

The significance of HBD-3 and fluorescent composite carriers in the process of bone formation in rats infected with *Staphylococcus aureus*

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Abstract. – OBJECTIVE: The objective of the present study was to explore the significance of human β -defensin 3 (HBD-3) through establishment and evaluation of the model of implant-related biofilm infection of the femoral condyle of the outer knee using Sprague-Dawley (SD) rats.

MATERIALS AND METHODS: Age-matched SD rats were divided into three groups, the HBD-3 group, HBD-3 fluorescent liposome group, and the HBD-3 liposome-microbubble fluorescent composite carrier group. After biofilm infection for 24 h, the fluorescent composite vector was injected intraperitoneally 2 times/day. After the first injection, rats in each group were sacrificed on the 7th, 14th, and 28th day. The lower end of the femur bone was harvested after removing the surrounding soft tissue. H&E and immunohistochemical staining were applied and light microscopy was used for observation. Fluorescent markers including tetracycline and calcein were used to follow the formation of new bone *in vivo*. Undecalcified specimens were embedded in epoxy resin (thickness of roughly 150 μ m), and confocal microscopy was used for observation.

RESULTS: By assessing cell proliferation with cell counting kit-8, the proliferation ability of cells in the HBD-3 liposome-microbubble fluorescent composite carrier group was significantly increased compared with the other groups ($p < 0.05$). qPCR was used to measure the levels of alkaline phosphatase (ALP), type I collagen, osteocalcin (OCN), osteopontin (OPN), and bone sialoprotein (BSP) in each group. The levels of these genes in the HBD-3 liposome-microbubble fluorescent composite carrier group were significantly higher than those in other groups ($p < 0.05$).

CONCLUSIONS: The application of the HBD-3 liposome-microbubble fluorescent composite carrier can significantly promote osteogenesis in rats infected with *Staphylococcus aureus*, and increase the expression levels of ALP, type I collagen, OCN, OPN, and BSP.

Key Words

HBD-3, Fluorescent composite carrier, Osteogenesis.

Introduction

Presently, implant-related infection of bones and joints has become a serious challenge for orthopedic doctors¹. In the United States, the average cost for treatment of artificial joint infection is over \$50,000, and the annual cost could rise as high as \$1 billion¹. Persistent infection surrounding implants and chronic osteomyelitis often require thorough debridement, while surgical treatment can only remove the majority of bacteria and necrotic tissue that is visible to the naked eye. Even when implants are removed, remnants of bacteria and biofilms remain that could not be completely removed, which often become a recurrent source of infection.

Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis* are the most common types of drug-resistant bacteria that cause infection of orthopedic implants². The root causes of infections, which are difficult to treat, include the formation of bacterial biofilms on the surface of implants, the lack of immunity at the implant/tissue interface, and the presence of biofilms, which leads to bacterial variation³. Studies⁴ showed that once biofilms form after the bacterial invasion, the dosage of antibiotics required to remove the bacteria was 500-1000 times that used to eliminate bacteria from body fluids. Our previous research⁵ showed that human β -defensin-3 (HBD-3) used at twice the minimum inhibitory concentration not only inhibited the formation of MRSA and had a significant bactericidal effect on bacteria in the biofilm, but also inhibited the formation of biofilms and the expression of drug resistant genes. However, our previous study⁵ did not find a suitable carrier for HBD-3, which is required to carry as much HBD-3 as possible to sites of inflammation and release it to treat infection and promote osteogenesis.

By increasing drug coating ability, this investigation combined fluorochromes with microbubble-liposomes to specifically induce targeted fluorescence imaging and targeted release of HBD-3 at the site of inflammation. The aim of the present work was to further explore new and improved methods for the diagnosis and treatment of biofilm infection by common drug-resistant bacteria at the bone joint.

Materials and Methods

Establishment of the Model of Implant-Related Biofilm Infection of the Femoral Condyle of the Outer Knee

A lateral longitudinal incision exposed the cortical bone of the femoral condyle of rats, a hole was drilled, and a titanium nail with biofilm formation on its surface was inserted to establish and evaluate the model of implant-related biofilm infection of the femoral condyle of the outer knee.

Animal Grouping and Experiments

Age-matched Sprague-Dawley (SD) rats were divided into the HBD-3 group, the HBD-3 fluorescent liposome group, and the HBD-3 liposome-microbubble fluorescent composite carrier group. After biofilm infection was established for 24 h, the fluorescent composite vector was injected intraperitoneally 2 times/day. After the first injection, rats in each group were sacrificed on the 7th, 14th, and 28th day, and the lower end of the femur bone was harvested after removing the surrounding soft tissue. The study was approved by the Ethics Committee of Anhui Provincial Hospital of Anhui Medical University.

Osteoblast Culture

The parietal bone tissue of SD rats was cultured in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY, USA). Osteoblasts between passages 3-5 were used for experiments.

Identification of Osteoblasts

An inverted phase contrast microscope (Olympus, Tokyo, Japan) was used to observe osteoblast morphology, an alkaline phosphatase (ALP) kit (Nanjing Jiancheng, Nanjing, China) was used to stain osteoblasts; a bone Gamma-carboxyglutamic acid-containing protein radioimmunoassay (BGP RIA) kit (Nanjing Jiancheng, Nanjing, China) was used to measure the levels of osteo-

calcin and the tetracycline fluorescence method (Nanjing Jiancheng, Nanjing, China) was used to measure osteogenic activity of cells.

The Detection of Proliferation and Differentiation of Osteoblasts and Related Genes

Different dilutions of the HBD-3 liposome-fluorescent microbubble composite carrier were prepared and added to cell suspensions of 10⁴ cells/ml for incubation. An inverted microscope was used to observe cell morphology of the control group, and MMT assay (Bio-sharp, Hefei, China) was used to measure cell proliferation. Osteoblasts were cultured *in vitro* in contact with different concentrations of the fluorescent composite carrier. After 1 day, 4 days, 8 days and 16 days, the ideal conditions for allowing optimal concentrations of the fluorescent compound carrier to enter cells was determined as follows: (a) observation by transmission electron microscopy and fluorescence microscopy; (b) a cell counting kit (CCK-8) (Bio-sharp, Hefei, China) was used to detect cell proliferation; (c) the ρ -nitrophenyl phosphate (PNPP) method was used to detect ALP; (d) qPCR was used to measure the levels of osteogenic genes such as ALP, type I collagen, osteocalcin (OCN), osteopontin (OPN), and bone sialoprotein (BSP).

Statistical Analysis

SPSS 19.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. All quantitative data were expressed as mean $\pm$ standard deviation. The comparison between groups was done using One-way ANOVA test followed by LSD (Least Significant Difference). Percentage (%) was used to express the enumeration data and χ^2 -test was used for data analysis. *p*-values <0.05 were considered statistically significant.

Results

ALP Calcium-Cobalt Staining

New bone synthesis in rats was detected by conventional calcium-cobalt staining⁶, and the cytoplasm of most cells was shown in black. Cells transfected with HBD-3 liposome-microbubble fluorescent composite carrier-mediated by ultrasound were mostly triangular and polygonal, indicating that the cellular ALP content was higher, and new bone synthesis increased. Compared with the other groups,

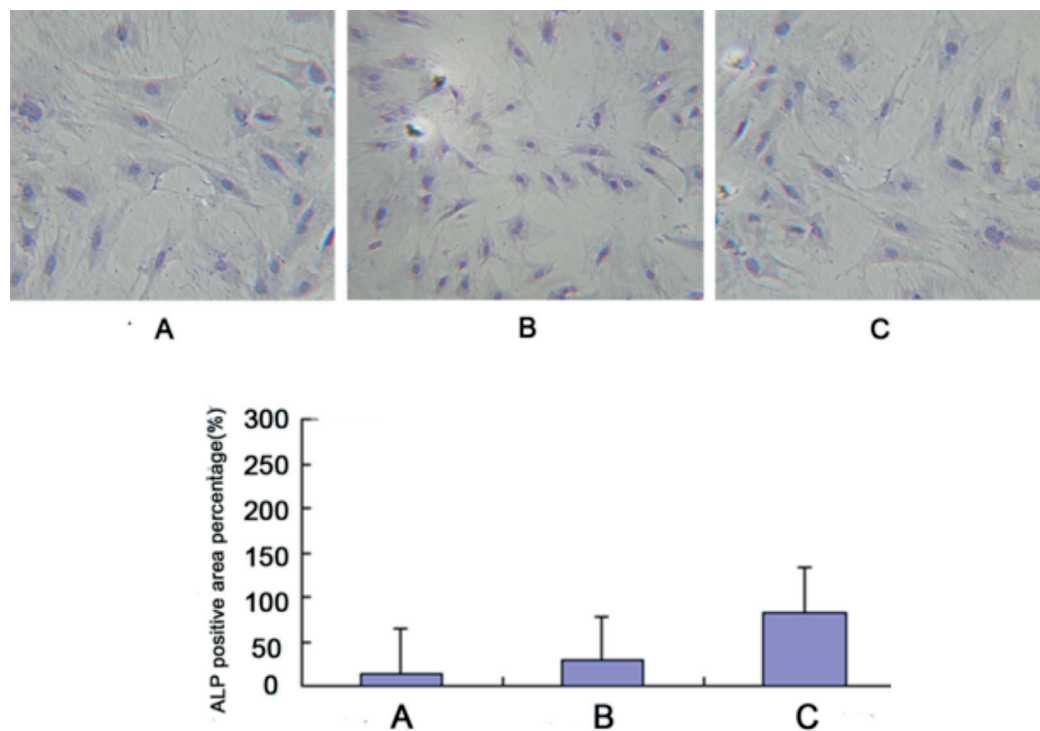


Figure 1. Inverted microscopy (200×). ALP staining in each group after osteoblasts from SD rats was cultured for 48 h. **A**, HBD-3; **B**, HBD-3 fluorescent liposome group; **C**, HBD-3 liposome-microbubble fluorescent composite carrier group.

the percentage of positive areas of cells in the HBD-3 liposome-microbubble fluorescent composite carrier group was significantly higher ($p < 0.05$) (Figure 1).

Fluorescent Staining Under the Inverted Microscope

To further examine osteoblast proliferation in rats, DAPI staining was used to observe cells under an inverted fluorescence microscope. Compared with the other groups, the number of osteoblasts was highest in the ultrasound

combined HBD-3 liposome-microbubble fluorescent composite carrier group, and the difference was statistically significant ($p < 0.05$) (Figure 2).

The Detection of Cell Proliferation by MTT Assay

To observe the proliferation of osteoblasts in the different groups, cells were cultured for 48 h and cell proliferation was determined by MTT assay. The results showed that cell proliferation in the ultrasound combined HBD-3 liposome-mi-

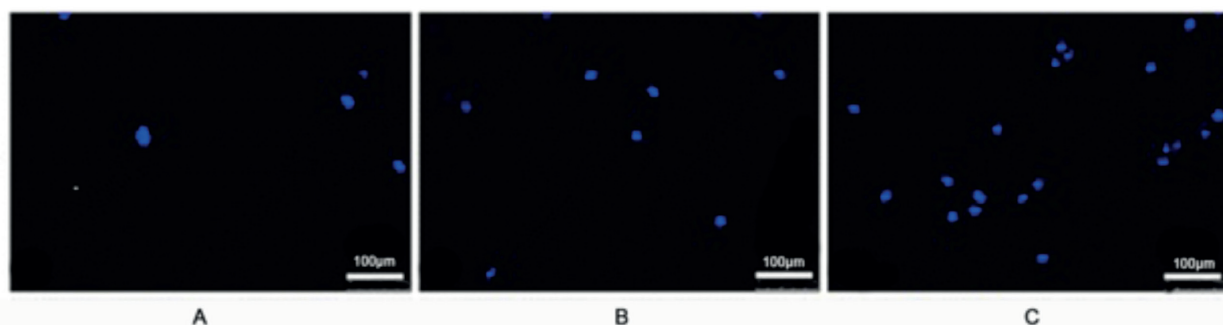


Figure 2. 200×, scalebar: 100 μm. DAPI staining, 37°C, 5% CO₂ culture for 48 h. Inverted fluorescence microscopy was used to visualize staining. **A**, HBD-3; **B**, HBD-3 fluorescent liposome group; **C**, HBD-3 liposome-microbubble fluorescent composite carrier group.

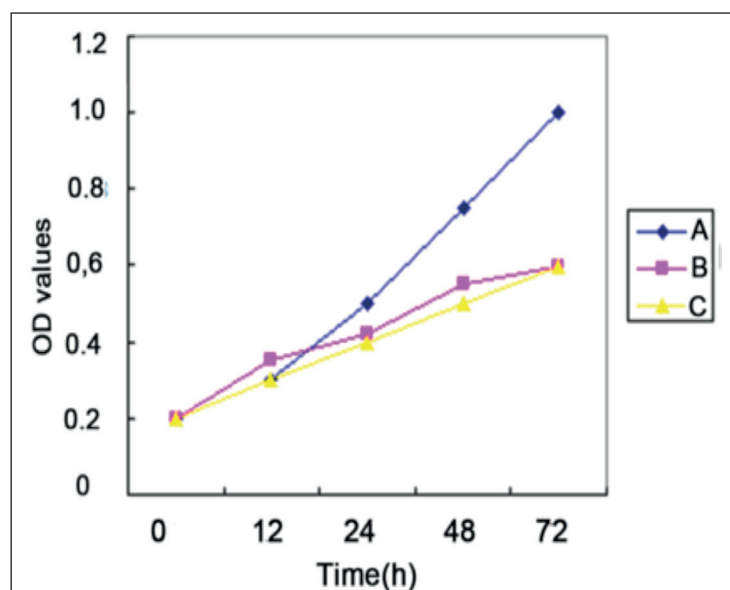


Figure 3. Cell proliferation in each group was measured by MTT assay. **A**, HBD-3; **B**, HBD-3 fluorescent liposome group; **C**, HBD-3 liposome-microbubble fluorescent composite carrier group.

microbubble fluorescent composite carrier group was significantly enhanced, the G2/M ratio was significantly increased, and the differences were statistically significant ($p < 0.05$) (Figure 3).

We also examined the cell cycle of rat osteoblasts. We found that in the HBD-3 liposome-microbubble fluorescent composite carrier group, the number of cells in the G2 phase was significantly increased. Compared with the other two groups, the difference was statistically significant ($p < 0.05$) (Figure 4).

qPCR Detection of ALP, Type I Collagen, OCN, OPN, and BSP

qPCR was used to measure the levels of ALP, type I collagen, OCN, OPN, and BSP in each group. The levels of these genes in the HBD-3 liposome-microbubble fluorescent composite carrier group were significantly higher than those in the other groups ($p < 0.05$) (Figure 5).

Discussion

HBD-3 is mainly expressed in the skin and mucosal epithelial tissue, and plays an important role in the primary barrier to external infection. HBD-3 has a strong effect in killing Gram-positive and Gram-negative bacteria, enveloped viruses, and fungi. HBD-1 and HBD-2 have a strong ability to kill Gram-negative bacteria. HBD-3 kills Gram-negative bacteria, while Gram-positive bacteria are even more susceptible to its

effects. Even low concentrations of HBD-3 can kill *S. aureus* with multiple drug resistance and Vancomycin-resistant enterococci^{5,7,8}. Harder et al⁹ isolated and cloned HBD-3 (the third member of the β -defensin family) from the dander of psoriasis in 2001.

Liposomes are composed of phospholipids, which disperse in water to form single- or multi-layered film structures, and fat-soluble or water-soluble drugs can be encapsulated within them¹⁰. Liposomes combined with antibiotics have good effects on targeting bacterial biofilms, which help to solve the problem of low concentrations of hydrophilic antibiotics around the biofilm. Liposomes can also mediate the entering of antibiotics into cells to achieve intracellular anti-bacterial activity. Liposomes are effective controlled-release carriers, which can be applied for maintaining high inhibitory concentrations *in vivo* over long periods of time to prevent drug degradation. Liposomes combined with ultrasound can increase the transfection efficiency of plasmids and enhance the anti-tumor effects of chemotherapy drugs^{11,12}. SonoVue commercial microbubbles are composed of phospholipids and contain sulfur hexafluoride gas. They have already been approved by the Food and Drug Administration for a wide range of uses in clinical ultrasound imaging¹³. Targeted imaging of the lesion at the molecular level was obtained through binding of the specific ligand on the surface of the contrast agent, and targeted therapy was achieved using microbubbles, which carried the drug¹⁴.

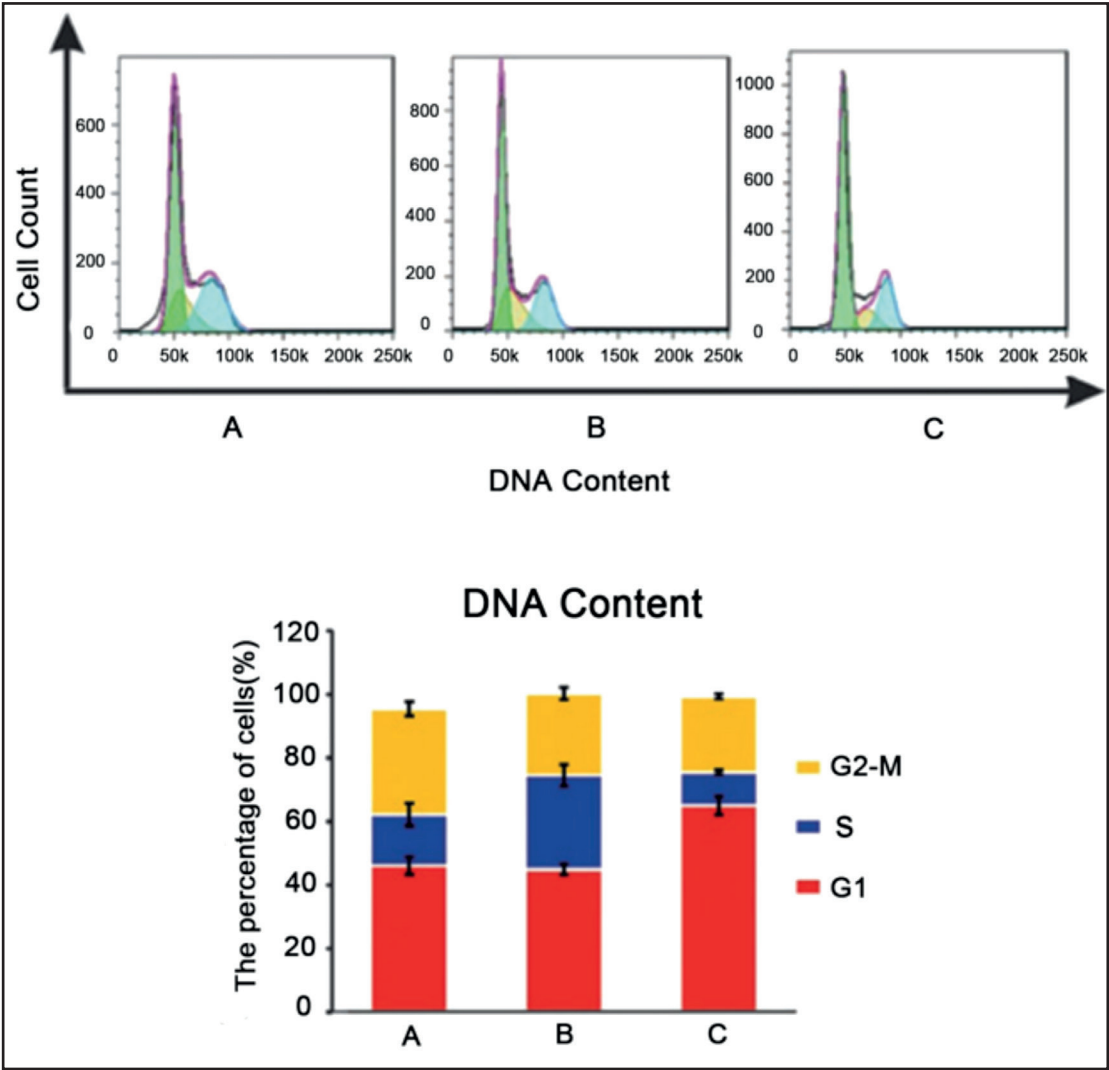


Figure 4. The determination of cell cycle after culture for 48 h. **A**, HBD-3; **B**, HBD-3 fluorescent liposome group; **C**, HBD-3 liposome-microbubble fluorescent composite carrier group.

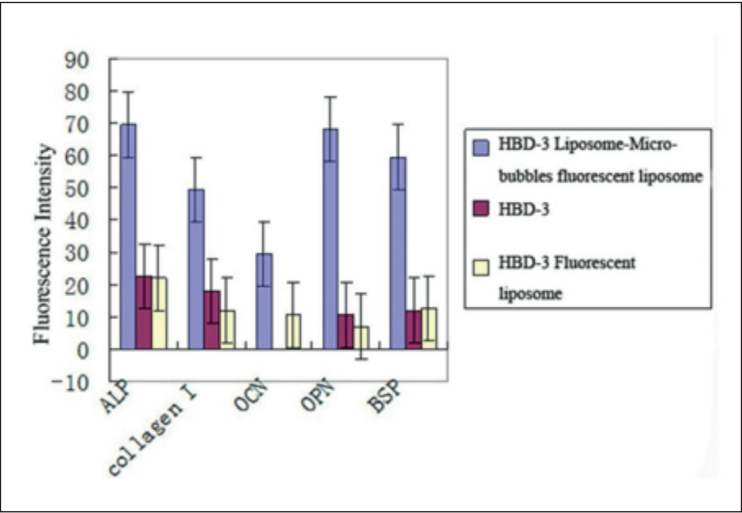


Figure 5. Measurement of ALP, type I collagen, OCN, OPN, and BSP by qPCR.

In our study, we found that cell proliferation in the HBD-3 liposome-microbubble fluorescent composite carrier group was significantly increased ($p < 0.05$). qPCR was used to measure the levels of ALP, type I collagen, OCN, OPN, and BSP in each group, and the levels of these genes in the HBD-3 liposome-microbubble fluorescent composite carrier group were significantly higher than those in the other groups ($p < 0.05$). In the inflammatory state, the secretion of β -defensin from osteoblasts is significantly increased, and it can activate and regulate the internal immune system of bone¹⁵. Preliminary results¹⁶ also showed that when mouse osteoblasts were treated with *S. aureus* supernatant, the p38 MAPK and NF- κ B signaling pathways were activated, which caused the secretion of mouse β -defensin-14, which is the homologue of HBD-3 with similar structure and function. Furthermore, the expression of HBD-3 increased in bone tissue with acute infection and the synovial membrane or membrane tissues with periprosthetic infection. Therefore, we believe that endogenous antimicrobial peptides are the first line of defense after bacterial invasion of bone tissue, which also plays a central role in exogenous bacterial infections¹⁷⁻¹⁹.

Conclusions

We suggest that the application of the HBD-3 liposome-microbubble fluorescent composite carrier can significantly promote osteogenesis in rats infected with *S. aureus*, and increase the expression levels of ALP, type I collagen, OCN, OPN, and BSP.

Conflict of Interest

The authors declare no conflict of interest.

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