quantitative measurements.

Statistical methods

The data in this research is reported as the mean ± standard deviation of the mean. SPSS software (Version 25, IBM) was used to analyze the differences between groups by ANOVA, followed by Tukey's post-hoc test. A *P*-value of more than 0.05 was considered statistically significant.

Partial least squares structural equation modeling (PLS SEM) was used to analyze the pathway mechanism of SCI regeneration through analysis of the outer model, inner model, and hypothesis testing. The outer model measurement of PLS SEM is based on convergent validity, discriminant validity, and composite reliability. Convergent validity can be determined from the value of the loading factor and average variance extracted (AVE). If the loading factor value is more than 0.7, the correlation between indicator and variable is valid. If the AVE value more than 0.5, the ability of the variable value to represent the original data score is valid. Discriminant validity testing the construct indicator has a higher cross-loading value than other construct indicators, whereas composite reliability is used to measure the consistency of variables. If the composite reliability value is more than 0.7, it is declared valid.

Path bootstrapping analysis is a description of the inner model and the results of the path analysis hypothesis test, based on the original sample value and the statistical T value. If the statistical T value is greater than 1.9 (T table value) or the *P* value is less than 0.05, the direct effect of the latent variable/construct is stated to be significantly different. The relationship between latent variables in the inner model can be tested with R square (coefficient of determination on endogenous variables), path coefficients, F square (effect size), and Q square (prediction relevance).

RESULTS

The mechanism of SCI regeneration

The SCI regeneration mechanism was analyzed using PLS SEM. The test results of the measurement model (outer model) are valid, based on the PLS algorithm (Figure 2), the analysis of convergent validity is more than 0.7 (Table 1), the AVE value is more than 0.5 (Table 2), the Cronbach's Alpha value is more than 0.5 (Table 2), composite reliability is more than 0.7 (Table 2), and discriminant validity is good (Table 3).

The results of the analysis of the inner model (structural model) using bootstrapping and blindfolding PLS SEM procedures are valid and show the path coefficients that are in accordance with the hypothesized theory, significant with T-statistics greater than 1.9 (T-table) and *P* value less than 0.05 (Figure 3, Table 4). The relationship between latent variables in the inner model was analyzed, with F square (effect size) more than 0.05 (Table 5), Q square (prediction relevance) more than 0 (Table 6), and path coefficients positive. For R square (coefficient of determination on endogenous variables), the anti-inflammatory value of 0.860 indicates an effect of 86%, the anti-apoptotic value of 0.680 indicates an effect of 68%, neuroangiogenesis of 0.776 indicates an effect of 77%, neurogenesis of 0.444 indicates an influence of 44%, and locomotory of 0.536 indicates an influence of 53% (Table 7).

Locomotor by BBB score

The rats were examined for eight weeks to assess the recovery of their motor function. Locomotor recovery recorded on day seven and continued until day 56. The treatment group's mean BBB score was

Table 1 Loading factor value

	Anti apoptotic	Anti inflammatory	Lokomotorius	Neuroangiogenesis	Neurogenesis	Pro inflammatory
Anti apoptotic	1.000					
Anti inflammatory 1		0.978				
Anti inflammatory 2		0.978				
Lokomotorius			1.000			
Neurogenesis 1					0.932	
Neurogenesis 2					0.928	
Neurogenesis 3					0.892	
Neuroangiogenesi				1.000		
Pro inflammatory 1						0.940
Pro inflammatory 2						0.901
Pro inflammatory 3						0.936
Pro inflammatory 4						0.770

Table 2 Average variance extracted value

	Cronbach's alpha	rho_A	Composite reliability	Average variance extracted
Anti apoptotic	1.000	1.000	1.000	1.000
Anti inflammatory	0.954	0.954	0.978	0.956
Lokomotorius	1.000	1.000	1.000	1.000
Neuroangiogenesis	1.000	1.000	1.000	1.000
Neurogenesis	0.907	0.915	0.941	0.842
Pro inflammatory	0.917	0.979	0.938	0.792

Table 3 Discriminant validity values

	Anti apoptotic	Anti inflammatory	Lokomotorius	Neuroangiogenesis	Neurogenesis	Pro inflammatory
Anti apoptotic	1.000	0.697	0.271	0.850	0.815	0.085
Anti inflammatory 1	0.663	0.978	-0.293	0.714	0.704	0.642
Anti inflammatory 2	0.699	0.978	-0.140	0.796	0.739	0.532
Lokomotorius	0.271	-0.220	1.000	0.064	0.072	-0.869
Neurogenesis 1	0.725	0.691	-0.037	0.961	0.932	0.369
Neurogenesis 2	0.776	0.612	0.313	0.862	0.928	-0.006
Neurogenesis 3	0.748	0.736	-0.084	0.804	0.892	0.338
Neuroangiogenesi	0.850	0.772	0.064	1.000	0.958	0.319
Pro inflammatory 1	0.078	0.580	-0.865	0.268	0.201	0.940
Pro inflammatory 2	-0.073	0.496	-0.760	0.192	0.118	0.901
Pro inflammatory 3	0.257	0.654	-0.740	0.455	0.392	0.936
Pro inflammatory 4	-0.206	0.153	-0.881	0.012	0.046	0.770

19.93, whereas the control group's score was 10.33. The mean difference in the BBB scores was 9.6 (BBB score 0-21). Based on the Tukey HSD test, control and treatment groups were different, with a significance value of $P = 0.001$ ($P < 0.05$). The treatment group demonstrated a higher effect on improving the value of locomotor recovery in the rat SCI subacute contusion-compression model (Figure 4).

Table 4 Results of bootstrapping and blindfolding partial least squares structural equation modeling

	O	M	STDEV	T statistics (|O/STDEV|)	*P* values
Anti apoptotic - > lokomotorius	0.109	0.129	0.210	4.519	0.000
Anti inflammatory - > anti apoptotic	0.697	0.692	0.145	4.818	0.000
Anti inflammatory- > neuroangiogenesis	0.772	0.768	0.125	6.196	0.000
Neuroangiogenesis - > neurogenesis	0.176	0.183	0.258	4.682	0.000
Neurogenesis - > lokomotorius	0.754	0.782	0.130	5.789	0.000
Pro inflammatory - > anti inflammatory	0.600	0.622	0.193	3.102	0.002

O: Original sample; M: Sample mean; STDEV: Standard deviation.

Table 5 F square value

	Anti apoptotic	Antiinflammatory	Lokomotorius	Neuroangiogenesis	Neurogenesis	Pro inflammatory
Anti apoptotic			0.197			
Anti inflammatory	2.126			3.458		
Lokomotorius						
Neuroangiogenesis					0.797	
Neurogenesis			0.563			
Pro inflammatory		6.163				

Table 6 Q square value

	SSO	SSE	Q^2 (= 1-SSE/SSO)
Anti apoptotic	15.000	8.131	0.458
Anti inflammatory	30.000	24.178	0.194
Lokomotorius	15.000	6.222	0.585
Neuroangiogenesis	15.000	13.410	0.106
Neurogenesis	45.000	44.724	0.006
Pro inflammatory	60.000	60.000	

Table 7 R square value

	R square	R square adjusted
Anti apoptotic	0.680	0.655
Anti inflammatory	0.860	0.850
Lokomotorius	0.536	0.459
Neuroangiogenesis	0.776	0.758
Neurogenesis	0.444	0.401

Spinal cord lession

The results of measurements size of spinal cord lesions in the control and treatment groups, successive mean values of 304.019 and 51.676, with the non-parametric test (Mann Whitney) found a significant difference in the size of the spinal cord lesion with a $P = 0.000$ (Figure 5).

Oxidative stress cytokine (F2-Isoprostanes)

The examination results of oxidative stress (F2-Isoprostanes) showed a significant decrease in the

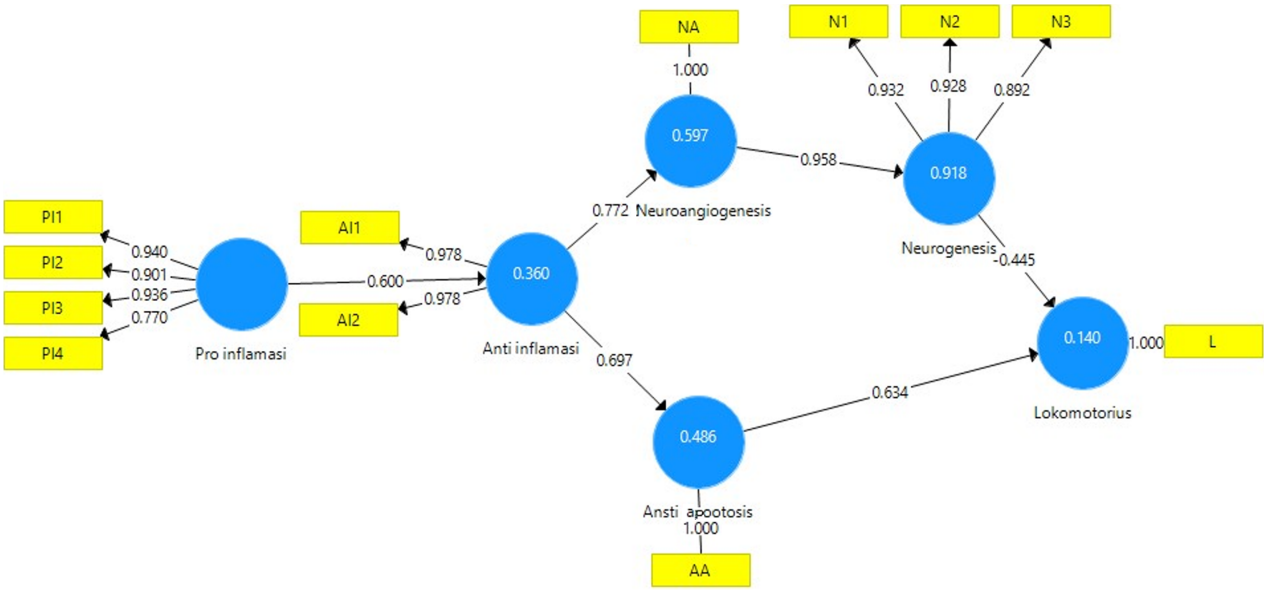

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Figure 2 Diagram outer model based on partial least squares algorithm.

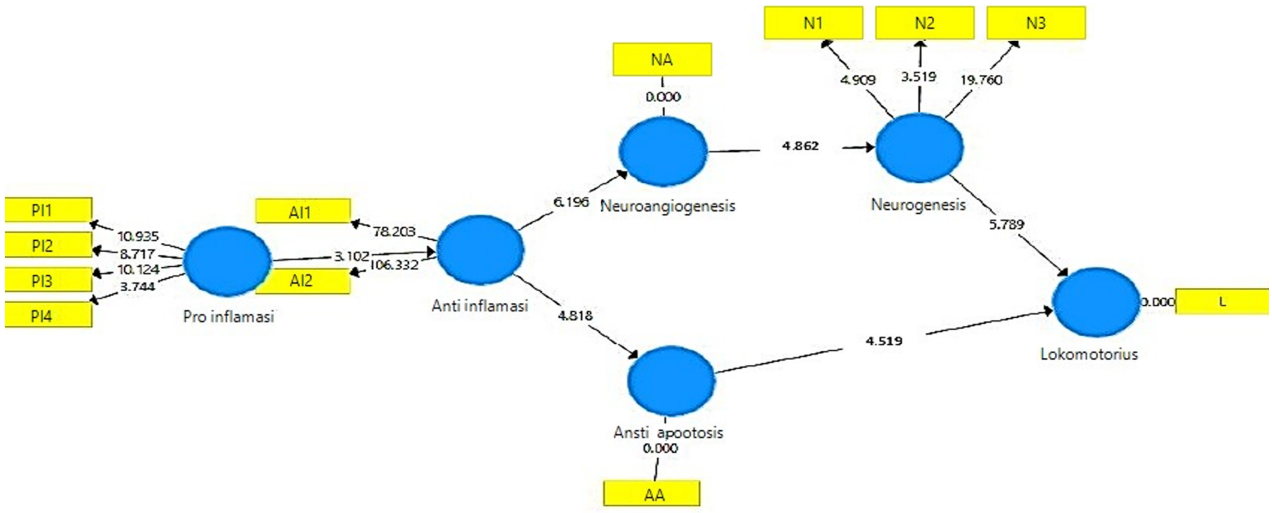

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Figure 3 Diagram inner model using bootstrapping and blindfolding partial least squares structural equation modeling.

treatment group compared to the control group, with significance values of $P = 0.001$. The level of F2-Isoprostanes in the treatment group 258.40, were smaller than the control groups 338.82 (Figure 6A, Table 8).

Pro-inflammatory cytokine (NF-κB, TNF-α, MMP9)

The examination results of neuro pro-inflammation biomarkers (NF-κB, TNF-α, and MMP9) showed a significant decrease in the treatment group compared to the control group, with successive significance values of $P = 0.000$, $P = 0.032$, and $P = 0.001$. The number of cells expressing NF-κB, TNF-α, and MMP9 in the treatment group, with successive mean values of 1.400, 171.85, and 1.19, were smaller than the control groups, with values of 2820, 215.1, and 3.09 (Figures 6A, 6B and Figure 7, Table 8).

Anti-inflammatory cytokine (IL-10, TGF-β)

The immunohistochemical examination of neuro anti-inflammation biomarkers (IL-10 and TGF-β) showed a significant increase in the treatment group compared to the control group, with successive significance values of $P = 0.022$ and $P = 0.047$. The number of cells expressing IL-10 and TGF-β in the treatment group, with successive mean values of 3.160 and 2.740, were greater than the control groups, with values of 1.900 and 1.840 (Figures 6B and Figure 8, Table 8).

Table 8 Tukey HSD test results of all biomarkers

	Biomarker	Group	Group	*P* value
Oxidative stress	F2-Isoprostanes	Treatment (mean = 258.40; SD = 12.45)	Normal (mean = 252.59; SD = 25.54)	0.938
			Control (mean = 338.82; SD = 36.87)	0.001
Pro-inflammatory	NF-kB	Treatment (mean = 1.400; SD = 0.254)	Normal (mean = 0.475; SD = 0.206)	0.003
			Control (mean = 2.820; SD = 0.531)	0.000
	MMP-9	Treatment (mean = 1.19; SD = 0.931)	Normal (mean = 0.160; SD = 0.213)	0.217
			Control (mean = 3.09; SD = 1.056)	0.001
	TNF-α	Treatment (mean = 171.85; SD = 35.84)	Normal (mean = 105.07; SD = 11.34)	0.002
			Control (mean = 215.14; SD = 15.38)	0.032
Anti-inflammatory	IL-10	Treatment (mean = 3.160; SD = 0.801)	Normal (mean = 0.240; SD = 0.167)	0.000
			Control (mean = 1.900; SD = 0.734)	0.022
	TGF-β	Treatment (mean = 2.740; SD = 0.684)	Normal (mean = 0.260; SD = 0.181)	0.000
			Control (mean = 1.840; SD = 0.572)	0.047
Neuroangiogenesis	VEGF	Treatment (mean = 5.12; SD = 0.878)	Normal (mean = 0.220; SD = 0.130)	0.000
			Control (mean = 2.120; SD = 0.889)	0.000
Anti-apoptotic	Bcl-2	Treatment (mean = 2.02; SD = 0.712)	Normal (mean = 0.160; SD = 0.151)	0.000
			Control (mean = 0.500; SD = 0.380)	0.001
Neurogenesis	Nestin	Treatment (mean = 1.96; SD = 0.610)	Normal (mean = 0.160; SD = 0.114)	0.000
			Control (mean = 1.000; SD = 0.524)	0.018
	BDNF	Treatment (mean = 2.01; SD = 0.576)	Normal (mean = 0.40; SD = 0.482)	0.000
			Control (mean = 0.57; SD = 0.468)	0.001
	GDNF	Treatment (mean = 3.420; SD = 2.480)	Normal (mean = 1.420; SD = 0.356)	0.000
			Control (mean = 2.480; SD = 0.788)	0.043

SD: standard deviation; NF-kB: Nuclear factor-kappa B; MMP9: Metalloproteinase matrix-9; TNF-α: Tumor necrosis factor-α; IL-10: Interleukin-10; TGF-β: Transforming growth factor-β; VEGF: Vascular endothelial growth factor; Bcl-2: B-cell lymphoma 2; BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell line-derived neurotrophic factor; F2-Isoprostanes: Free radical oxidative stress.

Neuroangiogenesis cytokine (VEGF): The number expressing VEGF in the treatment group significantly differed from the control group (P = 0.000). Moreover, the number of cells expressing VEGF in the treatment group (5.12) was greater than the control group (2.120) (Figures 6B and Figure 9, Table 8).

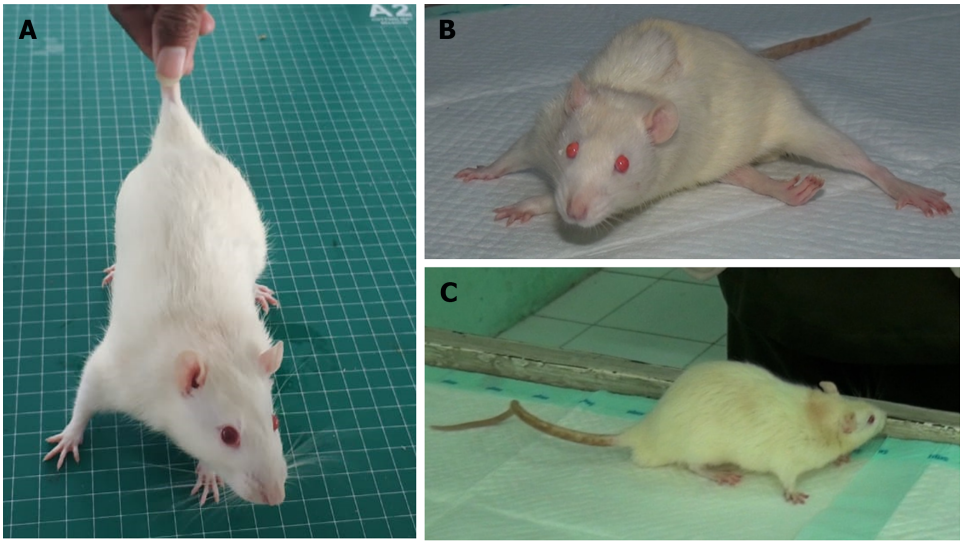

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Figure 4 Evaluation Basso, Beattie, Bresnahan scores in different rat groups. A: Normal group; B: Control group; C: Treatment group.

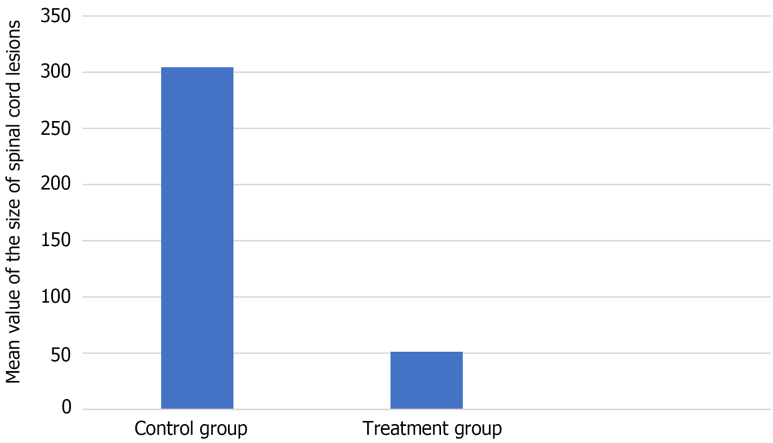

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Figure 5 The mean value of the size of spinal cord lesions in the control and treatment groups.

Anti-apoptotic cytokine (Bcl-2): The number expressing Bcl-2 in the treatment group significantly differed from the control group (P = 0.001). Moreover, the number of cells expressing Bcl-2 in the treatment group (2.02) was greater than the control group (0.500) (Figures 6B and 10, Table 8).

Neurogenesis cytokine (nestin, BDNF, GDNF): The results of the immunohistochemical examination of neurogenesis biomarkers (nestin, BDNF, and GDNF) showed a significant increase in the treatment group compared to the control group, with successive significance values of P = 0.018, P = 0.001, and P = 0.043. The number of cells expressing nestin, BDNF, and GDNF in the treatment group with successive mean values of 1.96, 2.01, and 3.420 were greater than the control groups, which had mean values of 1.00, 0.57, and 2.480 (Figures 6B and 11, Table 8).

DISCUSSION

After HNSCs-secretome intrathecal injections in model SCI post-laminectomy rats, the results showed that HNSCs-secretome increased locomotor function, decreased size of spinal cord lesion, increased GDNF, BDNF, nestin, VEGF, Bcl-2, TGF-β, IL-10, and decreased TNF-α, F2-Isoprostanes, MMP-9, NF-κB. The mechanism of SCI was valid, based on the analyzed outer model, inner model, and hypothesis testing. It began with pro-inflammation, anti-inflammation, anti-apoptotic, neuroangiogenesis, neurogenesis, and locomotor function.

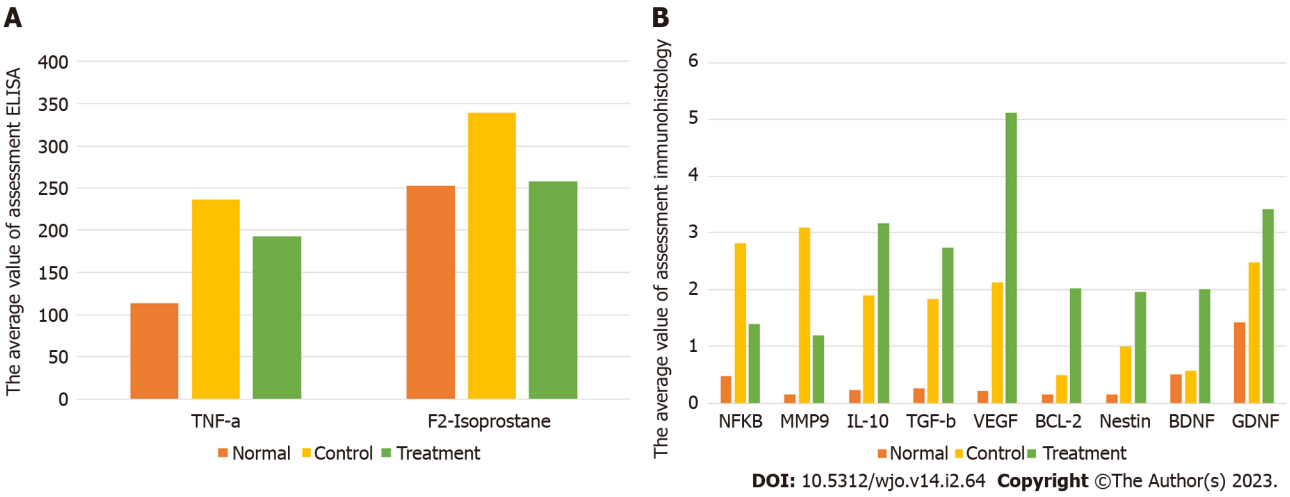

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Figure 6 The mean value of biomarker by enzyme-linked immunosorbent assay and immuhistochemichal assessment. A: Diagram showing the average value of enzyme-linked immunosorbent assay assessment; B: Diagram showing the average value of immuhistochemichal assessment. NF-kB: Nuclear factor-kappa B; MMP9: Metalloproteinase matrix-9; TNF-α: Tumor necrosis factor-α; IL-10: Interleukin-10; TGF-β: Transforming growth factor-β; VEGF: Vascular endothelial growth factor; Bcl-2: B-cell lymphoma 2; BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell line-derived neurotrophic factor; F2-Isoprostanes: Free radical oxidative stress; ELISA: Enzyme-linked immunosorbent assay.

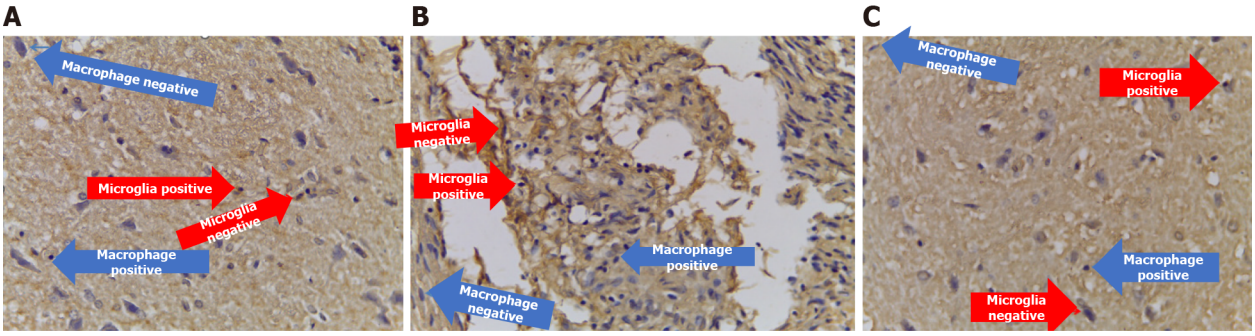

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Figure 7 We observed immunohistochemical matrix metalloproteinase 9 average value of 10 field of views, every field of view have 625 μ² with 400 × magnification. A: Treatment group; B: Control group; C: Normal group. Microglia (red arrow) are small round cells, solid nuclei and give a positive reaction with anti matrix metalloproteinase 9 (MMP9) indicated by brown color. While macrophage (blue arrow) cells are large, vesicular nucleus, and sometimes elongated resembling fibroblasts (macrophages like fibroblast) and give a positive reaction with anti MMP9 indicated by brown.

The results of this study are in accordance with findings from Cunningham *et al*[3] who stated that MSC-secretome in brain ischemia could modulate neurogenesis with an increase in BDNF, GDNF, and NT3. Kim *et al*[12] stated that adipose derived stem cell-secretome can provide anti-free-radical effects and reduce oxidative stress with the expression of F2-Isoprostane. Honmou *et al*[13] also stated that mononuclear stem cell secretome in SCI inhibited microvascular obstruction and thrombosis, promoted vasodilation, immunomodulation, and neuroprotection, while Santos *et al*[14] and Yang *et al*[15] stated that NSC-secretome in SCI stimulated the transformation of phenotype M1 to phenotype M2 of macrophages, microglia, and astrocytes through PgE2, IL-10, TGF-β, proliferator-activated receptor gamma[14,15]. Macrophage M2 secreted anti-inflammatory cytokines IL-4, IL-10, TGF-β, and hepatocyte growth factor, whereas macrophage M1 secreted pro-inflammatory cytokines IL-1, IL-6, IL-8, TNF-α, MMP9, and F2-Isophostane[14,15]. Miranpuri *et al*[5] stated that after trauma, there was an increase in inflammatory cells, such as macrophages, neutrophils, dendrites, and T-cells, as a result of ruptured blood vessels and increased vascular permeability.

Oxidative stress cytokine (F2-Isoprostane)

The results of the HNSCs-secretome in SCI study, there was a decrease in the oxidative stress cytokine F2-Isoprostane, accordance with previous research by Kim *et al*[12] who stated that NSC-secretome acts as an anti-free radical and anti-oxidative stress agent in mouse-model SCI. Santos *et al*[14] stated that NSC-secretome has an antioxidant role that reduces F2-Isoprostane by inhibiting endoperoxidase, arachidonic acid, and reactive oxygen species (ROS). Oxidative stress plays an important role in the secondary phase of SCI[16]. The high oxidative stress of F2-Isoprostan affects the production of pro-

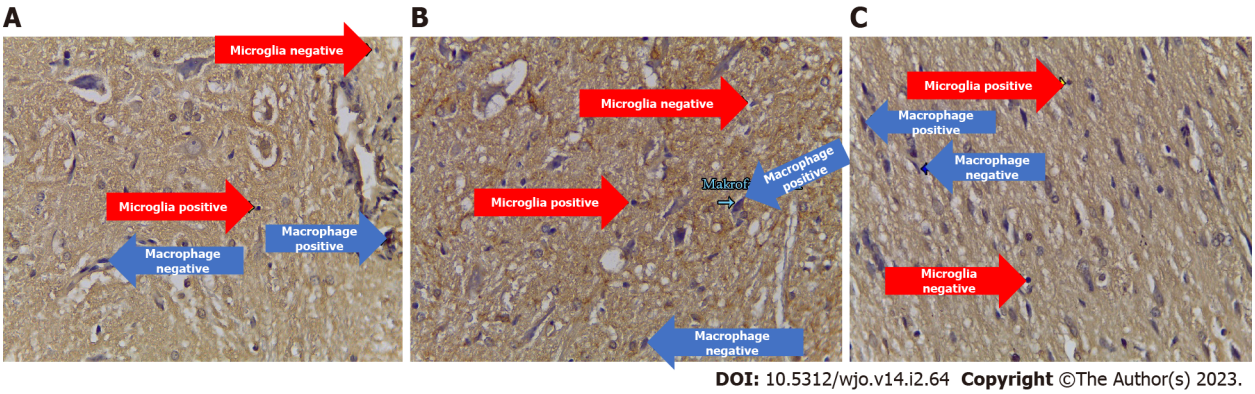

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Figure 8 We observed immunohistochemical transforming growth factor-β average value of 10 field of views, every field of view have 625 μ² with 400× magnification. A: Treatment group; B: Control group; C: Normal group. Microglia are small round cells, solid nuclei and give a positive reaction with anti-transforming growth factor-beta (TGF-β) indicated by brown color. While macrophage cells are large, vesicular nucleus,and sometimes elongated resembling fibroblasts (macrophages like fibroblast) and give a positive reaction with anti TGF-β indicated by brown.

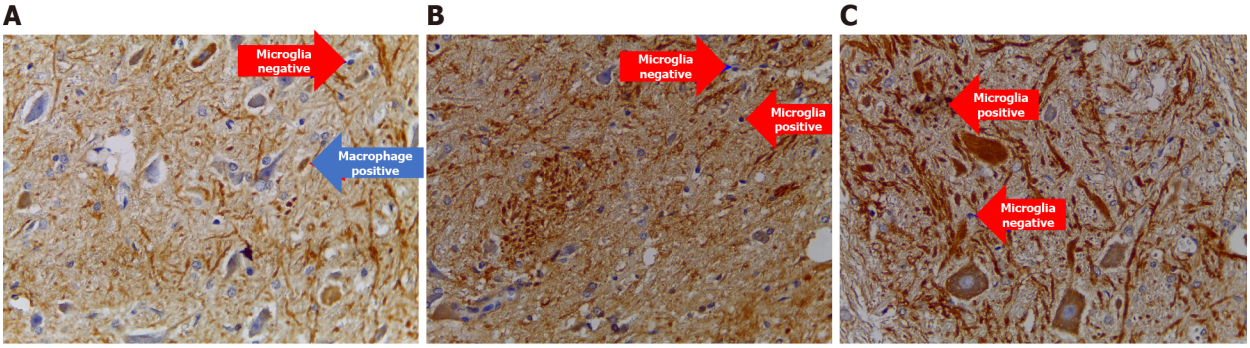

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Figure 9 We observed immunohistochemical vascular endothelial growth factor average value of 10 field of views, every field of view have 625 μ² with 400 × magnification. A: Treatment group; B: Control group; C: Normal group. Microglia are small round cells, solid nuclei and give a positive reaction with anti-vascular endothelial growth factor (VEGF) indicated by brown color. While macrophage cells are large, vesicular nucleus, and sometimes elongated resembling fibroblasts (macrophages like fibroblast) and give a positive reaction with anti-VEGF indicated by brown.

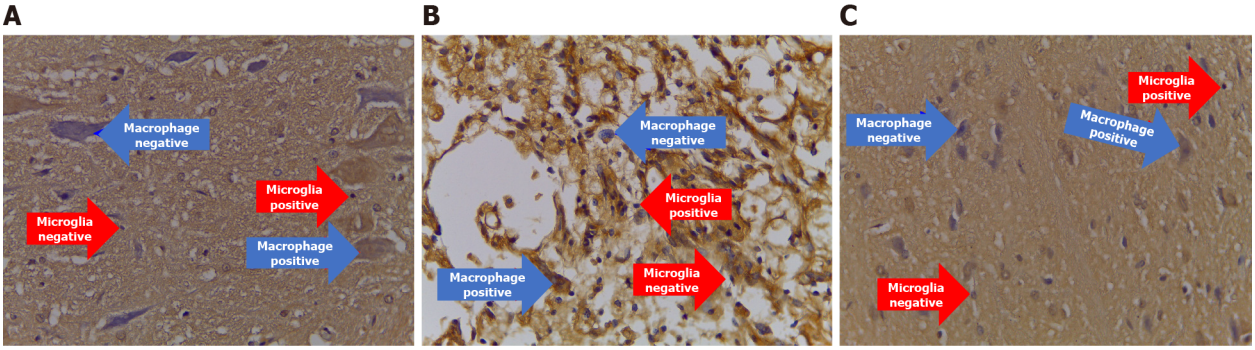

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Figure 10 We observed immunohistochemical B cell lymphoma-2 average value of 10 field of views, every field of view have 625 μ² with 400 × magnification. A: Treatment group; B: Control group; C: Normal group. Microglia are small round cells, solid nuclei and give a positive reaction with anti B-cell lymphoma 2 (Bcl-2) indicated by brown color. While macrophage cells are large, vesicular nucleus, and sometimes elongated resembling fibroblasts (macrophages like fibroblast) and give a positive reaction with anti Bcl-2 indicated by brown.

apoptotic proteins, inhibits the anti-apoptotic protein Bcl-2, damage to the function of the mitochondria, and affects DNA fragmentation, resulting in apoptotic[17]. The decrease in antioxidants also through neurotrophic NSC factors such as BDNF by increasing the activity of antioxidant enzymes super oxide dismutase, glutathione peroxidase, glutathione reductase, sulfiredoxin, and sestrin2[18]. Antioxidant activity reduces ROS, increases mitochondrial uncoupling protein 2, and restores the mitochondrial

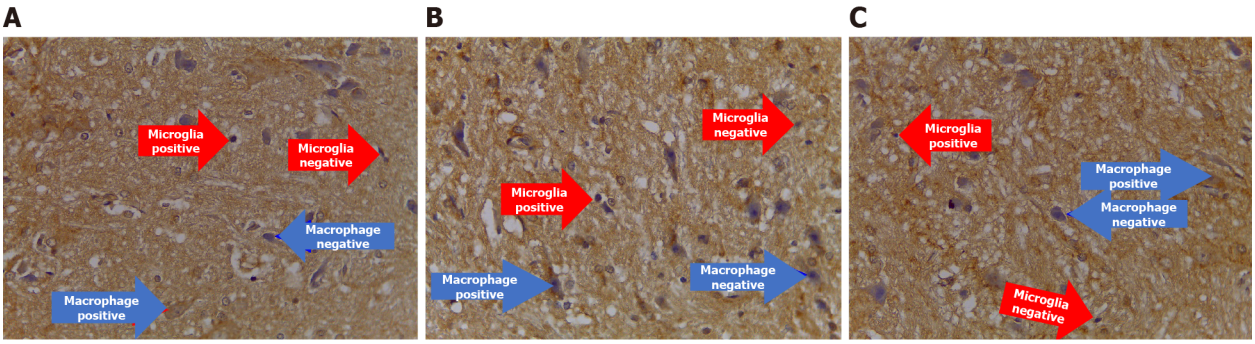

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Figure 11 We observed immunohistochemical brain derived neurotrophic factor average value of 10 field of views, every field of view have 625 μ² with 400 × magnification. A: Treatment group; B: Control group; C: Normal group. Normal group. Microglia are small round cells, solid nuclei and give a positive reaction with anti-brain derived neurotrophic factor (BDNF) indicated by brown color. While macrophage cells are large, vesicular nucleus, and sometimes elongated resembling fibroblasts (macrophages like fibroblast) and give a positive reaction with anti BDNF indicated by brown.

electron-coupling capacity to its original state by inducing the accumulation of phosphorylated cAMP response element binding protein in the mitochondrial matrix and membrane, assisting the synthesis of complex V mitochondria to protect apoptotic[13].

Pro-inflammatory cytokines (NF-κB, TNF-α, and MMP9)

After administering HNSCs-secretome to SCI-affected rats, there was a decrease in the pro-inflammatory cytokines NF-κB, MMP9, and TNF-α. Cheng *et al*[19] stated that NSC-secretome in SCI can suppress the inflammatory process by reducing the number of macrophages and microglia, decreasing inducible nitric oxide synthase by promoting SCI regeneration. Rong *et al*[20] stated that a decrease in proinflammatory cytokines occurred due to the autophagy activity of macrophages after administration of NSC-secretome. TNF-α, MMP9, and F2-Isoprostan decreased due to the transformation of macrophages phenotypes M1 to M2[14,15].

The decrease in NF-κB levels in this study is in accordance with research conducted by Wang *et al*[21] and Chen *et al*[22], which stated that there was a decrease in NF-κB levels after MSC-secretome intervention in mouse-model SCI. Chen *et al*[22] stated that a decrease in NF-κB will encourage axon regeneration through the phosphatase and tensin homolog/AKT/mammalian target of rapamycin pathway, where NF-κB serves to provide intracellular signals for macrophages to release pro-inflammatory cytokines such as MMP9 and TNF-α.

In this study, after administering HNSCs-secretome to SCI-affected rats, there was a decrease in the pro-inflammatory cytokine TNF-α, accordance with five previous studies by Huang *et al*[23], Cizkova *et al*[24], Huang *et al*[25], and Borhani-Haghighi *et al*[26], who stated that there was a decrease in TNF-α after MSC-secretome intervention in mouse-model SCI. The cytokine TNF-α is the most influential proinflammatory mediator in SCI, followed by other proinflammatory mediators such as interferon gamma, IL-6, and IL-8[27]. M1 phenotype macrophages secrete TNF-α through several mechanisms, namely NF-κB signaling, mitogen activated protein kinase, c-Jun N-terminal kinase, extrinsic apoptotic pathway, and extracellular signal-regulated kinase 1/2[28]. TNF-α influences the development of secondary injury by increasing inflammation, oxidative stress (F2-Isoprostane), and modulating apoptotic mechanisms[20]. TNF-α plays a role in increasing the endogenous migration of NSCs to the site of SCI by upregulating the chemokine receptors (CCR)2, CCR3, and CCR4 and motif C-C receptors [29].

This study showed that HNSCs-secretome in SCI could reduce MMP9 biomarkers, accordance with the research of Xin *et al*[30], who stated that there was a decrease in MMP9 and an increase in tissue inhibitor of metalloproteinases (TIMP) after administration of human bone marrow MSC (hBMSC) secretome in mouse-model SCI. MMP9 is inhibited by TIMP, while TIMP is inhibited by TGF-β[5]. This system modulates macrophage invasion and myelin destruction, which has an important role in neuropathic pain and contributes to glail scar formation[5].

Anti-inflammatory cytokines (IL-10 and TGF-β)

In this study, after administering HNSCs-secretome to SCI-affected rats, in addition to decreasing pro-inflammatory cytokines, there was also an increase in anti-inflammatory cytokines IL-10 and TGF-β. The increase in IL-10 cytokines accordance with a study conducted by Chudickova *et al*[31], who stated that there was an increase in IL-10 as an anti-inflammatory factor in the systemic immunological response after BMSC secretome intervention on SCI. IL-10 can decrease MMP9 synthesis, induce macrophage polarization from M1 to M2 phenotypes, reduce inflammatory response, and suppress inflammatory cells[32,33]. IL-10 can inhibit the initial effect of MMP9 in terms of the degradation of the basal lamina blood medulla spinal barrier matrix[5,32]. Previous studies have shown that systemic IL-10 injection

results in significant neuroprotection and greater functional improvement after SCI trauma[33]. IL-10 also provides anti-apoptotic support to neurons, reduction of lesion size, and improvement of locomotor function[33,34].

In this study, TGF-β increased in the treatment group compared to the control and normal groups, accordance with research by Cunningham *et al*[3], who state that there was an increase in TGF-β after MSC-secretome intervention in the ischemic brain. In addition to TGF-β, other anti-inflammatory agents, including BDNF, CXCL12, GDNF, hypoxia-inducible factor -1alpha (HIF-1α), IL-10, and VEGF, were also found. TGF-β also plays a role in overcoming matrix degradation, which is caused by the effect of MMP9[5]. TGF-β is involved in neuronal repair and regeneration and has been observed to inhibit neuronal damage and stimulate cell survival, growth, proliferation, differentiation, and invasion of neurons and glial cells[33].

Neuroangiogenesis cytokine (VEGF): The results of the HNSCs-secretome study in SCI showed an increase in the neuroangiogenesis cytokine VEGF, accordance with three previous studies by Cizkova *et al*[24], Liu *et al*[35], and Zhong *et al*[36], who stated that there was an increase in VEGF after MSC-secretome administration in mouse-model SCI. Cunningham *et al*[3] stated that in addition to VEGF, there was also an increase in other angiogenesis factors such as PDGF, BDNF, GDNF, basic fibroblast growth factor, CXCL12, Ang-1, Ang-2, and HIF-1α. Zhong *et al*[36] stated that administration of NSC-secretome in acute SCI can increase the expression of VEGF-A, which promotes axon proliferation and the migration of spinal cord microvascular endothelial cells from the third day post-injection, reduces lesion size, glial scars, and improves locomotor function in a mouse-model of SCI. VEGF is the highest protein found in the angiogenesis process, while VEGF-A is more commonly found in NSC-secretomes than in NSCs themselves[36]. VEGF plays a role in neuroprotection, with blood vessel formation starting on day 3 to 10 and optimally on day 14, where perfusion, oxygenation, and carbohydrate metabolism occur[24,35-37].

Anti-apoptotic cytokine (Bcl-2): In this study, there was an increase in Bcl-2 as an anti-apoptotic factor of HNSCs-secretome in a mouse model of SCI, consistent with three previous studies by Huang *et al*[23], Liu *et al*[35], and Zhou *et al*[27], who stated that there was an increase in Bcl-2 levels after MSC-secretome intervention in mouse-model SCI. Rong *et al*[20] stated that there was an increase in Bcl-2 and a decrease in caspase 3 due to the role of secretome anti-apoptotic factors in SCI regeneration. Bcl-2 functions as an anti-apoptotic factor by blocking the release of cytochrome-c from the mitochondria into the cytosol, thereby preventing the activation of caspase-3 and caspase-9[27,38,39].

Neurogenesis growth Factor (nestin, BDNF, and GDNF): The results of the HNSCs-secretome study in SCI, an increase in the neurogenesis growth factors nestin, BDNF, and GDNF. Cunningham *et al*[3] state that neurogenesis in brain ischemia is influenced by an increase in BDNF and GDNF after MSC-secretome administration.

Červenka *et al*[40] state that HNSCs-secretome increases nestin levels in brain and spinal cord trauma, and nestin is a more significant biomarker than SRY-box 2, doublecortin, tubulin-3 chain, and microtubule-associated protein 2 in identifying the differentiation of NSCs from pre-progenitor NSCs. Accordance with the research of Zhong *et al*[36], who found that the presence of nestin growth factor is a marker of the presence of neuron cells. Pajer *et al*[8] and Gilbert *et al*[41] also state that nestin is a neurotrophic factor that expresses the presence of NSC progenitors.

In this study, BDNF increased in the treatment group compared to the control and normal groups. Chudickova *et al*[31] and Gu *et al*[42] state that NSC-secretome in animal models with SCI, there was an increase in BDNF at week 1 and a maximum increase at week 6 that could reduce lesion size, minimize glial scar formation, and promote axon regeneration. BDNF is produced mostly by neuronal cells and is a neurotropin that is important in the regulation of neurogenetic processes such as increased axon collateral growth, nerve branching, dendrite formation, and synaptic plasticity[43]. BDNF works through cannabinoid receptor type 1 (CB1R) and CB2R receptors to promote neuronal differentiation and prevent nuclear degeneration[44]. In addition, BDNF also works through the tropomyosin kinase B receptor and low-affinity nerve growth factor receptor (GFR) commonly called p75[45]. Shahsavari *et al*[46] state that BDNF has neurophysiological functions such as nociception, cognition, and memory.

In this study, GDNF increased sharply in the treatment and control groups compared to the normal group, and the treatment group was slightly higher than the control group. Cheng *et al*[19] and Zhong *et al*[36] found that NSC-secretome increase the occurrence of axon regeneration, collateral formation, and the occurrence of new circuits in axon pathways by activating neurons and glial cells. Rosich *et al*[47] state that GDNF plays a role in the spinal cord in reducing lesion size, cystic cavity, increasing locomotor function improvement, nerve differentiation, chemoattractant, migration, neuroprotectant, neuroplasticity, and axon regeneration. GDNF also exerts a substantial neuroprotective effect by increasing the number of neurons in the SCI and the supraspinal central canal area[48]. GDNF acts through GFRα 1-4 receptors and is rearranged during transfection tyrosine kinase[49]. GDNF is a neurotropin involved in increasing the number of motor neurons, regenerating distal nerve axons, forming synapses, and myelination[48].

Locomotor function BBB score: Locomotor function is one of the most significant therapeutic intervention goals demonstrating the efficacy of administering HNSCs-secretome treatment in subacute SCI. Administration of HNSCs-secretome significantly improved locomotor function starting on day 7 and continuing until day 56, with mean value is 19.93 and standard deviation is 6.28. This is in accordance with previous studies that showed an increase in locomotor function improvement after NSC-secretome intervention in three studies[19,20,36].

Spinal cord lession: The treatment group showed significant differences where the treatment group showed smaller lesion sizes compared to the control group, successive mean values of 304.019 and 51.676. This is in accordance with previous studies that showed an descreased size of spinal cord lesion after MSC-secretome intervention in three studies[24,31,37].

Mechanism of SCI regeneration: The mechanism of SCI regeneration is still uncertain[3,8]. In this study, we found that analysis of the outer model, inner model, and hypothesis testing were valid. SCI regeneration begins with pro-inflammation and continues with anti-inflammatory, anti-apoptotic, neuroangiogenesis, neurogenesis, and locomotor function. Inner model by path bootstrapping analysis found that all pathways had positive original sample values, with T-statistics more than 1.96 and *P*-values more than 0.05, determined to be significantly different. The relationships between latent variables in the inner model were valid based on an F square (effect size) more than 0.05, Q square (prediction relevance) more than 0, and positive path coefficients. The R square (coefficient of determination on endogenous variables) anti-inflammatory value of 0.860 indicated an effect of 86%, the anti-apoptotic value of 0.680 indicated an effect of 68%, the neuroangiogenesis value of 0.776 indicated an effect of 77%, the neurogenesis value of 0.444 indicated an influence of 44%, and the locomotory value of 0.536 indicated an influence of 53%. The outer model was valid based on the PLS SEM algorithm, the convergent validity value was more than 0.7, the AVE value was more than 0.5, and the Cronbach's alpha value was more than 0.5. The discriminant validity based on cross-loading indicator was higher than the other construct variable indicators, while composite reliability was more than 0.7.

Assinck *et al*[50] state that spinal cord regeneration has five mechanisms: neuroprotection, immunomodulation, axon growth/regeneration, human neural relay formation, and myelin regeneration. Anjum *et al*[6] also state that tissue regeneration is divided into three overlapping phases, namely, cell death and inflammation, cell proliferation and tissue replacement, and tissue remodeling. Vizoso *et al*[11], and Cunningham *et al*[9], stated that advantages of secretome (cell free therapy) compared to stem cell, are the secretome solves problems that have so far arisen in stem cell applications, namely from the aspect of live cell transplantation to donors (immune compatibility, tumorigenicity, embolism formation, and infection transmission); storage is easier by not giving it a toxic cryopreservative agent for a long time; it is more practical and economical because it does not use invasive cell retrieval procedures compared to stem cell based therapy; mass production is more possible; time and maintenance costs for stem cell culture can be reduced, it is said that it takes hundreds of millions of MSCs each time for therapy, and cell expansion *in vitro* is needed for 10 wk before implantation, implantation time is also said to take quite a long time to be effective; in one study it was said that < 1 percent of MSCs survived after systemic administration; and it is also said that the main factor in stem cell therapy is through the paracrine effect, which is owned by the secretome[3,11]. The key limitations of study do not cover the chronic SCI model. HNSCs-secretome is expected to be the basis for use in SCI cases in the primary research stage, translational research, and neurological research for the benefit of managing SCI disease problems.

CONCLUSION

These findings may identify HNSCs-secretome as a neuroprotective-neuroregenerative agent for treating SCI. The SCI regeneration mechanism started with pro-inflammation and continued with anti-inflammation, anti-apoptotic, neuroangiogenesis, neurogenesis, and locomotor function.

ARTICLE HIGHLIGHTS

Research background

Globally, complete neurological recovery of spinal cord injury (SCI) is still less than 1%, and 90% experience permanent disability. The key issue is that a pharmacological neuroprotective-neuroregenerative agent and SCI regeneration mechanism have not been found. The secretomes of stem cell are an emerging neurotrophic agent, but the effect of human neural stem cells (HNSCs) secretome on SCI is still unclear.

Research motivation

HNSCs-secretome is expected to be the basis for use in SCI cases in the primary research stage, translational research, and neurological research for the benefit of managing SCI disease problems.

Research objectives

To investigate the effects of HNSCs-secretome and the regeneration mechanism on subacute SCI in rats.

Research methods

An experimental study was conducted with 45 Rattus norvegicus, divided into 15 normal, 15 control (10 mL physiologic saline), and 15 treatment (30 μL HNSCs-secretome, intrathecal T10, three days post-traumatic). The strategies to increase the HNSCs-secretome production capacity include hypoxic preconditioning, tissue engineering, and growth medium composition. Locomotor function was evaluated weekly by blinded evaluators. Fifty-six days post-injury, specimens were collected, immunohistochemical-enzyme-linked immunosorbent assay assessment, and hematoxylin-eosin staining. We analyzed free radical oxidative stress (F2-Isoprostanes), nuclear factor-kappa B (NF-κB), matrix metallopeptidase 9 (MMP9), tumor necrosis factor-alpha (TNF-α), interleukin-10 (IL-10), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), B cell lymphoma-2 (Bcl-2), nestin, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and spinal cord lesion. The regeneration mechanism of SCI was analyzed using partial least squares structural equation modeling (PLS SEM).

Research results

The regeneration mechanism of SCI is valid by analyzed outer model, inner model, and hypothesis testing in PLS SEM, started with pro-inflammation followed by anti-inflammation, anti-apoptotic, neuroangiogenesis, neurogenesis, and locomotor function. HNSCs-secretome significantly improved locomotor recovery, reduced spinal cord lesion size, increased neurogenesis (nestin, BDNF, and GDNF), neuroangiogenesis (VEGF), anti-apoptotic (Bcl-2), anti-inflammatory (IL-10 and TGF-β), but decreased pro-inflammatory (NF-κB, MMP9, TNF-α), F2-Isoprostanes.

Research conclusions

HNSCs-secretome as a potential agent for the treatment of SCI and uncover the SCI regeneration mechanism.

Research perspectives

Future research investigating the chronic phase of SCI models may provide further evidence regarding the mechanism of SCI regeneration given HNSCs-secretome injection.

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FOOTNOTES

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LETTER TO THE EDITOR

Effect of SARS-CoV-2 infection on trauma throughput to alternative elective care approaches

Beuy Joob, Viroj Wiwanitkit

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Abstract

In response to the paper on coronavirus disease 2019's effects on trauma throughput, elective care models should be modified. Concerns about the relevant factors and their potential therapeutic applications are brought up and looked into.

Key Words: Trauma; Model; Adaptive care; COVID-19

Core Tip: This letter to the editor is in reaction to the article: The influence of coronavirus disease 2019 (COVID-19) on trauma throughput and the adaptation of elective care paradigms. Concerns are raised and examined concerning the factors involved and their therapeutic application. The model's influence may be limited to the COVID-19 pandemic phase and may not be applicable to the post-COVID-19 period.

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TO THE EDITOR

We read with interest a case report on "Utilising the impact of COVID-19 on trauma throughput to adapt elective care models for more efficient trauma care" by Kulkarni *et al*[1]. Kulkarni *et al*[1] investigated the effect of severe acute respiratory syndrome

coronavirus 2 infection on service delivery. A comparison between throughput and productivity parameters during the pandemic with those observed in the previous years was performed in order to search for successful, cost-effective, and long-term differences in practice[1]. Coronavirus disease 2019 (COVID-19) has resulted in a practical change in the delivery and access to care, according to Kulkarni *et al*[1], with many changes and adaptations anticipated to affect healthcare services in the future.

We all believe that coronavirus disease 2019 (COVID-19) necessitates medical care adjustments. During an emergency, adjustments may be made, but it should be recognized that the standards of care must still be met. Kulkarni *et al*'s recent report may reflect their experience during the pandemic[1]. However, if the models are to be employed in the post-crisis period, they must be carefully considered. In the absence of an emergency, resuming full-scale normal treatment may be necessary. Some options, such as delayed case management and telemedicine management, may be avoided. While some studies have demonstrated that different orthopaedic surgeries may be considered elective, medically required surgery must continue in areas with minimal medical care[2]. This could be the fundamental medical notion of first doing no harm to the patient. Furthermore, the COVID-19 period's epidemiological pattern of the medical problem may differ from the pre-COVID-19 period. The model's effect may differ depending on the disease pattern[3]. The model's influence may be limited to the COVID-19 pandemic phase and may not be applicable to the post-COVID-19 period. In order to assess the exact effect of adapting elective care models, there should be a long term follow-up, and the relationship with the changing background situation should also be assessed. Finally, in addition to the present clinical outcome measurement, it should place a greater emphasis on the patient's perspective on the change. This is a point that is frequently overlooked in many investigations.

FOOTNOTES

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LETTER TO THE EDITOR

New classification for septic arthritis of the hand

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Abstract

The severity of septic arthritis of the hand and the prospects for restoration of joint function are determined by a complex of factors. Among them, the leading role belongs to local changes in tissue structures. This includes the destruction of articular cartilage and bone tissue with the development of osteomyelitis, the involvement of paraarticular soft tissues in the purulent process, and the destruction of the flexor/extensor tendons of the fingers. The currently missing specialized classification of septic arthritis could help in systematizing the diseases, determining treatment tactics, and predicting the results of treatment. The classification of septic arthritis of the hand proposed for discussion is based on the following principle: Joint–Wound–Tendon ($J_xW_xT_x$); J_x characterizes damage to the osteochondral structures of the joint, W_x is the presence of paraarticular purulent wounds or fistulas, and T_x is destruction of the flexor/extensor tendons of the finger. The classification of the diagnosis makes it possible to assess the nature and severity of damage to the structures of the joint and may also be useful when comparing the results of treatment of septic arthritis of the hand.

Key Words: Hand; Septic arthritis; Classification; Osteomyelitis; Paraarticular wounds

Core Tip: The absence to date of a specialized classification of septic arthritis of the hand determines the relevance of its development. The proposed classification is based on the principle JOINT-WOUND-TENDON and reflects in aggregate the lesion of the osteo-cartilaginous structures of the joint, paraarticular soft tissues, and tendons of the flexor/extensor of the finger.

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TO THE EDITOR

Septic arthritis of the hand is a common infectious pathology of the joints and ranks second in frequency after inflammation of the knee joint[1]. Given the high functional significance of the small joints of the hand, their inflammation often leads to serious consequences, sometimes ending in limitation or even disability. The most common cause of septic arthritis of the hand is various penetrating wounds. The pathogenic microflora that has entered the joint cavity causes the development of an infectious process, which, if surgical care is not provided in time, leads to the destruction of the articular cartilage and the development of osteomyelitis. In septic arthritis of the hand, a purulent process often occurs in paraarticular tissues with the formation of wounds and fistulas, which has a significant impact on the extent of surgical intervention and the results of treatment in general. The inflammatory process in the small joints of the hand, proceeding with purulent-necrotic lesions of the surrounding soft tissues, may be accompanied by destructive changes in the area of the flexor/extensor tendons of the finger, which negatively affects the prospects for restoring movements in the joint. This fact distinguishes the course of septic arthritis of the hand from purulent arthritis of large joints, in which tendon damage is uncharacteristic[2].

Thus, the course of septic arthritis of the hand and the features of treatment and its results are determined by a complex of pathological changes in the osteochondral apparatus, paraarticular soft tissues, and flexor/extensor tendons of the finger. While analyzing the literature on septic arthritis of the hand (published in the *World Journal of Orthopedics* 2022; 13: 622-630)[3], we faced problems comparing the results of surgical treatment presented by different authors.

To obtain reliable results, it is necessary to understand the nature and extent of tissue damage in the observations that we compare.

Evaluating the response to treatment of isolated septic arthritis, septic arthritis with osteomyelitis, purulent process in the paraarticular soft tissues, and tendon destruction can hardly be justified. However, in most cases, the authors use the term "septic arthritis of the hand" when formulating the diagnosis, only occasionally highlighting cases with osteomyelitis[4].

As a consequence, comparison of the results of treatment presented by different experts may be incorrect since the severity of the disease was initially different. In this regard, the use in clinical practice and in the course of scientific analysis of the classification of the pathological process is of great importance. However, to date, there has been no specialized classification of septic arthritis of the hand. According to the most common classification of hand infection given by Brown[5], only two forms can be attributed to the characteristics of purulent arthritis: Osteomyelitis and septic arthritis. However, this is clearly not enough in view of the above arguments[5]. This can be partially compensated for by using the classification of Tan *et al*[6] for infection of the joints of the hand, which was developed to characterize the inflammatory process in large joints[6]. It presents information about the damage to the osteochondral structures of the joint and paraarticular soft tissues (*isolated septic arthritis; septic arthritis with soft-tissue extension, but no osteomyelitis; septic arthritis with contiguous osteomyelitis*). At the same time, this classification does not reflect the presence/absence of destruction of the tendon apparatus, which is rare in arthritis of large joints but is extremely important in septic arthritis of the hand. In addition, the classification of Tan *et al*[6] is quite voluminous, which makes it difficult to use it in wide clinical practice.

We have significant experience in the treatment of patients with septic arthritis of the hand and would like to propose our classification of septic arthritis of the hand.

The classification is based on the principle of Joint−Wound−Tendon ($J_xW_xT_x$) and takes into account the presence of damage to the osteochondral structures of the joint, paraarticular soft tissues, and tendon apparatus.

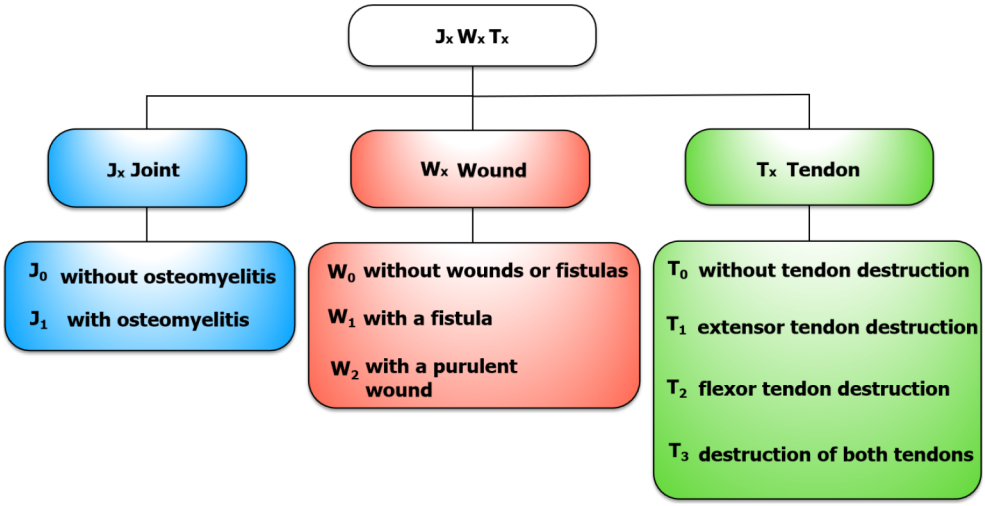

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Figure 1 New classification for septic arthritis of the hand. $J_xW_xT_x$: Joint−Wound−Tendon.

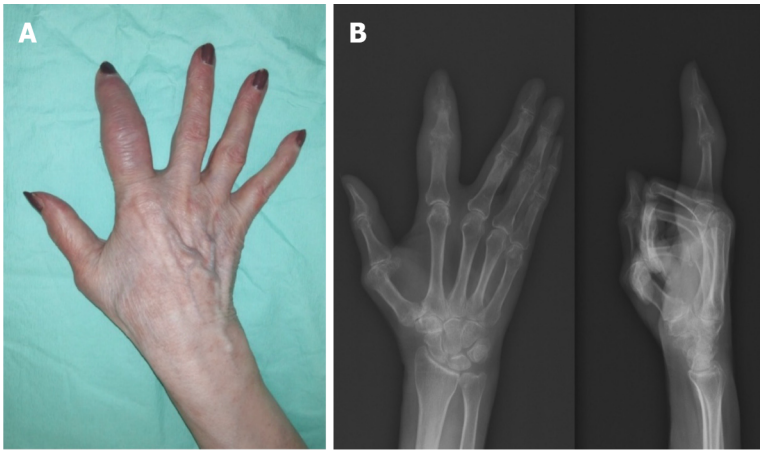

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Figure 2 Septic arthritis of the proximal interphalangeal joint of the index finger ($J_1W_0T_0$). A: Hand after repeated treatment; B: X-ray.

The symbol J_x characterizes the lesion of the osteochondral structures of the joint, where the index "x" determines the presence or absence of a sign: J_0–without osteomyelitis; J_1–with osteomyelitis.

The symbol W_x characterizes the presence of purulent wounds or fistulas in the paraarticular region: W_0–without wounds or fistulas; W_1–with a fistula; W_2–with a purulent wound.

The symbol T_x characterizes the destruction of the flexor/extensor tendons: T_0–without tendon destruction; T_1–extensor tendon destruction; T_2–flexor tendon destruction; T_3–destruction of both tendons.

Schematically, this classification can be represented below (Figure 1).

Examples of the application of this classification in the formulation of a diagnosis are shown below (Figures 2-4).

A 58-year-old woman pricked the index finger of her right hand with a needle while doing embroidery 3 wk ago. A day later, she noticed swelling of the finger and aching pain, aggravated by bending the finger. She was examined by a polyclinic surgeon and received a course of antibacterial drugs with some positive effect. Despite this, finger edema and pain on flexion persisted, which served as the basis for a second visit to the doctor. Upon further diagnostic testing, X-ray showed osteomyelitis in the proximal interphalangeal joint. On magnetic resonance imaging, there was purulent inflammation in the joint without destruction of the capsule and tendon apparatus (Figure 2).

A 54-year-old woman pricked the index finger of her left hand with a plant thorn while gardening a month ago. The next day, she noted swelling of the nail phalanx and reddening of the skin. She was self-treated using topical antiseptics and oral antibiotics (amoxicillin). The inflammation subsided, but a week ago, swelling and redness of the skin and pain reappeared in the same area. After using ointment dressings, a purulent fistula opened. X-ray showed signs of osteomyelitis in the area of the distal interphalangeal joint (DIP). On examination, there was a purulent fistula in the DIP projection. Intraop-

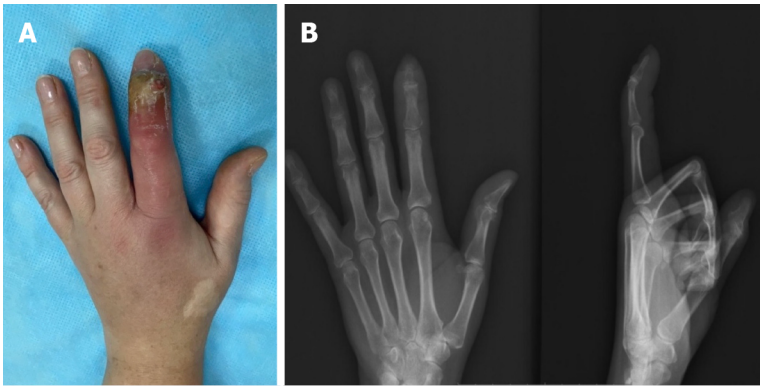

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Figure 3 Septic arthritis of the distal interphalangeal joint of the index finger ($J_1W_1T_1$). A: During hospitalization; B: X-ray.

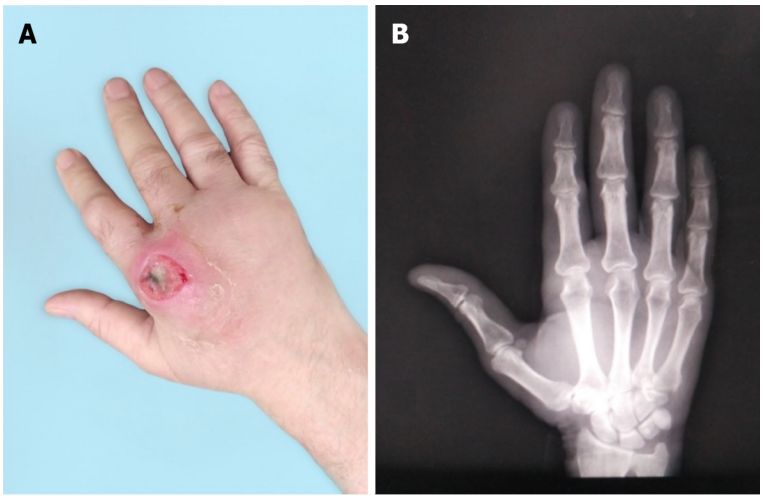

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Figure 4 Septic arthritis of the 2nd metacarpophalangeal joint of the right hand ($J_0W_2T_1$). A: During hospitalization; B: X-ray.

erative finding included the destruction of the joint capsule and extensor tendon (Figure 3).

A 30-year-old man injured his right hand as a result of a blow with a fist in a fight 10 d ago. The patient did not consult a doctor. He treated himself independently with topical antiseptics. During the last few days, the pain increased, the swelling of the hand increased, and the discharge from the wound became purulent. Wound revision revealed damage to the extensor tendon of the finger and a violation of the integrity of the capsule of the metacarpophalangeal joint and purulent exudate in the joint cavity. Upon further diagnostic testing, X-ray showed no evidence of osteomyelitis (Figure 4).

Thus, a specialized classification of septic arthritis of the hand takes into account the main pathological changes that occur in this disease: Destruction of bone and cartilage structures, paraarticular soft tissues, and tendon apparatus. Its use can help in predicting the course of the inflammatory process, determining treatment tactics, and determining the nature and timing of the start of rehabilitation measures. The use of the classification will make it possible to objectify the comparison of the results of treatment of septic arthritis of the hand presented by various authors.

FOOTNOTES

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