Original Article

EVALUATION OF ANTI-BACTERIAL AND ANTI-BIOFILM PROPERTY OF NARINGIN LOADED NANO-HYDROXYAPATITE AGAINST STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI

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Abstract

Bacterial infections are one of the most often occurring complications with medical implants and reconstructive bone surgery, and they still remain to be one of the leading causes of failure following bone repair. One of the major challenges is treating the infection caused by microorganisms. To overcome this, systemic antibiotics are crucial for successful bone regeneration surgery. Regardless of extensive, high-dose therapies these drugs cannot reach the target surgical area and also antibiotic resistance develops. Hence, the purpose of this research was to develop a biomaterial with antibacterial potential for bone tissue engineering. The hydroxyapatite nanoparticles (n-HA) is widely employed in bone therapy owing to its good biocompatibility, bioactivity, osteoconductivity and has a topography that resembles the architecture of natural bone. Here, we present the experimental findings of the antibacterial activities of pure n-HA and n-HA+NA biocomposite compounds as assessed by Gram- positive and Gram-negative bacterial strains. According to the findings, HA nanoparticlesthat have been functionalized with naringin exhibit excellent antibacterial activity and may find use in the medical and dental fields as a bone-regenerating material.

Keywords: Nano-hydroxyapatite, Naringin, Bone tissue engineering, Anti-bacterial activity, Bone regeneration

Introduction:

Infections of the bone and tooth such as osteomyelitis, periodontitis etc., are invariably followed by inflammation and connective tissue breakdown which leads to bone loss.[1]Bacterial infections are one of the most often occurring complications with medical implants and reconstructive bone surgeries and still remain one of the leading causes of failure following bone repair. Therefore, systemic antibiotics are crucial for successful bone regeneration surgery. Regardless of extensive, high-dose therapies these drugs cannot reach the target surgical area and antibiotic resistance additionally develops in almost all thebacteria, including the most

prevalent bacteria such as S. aureus. In response to the aforementioned issues, local drug delivery devices that targets bones have been developed.[2] Though bacterial biofilm is the key etiological factor in periodontitis, the degradation of the extracellular matrix triggered by the host-mediated responses are responsible for the resultant inflammation and bone loss.[3] As a result, designing a local drug delivery system that address all of the aforementioned issues namely anti-inflammatory, anti-microbial, and bone regeneration, is more appropriate for the comprehensive treatment of periodontitis.

Nano-Hydroxyapatite (n-HA) is widely employed in bone therapy, controlled drug release and dental implant coatings owing to its good biocompatibility, bioactivity, andosteoconductivity.[4] Owing to its porous nature, n-HA facilitates adequate loading of drugs and their long-term release, which is essential for the antibacterial efficacy of a local drug delivery system. In general, n-HA has only osteoconductive potential and does not exhibit antibacterial properties.[5] Therefore n-HA can be mixed with additives such as biologically active components like collagen, growth factors etc., which can impart osteoinductive or even osteogenic properties to these materials. However, currently available osteogenic agents are particularly associated with exorbitant costs and detrimental side effects that limit their widespread application.[6]Therefore the fabrication of n-HA composites with antibacterial properties and osteoinductive potential might be an intriguing solution.

Recently, herbal medicines have gained popularity as an alternative treatment option for chronic conditions such as rheumatoid arthritis. In China, "Rhizoma drynariae," a herbal remedy, is widely used to treat orthopaedic issues, with promising results in bone healing. The primary active natural element found in a variety of Chinese herbal remedies and citrus fruits is the flavanone glycoside "Naringin" (NA).[7,8]Based on a detailed literature search, Naringin exhibits antioxidant, anti-bacterial, anti-inflammatory, anti-osteoporotic and anti- carcinogenic properties.[8] It (0.01–100mg/L) also stimulates human periodontal ligament cells proliferation by altering osteoprotegerin levels and alkaline phosphatase activity.[9] It (0.0625–

0.25 g/mL) also disrupts the growth of periodontal pathogens such as Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis.[10]

Naringin regulates the activity of osteoblasts and osteoclasts. Naringin has been foundto stimulate osteoblastic cell proliferation and differentiation[8,11] while suppressing osteoclast formation.[12-14] It also inhibits HMG-CoA reductase, activates the promotor region of bone morphogenic protein-2 (BMP-2) resulting in increased bone formation[15], and induces osteoblasts to produce osteogenic markers. Chen et al.,[14] discovered that Naringin reduces the number of osteoclasts while increasing alkaline phosphatase activity, osteocalcin levels, and bone mineral density. Furthermore, Naringin has been shown to improve bone quality in rats with retinoic acid-induced osteoporosis.[17]

In this study, we investigated the antimicrobial properties of Naringin functionalized Nano-hydroxyapatite using Gram-positive and Gram-negative bacterial strains, in order to develop novel bone repair materials by local administration of the flavonoid.

Materials and methods

Synthesis of Nano-hydroxyapatite (n-HA)

The wet-chemical precipitation technique was employed for the synthesis of nano- hydroxyapatite (n-HA) powder.[18]In this procedure, 250 ml of an aqueous solution of H_3PO_4 (0.6 M) was added gradually to 250 ml of an aqueous suspension of $Ca(OH)_2$ (1 M) and agitated with a magnetic stirrer for 2 hours at room temperature. Then concentrated NaOH was added until the pH reached 11. After rinsing with deionized water, the white suspension was dried in an oven at 80°C for 24 hours.

Synthesis of n-HA/ NA Composite

To load the Naringin molecules into the hydroxyapatite nanoparticles, the Naringin and Nano-hydroxyapatite powders (in the same weight ratio) are taken in a separate beaker. Both powders were dissolved using ethanol under constant stirring at 350 rpm for 40 minutes. The Naringin solution was then added using a dropper into n-HA solution under constant stirring for another 40 minutes. After centrifuging the suspension (2000 rpm, 5 minutes), the supernatant and precipitate were separated and dried.

Antibacterial activity

Agar well diffusion method, colony counting method and biofilm assays were used to check the antibacterial activity of n-HA and n-HA/NA nanocomposites with two different bacterial species (Gram positive S.aureus and Gram negative E.coli).

Agar well diffusion Method

The antibacterial analysis of pure n-HA and n-HA/NA nanocomposites were tested independently with two distinct bacterial species (S. aureus and E. coli) using agar well diffusion technique. Different microorganisms were initially inoculated in a Nutrient agar plate. Using a well borer, 6mm wells have been made on the nutrient agar surface and pure HA nanoparticles at various concentrations (50 mg/ml, 100 mg/ml, 150 mg/ml and 200 mg/ml) were poured into the wells and incubated for 24 hours at a temperature of 37°C. The antibacterial analysis for HA/NA nanocomposites was performed using the same procedure as previously described. The development of the inhibitory zone was assessed independently. The study was replicated thrice.[19]

Colony counting method

S. aureus and E. coli colony count assays were performed with HA nanoparticles (200mg/ ml) & n-HA/NA (200mg/ml) nanocomposite using serial dilution method. Distilledwater was used to make a 1% phosphate buffer solution. A predetermined amount of samples were dissolved in water and added to a PBS-containing bacterial inoculum. This solution was serially diluted 10^6 times with PBS and maintained at 37°C in a shaking incubator. Then 1ml of final serially diluted solution was poured over the agar culture plates and incubated at 37° C for 24 hours. The same method was repeated without adding the samples, to visualize the variations in bacterial colony development between the control group and nanocomposites. The study was replicated thrice.[19]

Biofilm Assay

The biofilm analysis was carried out using a plate technique. The bacterial cultures of S. aureus and E. coli were inoculated on nutrient agar plates and treated with nanocomposites of pure n-HA (200 mg/ml) and n-HA/NA (200 mg/ml). Then they were incubated at 37°C for 24 hours. To eliminate planktonic and weakly adherent bacteria on the surface of the culture plates, they were rinsed with 0.1 % Phosphate Buffer Solution (PBS). For assessment of residual biofilm, the washed surface was stained with 75 µl of 0.1 % crystal violet (CV) and incubated for 20 minutes. To remove the excess stain, the surface was washed with PBS several times and air dried. Using a fluorescence microscope, the plates were examined and observed. The study was triplicated.[20]

Results and Discussion:

Antibacterial Analysis:

Infections of bone graft pose a challenge in everyday clinical practice and are regarded as the most serious complications following bone regenerative surgeries. The bone grafts act like a foreign object that is initially devoid of blood supply and with low bacterial resistance.[21]Previous studies have shown that graft contamination is unavoidable and happens during surgery or during wound healing phase through the surgical incision or via bloodstream .[22,23] In addition, several studies show that contamination of the transplanted bone graft material by bacteria occurs at a rate of 0.7% and 13%, suggesting that the bacterial contamination of bone graft provokes wound infection.[24,25]

The bacteria causing bone graft contamination and wound infection are primarily organized in surface-associated biofilms that are well-structured. Gram-positive cocci, such as staphylococci as well as Gram-negative rods predominate in the biofilm found on the bone surface.[26] The pathogens such as S. aureus, P. aeruginosa, E. coli and E. faecalis have often been isolated from various biomaterial-associated infections. develop a bone graft material that is antibacterial potential as Hence, there is a need to well as osteogenic. In the present study, the research was focused on evaluating the antimicrobial potential of n-HA functionalized with naturally derived Naringin against two distinct bacterial strains (Gram positive S.aureus and Gram negative E.coli) by Agar well diffusion method, colony counting method and biofilm assay.

Agar well diffusion test:

It is one of the most widely used techniques to determine the antibacterial properties. However, this test does not totally reflect the antibacterial activity completely because it is less sensitive.[30] The findings of the test showed that after 24 hours of incubation, neither of the two nanocomposites could prevent bacterial growth or produce a growth inhibitory zone. (Figure.1) The reason for this might be that the solubility and diffusion capabilities of the nanocomposites in the agar have a significant impact on the test findings.[31]

Colony Counting Method:

The formation of biofilm begins with microbial adhesion to the surface of the biomaterial, and limiting this first step would protect various biomaterials against biofilm associated infections.[32,33] Figure 2b and 2c shows the CFUs of the initial bacterial adhesion following 24 hours incubation of the tested samples with two distinct microorganisms. In contrast to well diffusion method, the colony counting method demonstrated that both the tested biomaterials (n-HA and n-HA/NA) have inhibitory effects against both S.aureus and E.coli when compared to the control. CFUs appeared on both the control plate and the plate treated with the test samples (Figure:2b,2c).However, significant bactericidal activity was observed against S.aureus and E.coli after treatment with n-HA and n-HA/NA nanocomposites. Compared to pure n-HA, the antibacterial activity of n-HA/NA was superior, owing to Naringin's antibacterial potential. Previous studies have shown that 2′, 4′-dihydroxylation of B ring and 5, 7-dihydroxylation of the A ring are critical for significant inhibition of *Staphylococcus*.[34]Ohemeng et al. investigated the antibacterial activity of 14 flavonoids of different structure against *S.aureus, S.epidermidis* and *E. coli* and proposed thatthe inhibition of DNA gyrase was responsible for the antibacterial activity of flavonoids.[35]

Biofilm Assay:

Most bacteria that adhere irreversibly to the surface of a material or tissues have a high propensity to form a

biofilm. Biofilms are complex structures of microbial aggregation embedded in an extracellular polymeric substances (EPSs) that adhere to a solid surface.[36]The biofilms thus formed act as a protective layer against antibiotic penetration and innate immunity.[37] Therefore, anti-biofilm ability is one of the important property of biomaterials for obtaining the desired antibacterial efficacy. Nanoparticles (NPs) have recently emerged as a viable solution to this problem.[38] NPs; in particular have shown broad- spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria. Based on the existing research, the primary pathways underpinning the NPs antibacterial effects are as follows: 1) damage to bacterial cell membrane; 2) production of reactive oxygen species ;

3) penetration of the bacterial cell membrane; and 4) activation of intracellular antibacterial effects by interacting with DNA and proteins.[38]

In our study, the biofilm formation was seen in the control group, however the n-HA and n-HA/NA treated samples exhibited a substantial inhibitory effect.(Figure:3a,3b,c) Furthermore, n-HA/ NA composites outperformed the other samples in terms of antibacterial activity. These findings suggest that the incorporation of Naringin has improved the antibacterial activity of n-HA and can be employed as an antibacterial biomaterial. The mechanisms behind Naringin's antimicrobial activity are mostly unclear. It has been proposed that the presence of the hydroxyl side groups and pH values were connected with its antibacterial activity.[39] The nanocomposites (n-HA/NA) were more effective against S.aureus than E.coli which might be attributed to differences in their cell walls.

Numerous studies have proven that NPs are effective against Gram-positive bacteria than against Gram-negative bacteria. This is due to the fact that the cell wall of Gram- negative bacteria consists of lipopolysaccharides (LPS), lipoproteins, and phospholipids, which forms a barrier that selectively permits only macromolecules.[38] On the other hand, the cell wall of Gram-positive bacteria is made up of a thin layer of peptidoglycan, teichoic acid and contains numerous channels that permits foreign molecules to enter, leading to membrane damage and cell death. Furthermore, Gram-positive bacteria attract NP's more readily than Gram –negative bacteria owing to high negative charge on the surface of cell wall.[40]

Conclusion:

Overall, our results indicate that n-HA alone and when functionalized with Naringin, it showed better antibacterial activity. Therefore, n-HA/NA composite can be employed in bone implant and regenerative osseous surgeries as an antibacterial biomaterial for bone tissue engineering.

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List of Images:

Figure 1: Antibacterial activity using agar well diffusion method a) pure n-HA b) n-HA+NA

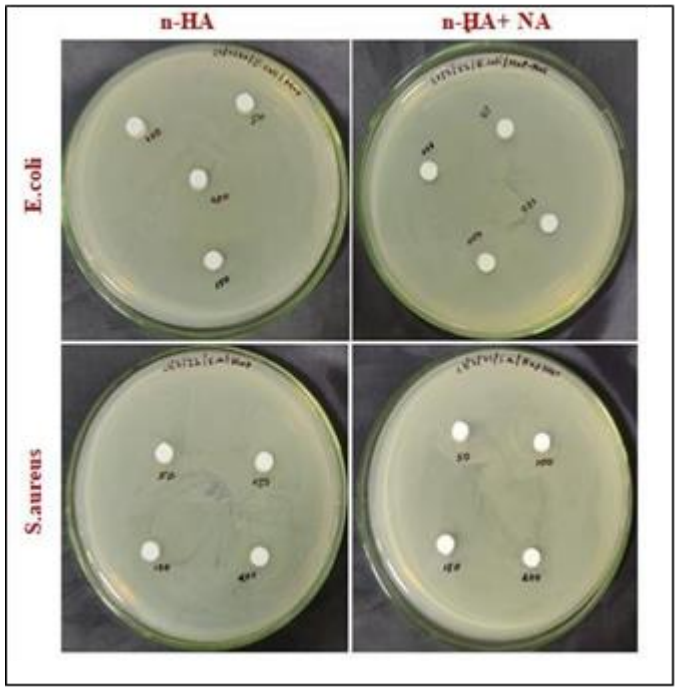

Figure 2: Antibacterial activity using colony count method a) Control b) n-HA c) n-HA+NA

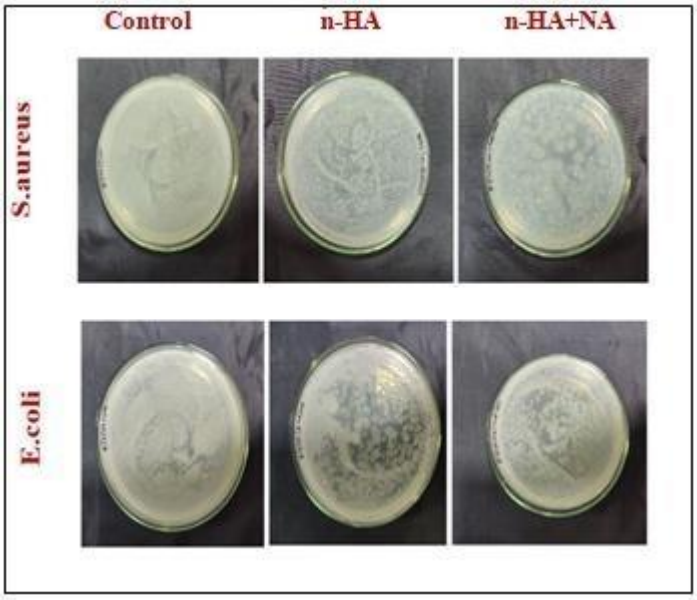

Figure 3 : Anti-bacterial activity using biofilm studies a) Control b) n-HA c) n-HA+NA