Local drug delivery in bone tuberculosis using an injectable biphasic calcium sulphate hydroxyapatite bone cement as a carrier

Irfan Qayoom[1], Deepak Bushan Raina[2], Prem Anand Murugan[1], Magnus Tägil[2], Saravanan Matheshwaran[1], Lars Lidgren[2], Ashok Kumar[1, *]

[1]Department of Biological Sciences and Bioengineering, Indian Institute of Technology Kanpur, Kanpur-208016, UP, India

[2]Department of Orthopedics, Medical Faculty, Clinical Sciences Lund, Lund University.

Email: Presenting author- sheikhirfan548@gmail.com ; * Correspondence: ashokkum@iitk.ac.in

Introduction

Bone and joints are infected in 3-5% of all the tuberculosis (TB) cases and is the most common form of extra-pulmonary tuberculosis. It mostly affects the vertebrae, metaphyseal bone and joints. It is accompanied by pain at rest and progressive bone, cartilage destruction, and commonly spinal deformity with neurological impairment (Li et al., 2015). In such cases, surgical intervention is important with complete eradication of the lesion if possible, often fixation and filling of remaining bone cavity. Emerging drug-resistant Mycobacteria necessitates long-term high dosage with multiple chemotherapeutic anti-tuberculosis drugs via systemic administration. Unfortunately, this reduces the bioavailability and adequate concentrations at the infection site and enhances the risk of re-infection. If a ceramic carrier could be used to locally deliver the TB drugs in a high, sustained and controlled manner and also have the potential to regenerate the bone simultaneously this could be advantageous. We have previously shown that an injectable biphasic calcium sulphate-hydroxyapatite (60:40) bone cement (NC) acts as a carrier for delivery of bone morphogenetic protein (BMP) and Zoledronate (ZA) and promotes the regeneration of critical sized bone defect ((Teotia et al., 2017). Here we hypothesise to use this material as a carrier of two first line anti-tuberculosis drugs, Rifampicin (RFP) and Isoniazid (INH) either alone or in combination. The controlled and sustained release of TB drugs may overcome the limitation of bioavailability giving low systemic levels and help in bone reconstruction.

Methodology

In vitro experiments were carried out after mixing the biphasic ceramic with different concentrations of RFP and INH either alone or in combination. The release kinetics of antibiotics was determined in PBS (PH=7.4) for 3 months. Biocompatibility of antibiotic impregnated NC was detected by MTT and cell material interaction was shown by SEM and PI imaging using pre-osteoblasts cell line MC3T3E1. Furthermore, antibacterial activity of antibiotic loaded NC was determined on a common test strain, *Mycobacterium Smegmatis* using disk diffusion assay, liquid inoculation assay and Resazurin assay. Intracellular killing of pathogen of the antibiotic containing NC was detected by infecting murine macrophages RAW264.7 with *M. Smegmatis* and GFP-vector transformed *M. Smegmatis* with multiplicity of infection (MOI) of 1:10. Intracellular killing was determined by counting CFU after lysis of infected cells and also by decrease or quenching of GFP expression by confocal microscopy.

Acknowledgements

IIT Kanpur (MHRD); DBT-TATA Innovation Fellowship
DBT-VINNOVA; DST-VR

Results and Discussions

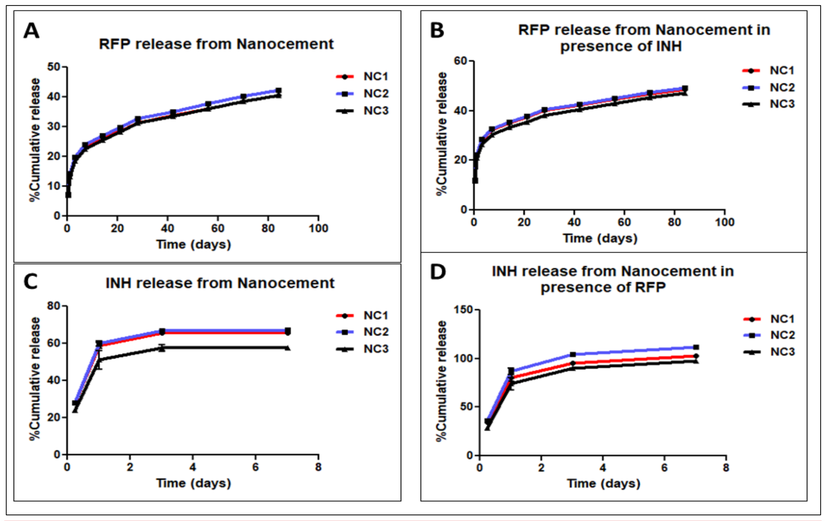

Figure 1: Release Kinetics. In-vitro release kinetics of Rifampicin (RFP) and Isoniazid (INH) when incorporated into biphasic calcium sulphate-nanohydroxyapatite bone cement (NC) for three months. The different ratios of α-CSH and nHAP (NC1, NC2 and NC3)** and it was found that this varied ratio doesn't change the release profile of either antibiotics. A) Release profile of RFP alone impregnated into NC with different ratios of α-CSH and nHAP shows that around 48% of drug was release upto 12 weeks, B) Release profile of RFP when incorporated along with INH into the different ratios of NC shows that around 55% of drug was released upto 12 weeks, C) Release profile of INH alone impregnated into different ratios NC shows that around 65% of drug was released upto one week only, D) Release profile of INH when incorporated along with RFP shows that 100% of drug was released in one week.

**NC1- 40:60 (α-CSH:nHAP)
NC2- 60:40 (α-CSH:nHAP)
NC3- 80:20 (α-CSH:nHAP)**

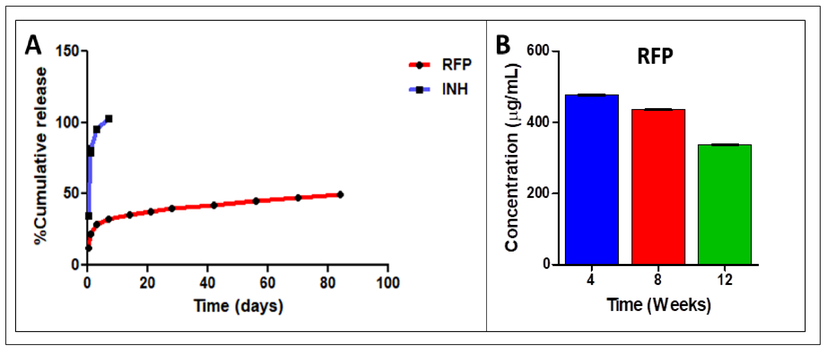

Figure 2: A) Percentage cumulative release profile of RFP and INH when both were impregnated into the NC. INH was released completely from NC within a week but RFP showed a sustained and controlled release and upto 12 weeks (3 months) only around 55% of drug was released. B) Concentration of RFP released from the NC at 4, 8 and 12 weeks. The concentration of released RFP at all time points even after 12 weeks is above the minimal inhibitory concentration (MIC) of this drug for Mycobacterium Tuberculosis (5-10 µg/ml).

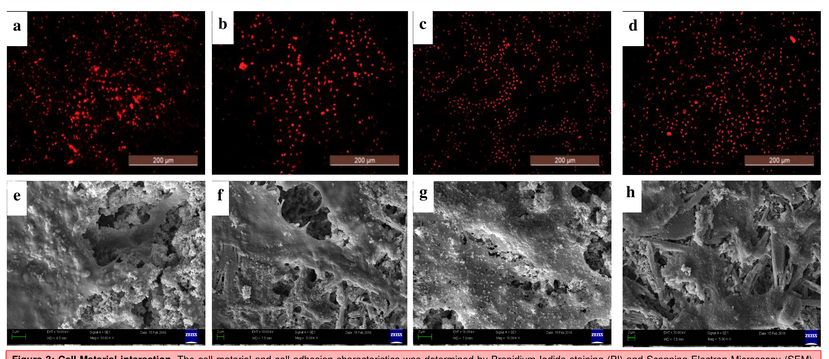

Figure 3: Cell-Material interaction. The cell material and cell adhesion characteristics was determined by Propidium Iodide staining (PI) and Scanning Electron Microscopy (SEM). MC3T3E1 were seeded onto the scaffolds and allowed to grown for 7 days. After which the cells were fixed with either 2.5% glutaraldehyde (for SEM) or with 4% Paraformaldhyde (for PI staining). PI imaging and SEM was done to evaluate the adhesion of cells onto the NC alone and NC impregnated with antibiotics. a, e) NC only, b, f) NC + INH, c, g) NC + RFP, d, h) NC + RFP + INH.

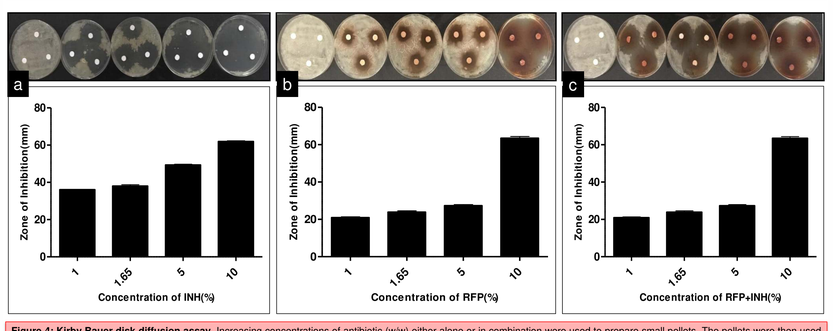

Figure 4: Kirby Bauer disk diffusion assay. Increasing concentrations of antibiotic (w/w) either alone or in combination were used to prepare small pellets. The pellets were then used to determine the Zone of Inhibition (ZOI) of *Mycobacterium Smegmatis mc2 155* that was grown for 72 hours on 7H9 agar base. a) ZOI of NC with increasing concentration of INH, b) ZOI of NC with increasing concentration of RFP, c) ZOI of NC with increasing concentration of INH and RFP.

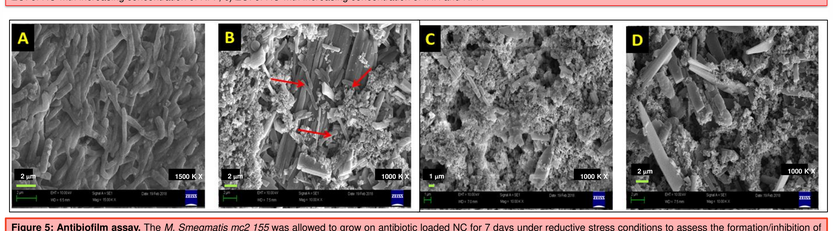

Figure 5: Antibiofilm assay. The *M. Smegmatis mc2 155* was allowed to grow on antibiotic loaded NC for 7 days under reductive stress conditions to assess the formation/inhibition of biofilm. After 30 hours, the SEM micrographs were procured and it was found that on A) NC only the bacteria formed the biofilm by secreting matrix, B) on NC + INH, few planktonic bacterium (red arrows) were seen but no biofilm formation was seen and on C) NC + RFP and D) NC + RFP + INH, no bacteria were found indicating complete death of bacteria.

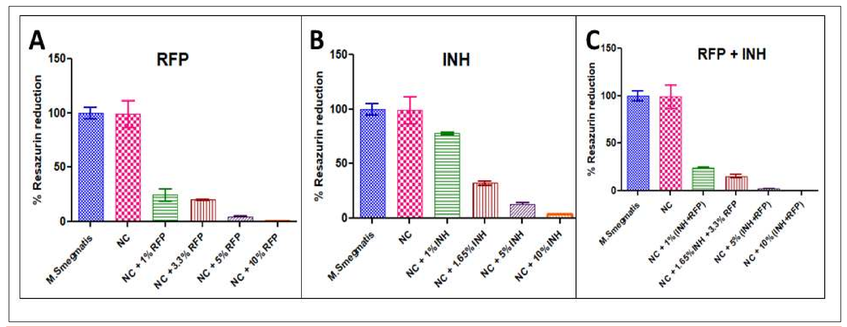

Figure 6: Resazurin assay. This assay is used to evaluate the viability of bacterial cells by assessing their respiratory potential. We used the increasing concentrations of antibiotic either alone or in combination impregnated into NC to validate the anti-mycobacterial activity of released pristine antibiotics. A) NC with increasing concentration of RFP, B) NC with increasing concentration of INH, C) NC with increasing concentration of both RFP and INH.

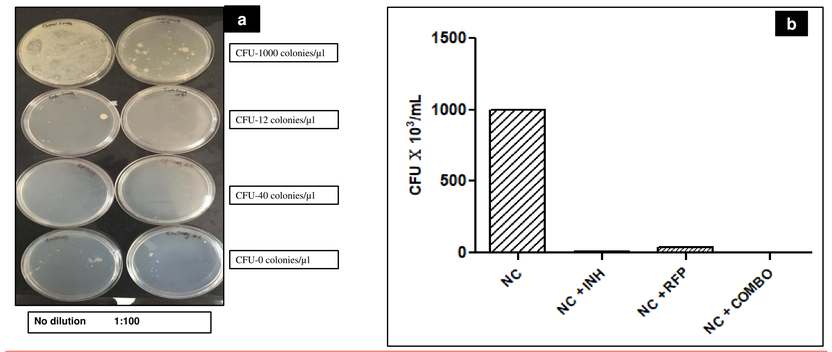

Figure 7: In-vitro Macrophage infection. Murine macrophages (RAW 264.7) cells were seeded on NC and NC containing antibiotics and infected with *M. smegmatis* for 4 hours. After 24 hours, the infected cells were lysed and cell lysate was stored. This cell lysate was serially diluted (1:100) and plated on 7H9 agar base for 72 hours. The number of colonies were counted to determine the intracellular killing ability of antibiotic impregnated nanocement. a) Colonies grown using cell lysate with dilution b) Decrease in CFU count of colonies grown using cell lysate when NC was impregnated with antibiotics.

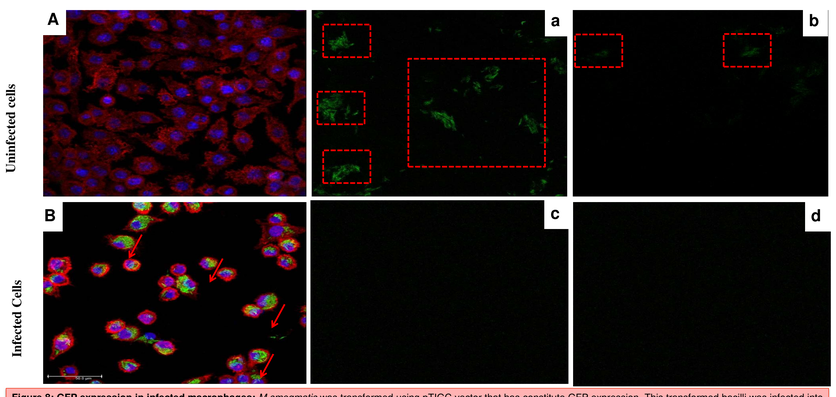

Figure 8: GFP expression in infected macrophages: *M.smegmatis* was transformed using pTIGC vector that has constitute GFP expression. This transformed bacilli was infected into murine macrophages RAW 264.7 cells for 4 hours followed by PBS-antibiotic washing to remove any extracellular bacteria. After 24 hours, the cells were fixed with 4% paraformaldehyde and stained with TRITC-phalloidin and DAPI and observed under confocal microscope. A) Uninfected macrophages B) Infected macrophages. a) NC b) NC + INH c) NC + RFP d) NC + INH + RFP)

Conclusion

In this study we have successfully evaluated the efficient activity of anti-mycobacterial antibiotics when impregnated into a biphasic calcium sulphate-nanohydroxyapatite bone cement (NC). The release of Rifampicin was found to be sustained and long term that could reduce the oral dosage of prognosis given to patients under general regimen (9-12 months). The ceramic based NC thus acts as a potent carrier of anti-tuberculosis drugs. Further, the released drugs are found to have a profound anti-bacterial activity as well as lead to elimination of intracellular bacilli from the infected macrophages under in vitro conditions. These scaffold were also found to disprove the formation of biofilm that is the main obstacle in implantation strategies and act as a prelude for revision surgeries in orthopaedic infections.

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