



Systemic rifampicin shows accretion to locally implanted hydroxyapatite particles in a rat abdominal muscle pouch model

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Abstract. Introduction: biomaterials combined with antibiotics are routinely used for the management of bone infections. After eluting high concentrations of antibiotics during the first week, sub-inhibitory concentrations of antibiotics may lead to late repopulation of recalcitrant bacteria. Recent studies have shown that systemically given antibiotics like tetracycline and rifampicin (RIF) could seek and bind to locally implanted hydroxyapatite (HA). The aim of this in vivo study was to test if systemically administered rifampicin could replenish HA-based biomaterials with or without prior antibiotic loading to protect the material from late bacterial repopulation. **Methods:** in vivo accretion of systemically administered RIF to three different types of HA-based materials was tested. In group 1, nano (n)- and micro (m)-sized HA particles were used, while group 2 consisted of a calcium sulfate/hydroxyapatite (CaS/HA) biomaterial without preloaded antibiotics gentamycin (GEN) or vancomycin (VAN), and in group 3, the CaS/HA material contained GEN (CaS/HA+GEN) or VAN (CaS/HA+VAN). The above materials were implanted in an abdominal muscle pouch model in rats, and at 7 d post-surgery, the animals were assigned to a control group (i.e., no systemic antibiotic) and a test group (i.e., animals receiving one single intraperitoneal injection of RIF each day (4 mg per rat) for 3 consecutive days). Twenty-four hours after the third injection, the animals were sacrificed and the implanted pellets were retrieved and tested against *Staphylococcus aureus* ATCC 25923 in an agar diffusion assay. After overnight incubation, the zone of inhibition (ZOI) around the pellets were measured. **Results:** in the control group, 2/6 CaS/HA+GEN pellets had a ZOI, while all other harvested pellets had no ZOI. No pellets from animals in test group 1 had a ZOI. In test group 2, 10/10 CaS/HA pellets showed a ZOI. In test group 3, 5/6 CaS/HA+GEN and 4/6 CaS/HA+VAN pellets showed a ZOI. **Conclusions:** in this proof-of-concept study, we have shown that a locally implanted biphasic CaS/HA carrier after 1 week can be loaded by systemic RIF administration and exert an antibacterial effect. Further in vivo infection models are necessary to validate our findings.

1 Introduction

In bone infections, targeted antibiotic delivery using a carrier has become an established treatment, as it gives high local concentrations and manages dead space with less systemic side effects (Hanssen and Spanghehl, 2004; Henry and Galloway, 1995). Following the introduction of antibiotic-containing polymethylmethacrylate (PMMA) bone cement, various resorbable and non-resorbable carriers were devel-

oped (Buchholz and Engelbrecht, 1970; Kanellakopoulou and Giamarellos-Bourboulis, 2000; McNally et al., 2016). Among the resorbable antibiotic carriers, biphasic calcium sulfate/hydroxyapatite (CaS/HA) bone void filler has shown encouraging clinical outcomes in the treatment of bone infections (McNally et al., 2016, 2022; Stravinskas et al., 2018). Antibiotic release kinetics is highly dependent on the chemical interactions between the antibiotic and the components of the carrier material (Funk et al., 2021; Stravinskas et

al., 2016; Stravinskas et al., 2019). After eluting high effective concentrations of antibiotics initially, non-degradable carriers release sub-inhibitory levels of antibiotics, which may lead to resistance development and infection recurrence (Anagnostakos et al., 2008, 2009; Gimza and Cassat, 2021; Neut et al., 2001; Sebastian et al., 2019). Especially PMMA was reported to become a prime surface for bacterial biofilm formation and infection recurrence (Neut et al., 2001; Sebastian et al., 2019). Currently, there is no method available for the clinicians to reload these carriers with antibiotics to treat lingering infections and to prevent bacterial attachment.

Using the unique binding properties of hydroxyapatite (HA), a major component in bone, recently Raina et al. (2020a) showed the concept of biomodulation, a novel method of targeted drug delivery, in a rat abdominal muscle pouch model. They demonstrated that a locally implanted particulate HA-containing biomaterial could act as a recruiting moiety for systemically administered HA-seeking drugs, including the antibiotic tetracycline (TET) (Raina et al., 2020a). In addition, using zoledronic acid, the authors showcased the possibility of drug reloading of a CaS/HA biomaterial. In a subsequent study, Sebastian et al. (2022a) demonstrated the antibacterial effect of locally implanted HA particles biomodulated with TET. Antibiotics other than TET like rifampicin (RIF) and daptomycin are capable of accretion to HA, potentially by chemically interacting with the calcium, phosphate, or hydroxyl groups on the HA crystal (Raina et al., 2020b). However, none of these previous studies reported systemic loading of a locally implanted carrier already containing an antibiotic.

RIF is a valuable part of the armamentarium in the treatment of osteomyelitis, prosthetic joint infections, and tuberculosis (Osmon et al., 2013). Bone infections caused by *Staphylococcus aureus* often include a minimum of 2–6 weeks treatment with oral RIF combined with an additional antibiotic given either intravenously or orally (Chen et al., 2022; Depypere et al., 2020; Osmon et al., 2013; Sebastian et al., 2021; Sendi and Zimmerli, 2017; Zimmerli and Sendi, 2019). A recent in vitro study showed that staphylococcal bone infection significantly impairs metabolic and bone formation activities of osteoblasts, and treatment with RIF may enhance bone regeneration (Alagboso et al., 2022). However, diffusion of systemically administered antibiotics into sclerotic bone is difficult (Chen et al., 2022). We recently published an in vitro study on the local addition of RIF to CaS/HA containing either gentamicin (GEN) or vancomycin (VAN) and verified extended antibacterial and biofilm effect with no viable bacteria (*S. aureus*) until study endpoint (35 d) (Sebastian et al., 2022b). The strains developed resistance when only RIF was added but not when combined with GEN or VAN (Sebastian et al., 2022b). However, no clinically approved RIF-containing carriers are today available for local delivery. In addition, there is a need for new techniques to improve the local concentration of cornerstone antibiotic for bone infections like RIF. Globally, there is an alarming in-

crease in antibiotic resistance, and all antibiotic treatment modalities should be guided by an active antibiotic stewardship.

The primary aim of this study was therefore to evaluate a novel method to load and protect an antibiotic carrier material from bacterial re-population by systemically administering RIF. In a non-infectious in vivo setting, pellets of commercially available and clinically used CaS/HA ceramic carrier containing GEN or VAN (Cerament® G or Cerament® V, Bonesupport AB, Sweden) were implanted in rat abdominal muscle pouches, and 7 d post operatively, systemic administration of RIF at clinical doses was given. The antibacterial effect of the antibiotic reloaded pellets was evaluated in vitro on planktonic bacteria. As a secondary objective, using the same methods, we also tested the RIF-seeking ability of synthetic, medical grade nano-/micro-sized HA particles (Fluidnova, S.A., Portugal) and clinically used CaS/HA biomaterial (Cerament® bone void filler, Bonesupport AB, Sweden) without any prior added antibiotic.

2 Materials and methods

2.1 Materials used

Nano (n)- and micro (m)-HA powder of < 50 nm and 10 µm particle size, respectively, were purchased from Fluidnova, S.A., Portugal. Medical grade rifampicin (Rifampicin Ebb, 600 mg; Sanofi S.p.A, Anagni, Italy) was purchased from the local pharmacy (Apoteket AB, Sweden). CaS/HA composites without any antibiotics (Cerament® bone void filler) or containing GEN (Cerament® G) or VAN (Cerament® V) were purchased from Bonesupport AB, Lund, Sweden.

2.2 In vivo biomodulation

2.2.1 In vivo biomodulation of synthetic HA particles (group 1)

Pellets of n-/m-HA were prepared by mixing 100 mg of n-/m-HA with 60 and 80 µL of hyaluronic acid (HAD, 1 mg mL⁻¹), respectively. Using the mixture, pellets were casted by transferring them to an elastic mold (Ø = 4.7 mm). Following an established muscle pouch model, eight male Sprague–Dawley (SD) rats (8–12 weeks old, average weight 363.5 g) were operated in the abdominal muscle, and each rat received n-HA (*n* = 8) and m-HA (*n* = 8) pellets on the right and left side of the abdominal midline, respectively (Fig. 1). At 7 d post-surgery, six animals received a single intraperitoneal injection of RIF each day (4 mg per rat) for 3 continuous days, and this group of animals formed the test group (*n* = 6), while the remaining animals were used as control animals (*n* = 2) and did not receive any RIF injection. Twenty-four hours after the third injection, the animals were sacrificed.

Implanted pellets devoid of muscle tissue were retrieved from the animals and placed in a 2 mL microcentrifuge tube.

Similarly, bone (trabecular and cortical), kidney, and liver tissues were collected from three animals and muscle tissue from all animals. All samples were placed in liquid nitrogen and immediately crushed to form a slurry. Using agar diffusion assay, antibacterial effect of the slurry was tested by placing them on the Muller Hinton agar plates inoculated with bacterial suspension of *Staphylococcus aureus* ATCC 25923 ($OD_{600} = 0.1$) which is sensitive to Gen, VAN, and RIF tested in this study. After overnight incubation at 37 °C, the diameter of the zone of inhibition (ZOI) around the paste was measured.

2.2.2 In vivo biomodulation of ceramic bone void fillers (group 2)

Pellets of CaS/HA were prepared by hand mixing 500 mg of CaS/HA powder in a 48-well plate by adding 215 µL iodine-based mixing solution (Iohexol[®], Bonesupport AB, Sweden), using a sterile wooden spatula for 30 s. Using the mixture, pellets were casted by transferring them to an elastic mold ($\varnothing = 4.7$ mm). Following the same surgical protocol mentioned previously, seven male SD rats (8–12 weeks old, average weight 484 g) were operated, and each rat received CaS/HA pellets on the right and left side of the abdominal midline, respectively. At 7 d post-surgery, five animals received a single intraperitoneal injection of RIF each day (4 mg per rat) for 3 continuous days (test group, $n = 5$), and remaining two animals did not receive any RIF injection (control group, $n = 2$). All animals were sacrificed 24 h after the third injection and implanted pellets devoid of muscle tissue were retrieved. Similarly, bone (trabecular and cortical), kidney, and liver tissues were collected from three animals and muscle tissue from all animals. All samples were placed in liquid nitrogen, immediately crushed to form a slurry, and then used for agar diffusion assay.

2.2.3 In vivo biomodulation of antibiotic eluting ceramic carries (group 3)

Pellets of CaS/HA containing antibiotics were prepared by hand mixing 500 mg of CaS/HA powder (Cerament G/V, Bonesupport AB, Sweden) in a 48-well plate with GEN (10.35 mg) dissolved in 283.5 µL saline (Bonesupport AB, Sweden) or VAN (24.57 mg) dissolved in 215 µL iodine-based mixing solution, using a sterile wooden spatula for 30 s. Using the mixture, pellets were casted as mentioned previously. Twelve male SD rats (8–12 weeks old, average weight 364 g) were operated, and each rat received CaS/HA-G ($n = 12$, approximately each pellet contains 2.6 mg of GEN) and CaS/HA-V ($n = 12$, approximately each pellet contains 8.2 mg of VAN) pellets on the right and left side of the abdominal midline, respectively. At 7 d post-surgery, six animals received a single intraperitoneal injection of RIF each day (4 mg per rat) for 3 continuous days (test group, $n = 6$), and remaining six animals did not receive RIF in-

jection (control group, $n = 6$). All animals were sacrificed 24 h after the third injection and implanted pellets devoid of muscle tissue were retrieved. Similarly, bone (trabecular and cortical), kidney, and liver tissues were collected from three animals and muscle tissue from all animals. All samples were placed in liquid nitrogen and immediately crushed to form a slurry and used for the agar diffusion assay.

2.3 Statistical analysis

All data were presented as mean \pm standard deviation (SD). Details of statistical analysis are indicated in each figure legend. All data processing was carried out on GraphPad Prism version 9.1.2 for MacOS (GraphPad Software, San Diego, CA, USA).

3 Results

3.1 In vivo biomodulation of synthetic HA particles (group 1)

The large ZOI ($23.3 \text{ mm} \pm 2.1$) shown by liver samples indicates the route of excretion of RIF and validity of the experimental model (Fig. 2b). No ZOI was observed with n-HA samples (Fig. 2a) and other tested tissue (Fig. 2b). Of the six m-HA samples, one showed a narrow ZOI (10 mm) (Fig. 2a). Overall, systemic RIF administration failed to biomodulate the tested synthetic HA particles (Fig. 2c).

3.2 In vivo biomodulation of ceramic bone void fillers (group 2)

None of the CaS/HA pellets (0/4) from two control animals without RIF administration showed a ZOI (Appendix A1). No muscle tissue samples showed ZOI, but, as expected, large ZOI was observed in the liver samples (Fig. 3a and b). Of the 10 CaS/HA pellets implanted in five animals (two pellets per animal), all of them showed ZOI ($13.3 \text{ mm} \pm 1.6$), confirming the recruitment of systemically administered RIF (Fig. 3a and b).

3.3 In vivo biomodulation of antibiotic eluting ceramic carries (group 3)

Both muscle and bone samples from animals with and without RIF injection showed no ZOI. Kidney samples from all animals showed ZOI indicating the route of excretion of GEN and VAN, whereas liver samples from animals that received systemic RIF injection only exhibited ZOI. Among the six animals without any systemic RIF injection, two CaS/HA-GEN samples showed ZOI ($5.2 \text{ mm} \pm 8$), but none of the CaS/HA-VAN showed any antibacterial effects (Fig. 4a–c). Of the animals that received systemic RIF, 5/6 ($9 \text{ mm} \pm 4.7$) and 4/6 ($5.5 \text{ mm} \pm 4.3$) CaS/HA-GEN and CaS/HA-VAN samples showed ZOI, respectively (Fig. 4a–c). Systemic RIF injection significantly improved

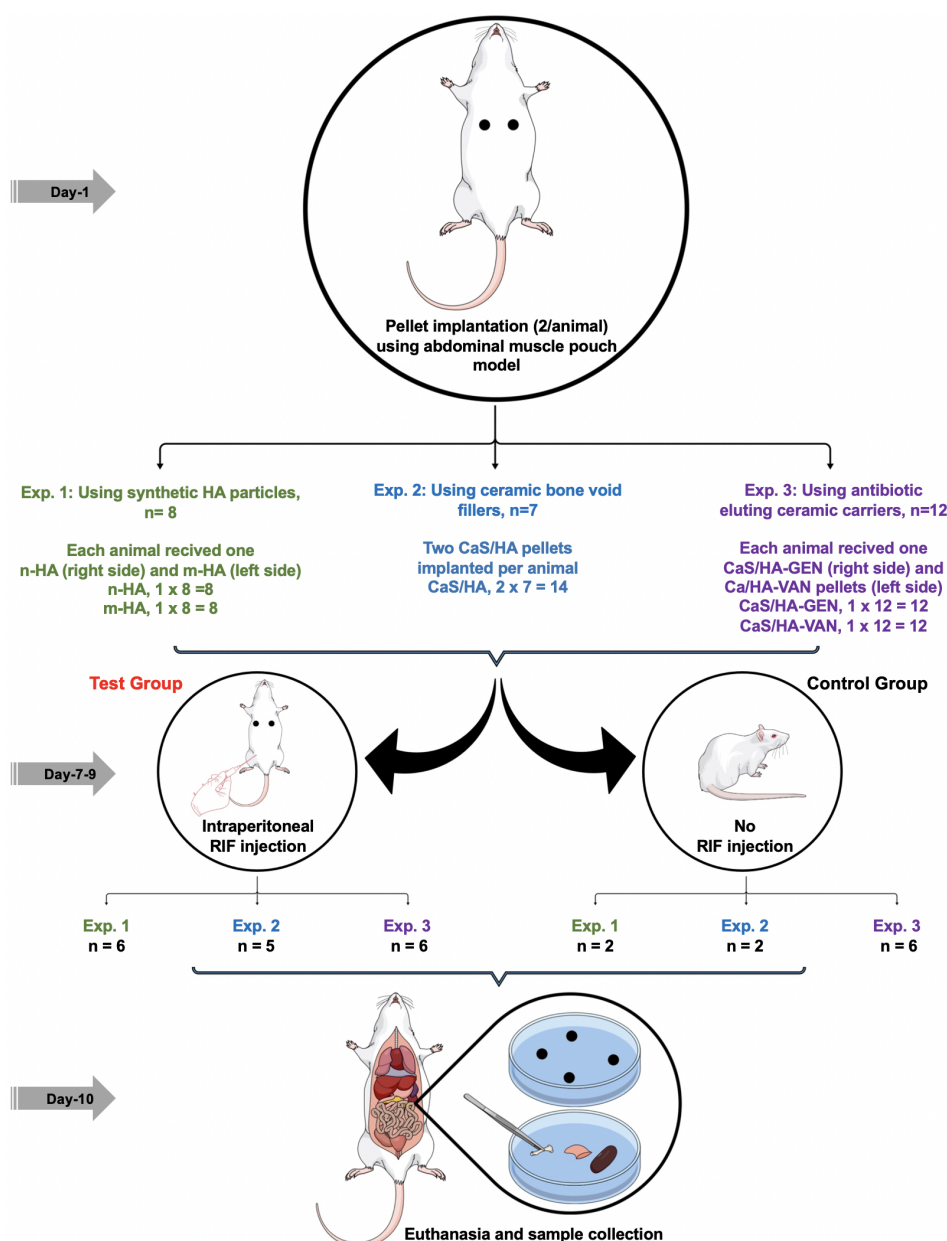


Figure 1. Details of the in vivo procedures performed in the study.

the antibacterial effect of CaS/HA-VAN. More CaS/HA-GEN samples showed antibacterial effect following systemic RIF injection, but the difference was not significant.

4 Discussion

Systemic loading of locally implanted HA-based carriers using an HA-seeking antibiotic is an attractive option to improve the treatment efficacy in bone infections (Chen et al., 2022; Raina et al., 2020a, b). In this study, we found that CaS/HA-based carriers could recruit an HA-seeking antibiotic like RIF to locally exert an adequate antibacterial ef-

fect at the targeted tissue. Considering the growing clinical use of biphasic ceramic carriers, our results provide new insights into obtaining extended effective local antibacterial levels beyond the first weeks. In addition, current findings open up a possibility of giving postoperative short-term systemic RIF to load a locally implanted antibiotic containing HA biomaterial, thereby increasing the efficacy. A recently published in vivo study reports that short-term administration of high dose RIF (human equipotent to $35 \text{ mg kg}^{-1} \text{ d}^{-1}$) ameliorated antimicrobial resistance in *Staphylococcus aureus* (Gordon et al., 2021). These results supplement our findings, and a higher but short-term postoperative RIF dos-

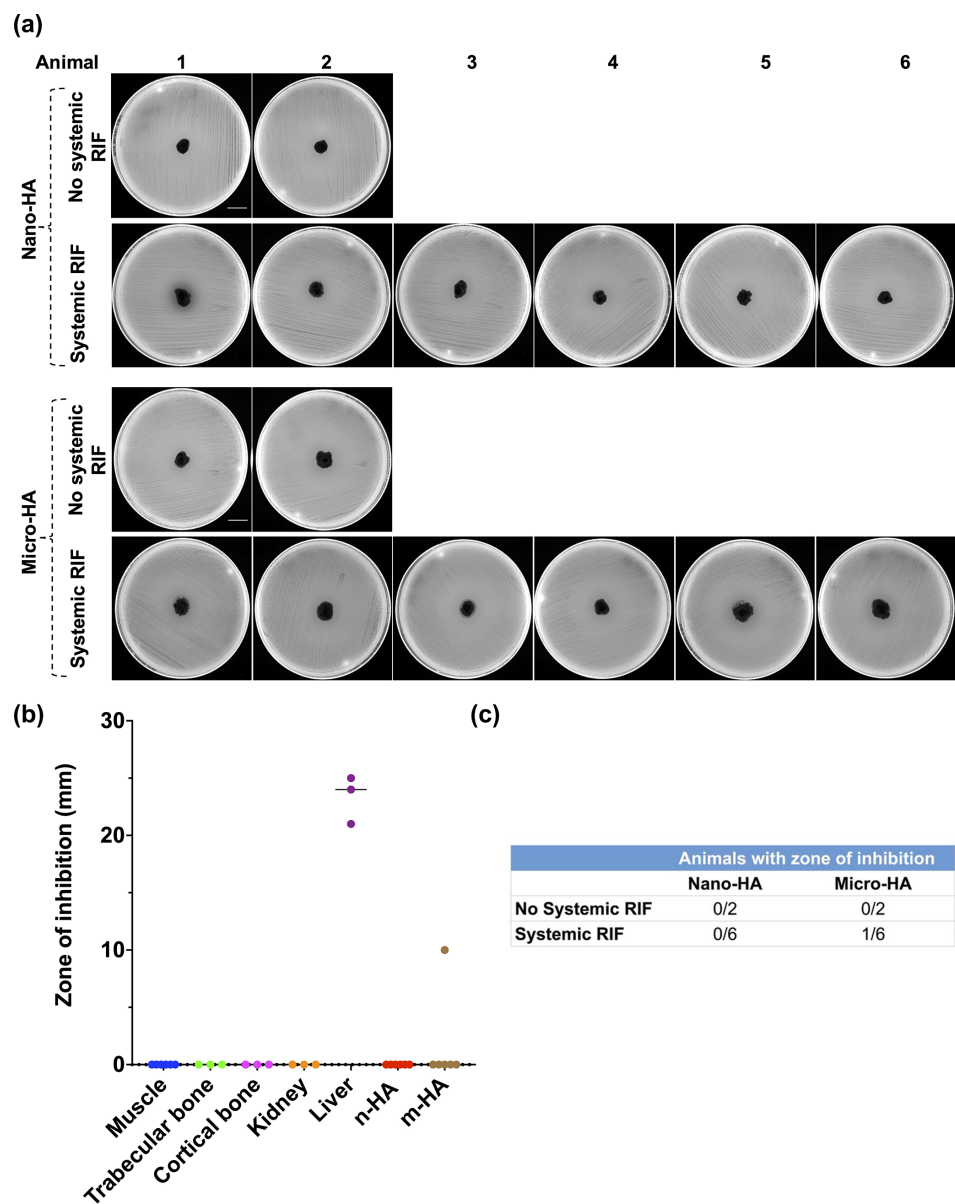


Figure 2. In vivo antibiotic biomodulation of synthetic n-/m-hydroxyapatite particles after systemic rifampicin administration. (a) Muller Hinton agar plates inoculated with *S. aureus* ATCC 25923 showing no zone of inhibition (ZOI) of both n- and m-HA particles, except one m-HA sample. Scale bar is 2.1 cm. (b) Graph showing the ZOI of tissue samples and HA particles harvested from the test animals that received systemic RIF. HA and muscle samples were collected from 6/6 animals, but bone, kidney and liver samples were collected only from 3/6. (c) Table showing the number of animals in the control and test groups wherein the implanted pellets of micro/nano HA exhibited a ZOI. Note: n-HA: nano-hydroxyapatite; m-HA: micro-hydroxyapatite.

ing regimen may further increase HA accretion and bacterial killing and thereby reduce the risk of developing resistance. Accretion to n- and m-HA particles implanted in abdominal muscle pouches of rats failed except in one case with m-HA. In a landmark study by Raina et al. (2020a), CaS/HA biomaterial containing m-HA particles in an ectopic implantation model recruited subcutaneously administered TET, an antibiotic known for its strong binding to HA particles, 2 weeks post-pellet implantation. By using a similar animal

model, Liu et al. (2021) showed the recruitment of a systemically given anticancer drug, doxorubicin, by locally implanted n- and m-HA particles. In a recent study, using an abdominal subcutaneous pouch model, Qayoom et al. (2020) reported RIF accumulation in locally implanted biphasic n-HA/CaS ceramic carrier following continuous oral RIF administration (12 mg/animal) for 4 weeks. Contrary to their report, none of our n-HA samples showed any signs of RIF accretion. However due to differences in the type of n-HA,

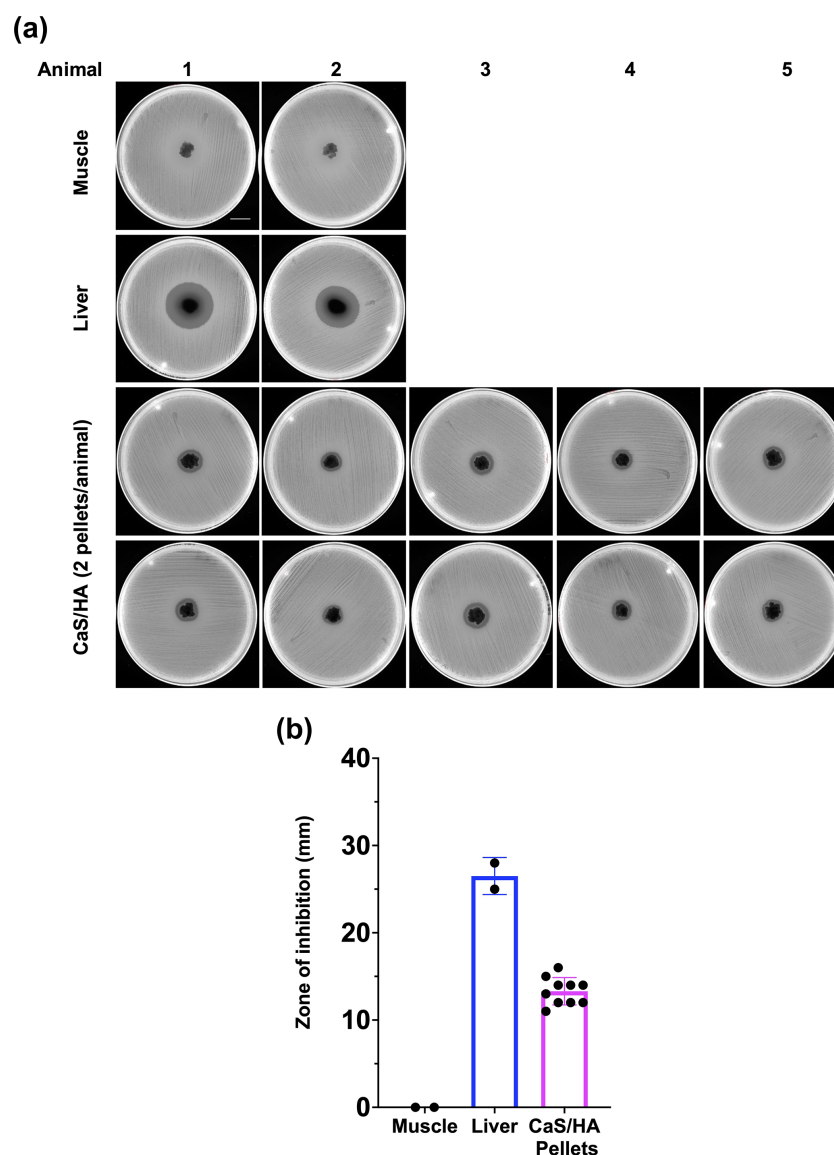


Figure 3. In vivo antibiotic biomodulation of ceramic bone void filler CaS/HA by systemic RIF administration. **(a)** Muller Hinton agar plates inoculated with *S. aureus* ATCC 25923 showing zone of inhibition (ZOI) by all RIF biomodulated CaS/HA samples. All HA samples from $n = 5$ animals ($n = 10$ pellets) were collected from rats in the test group. And $n = 2$ muscle samples and $n = 2$ liver samples were also collected from the test group rats as controls. Scale bar is 2.1 cm. **(b)** Graph showing the ZOI of tissue and CaS/HA samples harvested following RIF biomodulation.

amount, and duration of RIF administered, a direct comparison of the two studies is not possible (Qayoom et al., 2020; Raina et al., 2020a). In addition, it has been reported that the method for HA synthesis could significantly affect the chemical and structural properties (Hossain et al., 2021; Vandiver et al., 2005).

Several studies have reported successful outcomes of biphasic injectable ceramic bone substitutes for bone remodeling in clinical conditions like fractures or benign bone tumors (Iundusi et al., 2015; Kaczmarczyk et al., 2015; Kotrych et al., 2018). Although resorption of CaS occurs at

a fast phase during initial weeks, the HA particles have a slower resorption rate and they integrate with newly formed bone in a timespan of 6–12 months (Nilsson et al., 2013). This is important since we observed that HA particles can recruit systemically-given RIF and have an antibacterial effect. It has to be noted that contaminated open fractures always carry a high risk of getting a deep infection, especially using internal fixation devices (Metsemakers et al., 2018). One treatment approach would be to use the present standard, giving a conventional short-term antibiotic started early. In case a local antibiotic containing apatite is implanted in the frac-

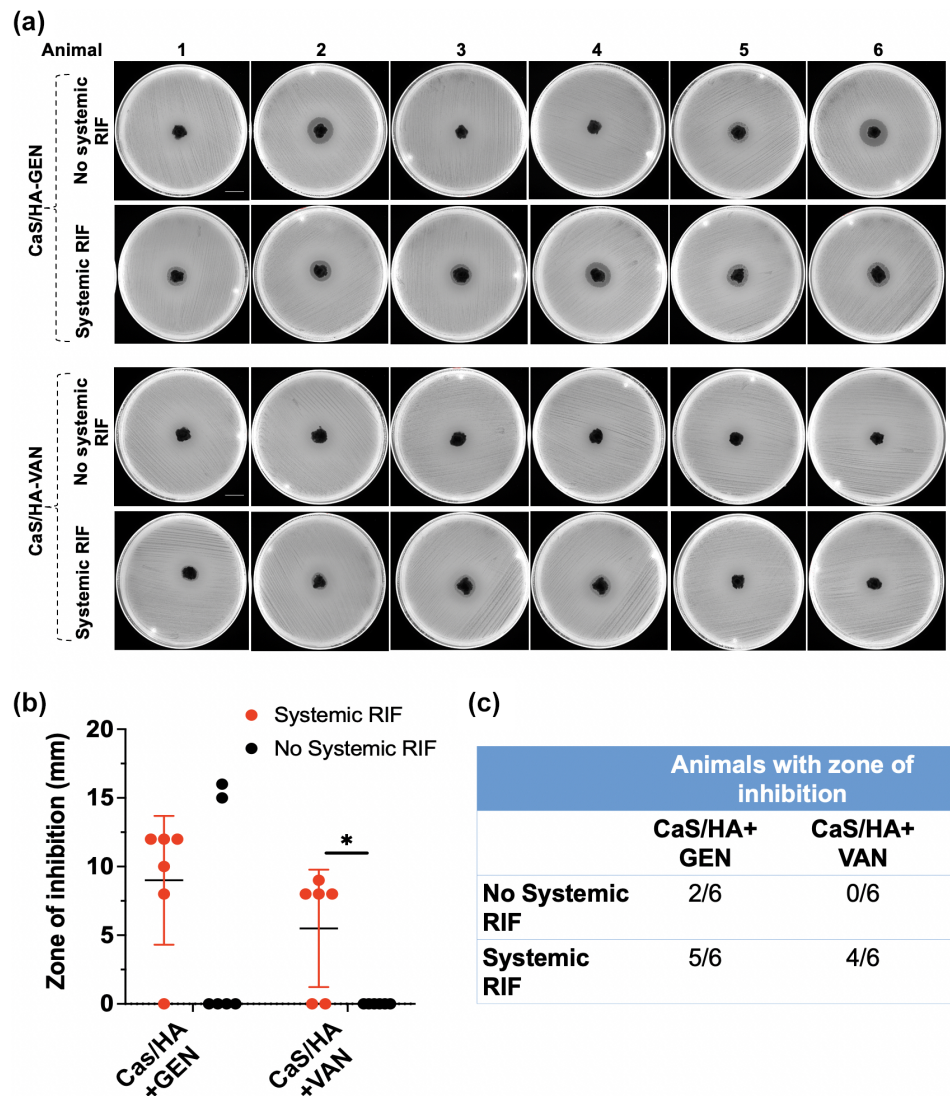


Figure 4. Differences in the in vivo antibiotic biomodulation of gentamicin (GEN) or vancomycin (VAN) eluting CaS/HA carriers (Cerament G/V) by systemic rifampicin (RIF) administration. (a) Agar diffusion assay showing the zone of inhibitions (ZOIs) exhibited by CaS/HA-GEN/VAN pellets from animals with or without RIF injections. Scale bar is 2.1 cm. A total of $n = 6$ CaS/HA+GEN and CaS/HA+VAN pellets were tested in the control group. Similarly, $n = 6$ pellets of CaS/HA+GEN and CaS/HA+VAN were tested in the experiment group. (b, c) Graph and table showing the difference in the ZOI exhibited by CaS/HA-GEN/VAN pellets with or without RIF injection. An unpaired t -test was used to compare the differences in ZOI. Note: * indicates $p < 0.05$.

ture void, by giving additional systemic RIF, an antibacterial effective concentration could be achieved locally due to HA accretion. Although RIF is a recommended second line treatment option in bone infections, due to risk of resistance, its potential “protecting the protector” role needs further research, and its HA biomodulation capability should be tested when given as combination therapy.

In a prospective observational study, patients with trochanteric hip fractures who underwent internal fixation using dynamic hip screws and application of CaS/HA-VAN, Stravinskas et al. (2019) measured VAN in urine for 20 d, indicating the elution of VAN from locally implanted CaS/HA.

Recently, an in vitro VAN release assay from CaS/HA-VAN pellets showed elution of VAN levels above the minimum inhibitory concentration of *S. aureus* for 14–21 d (Sebastian et al., 2022b). Contrary to these reports, in the present study, CaS/HA-VAN pellets did not show any antibacterial effects after day 10. However, because of the differences in the metabolic rates, physiological process, and pharmacokinetics, it has been reported that small lab animals do eliminate various antibiotics faster than humans (Freeman et al., 1997; Nair and Jacob, 2016). Since our study showed a significant antibacterial effect of CaS/HA-VAN pellets following RIF biomodulation, clinically the ideal time of RIF loading

could be after 7 d, but needs to be elucidated in further studies. Among six CaS/HA-GEN pellets obtained from animals without any systemic RIF injection, two of them showed antibacterial effects. This indicates that in spite of a faster elimination of aminoglycoside antibiotics like GEN in rats, the locally implanted CaS/HA still contains adequate levels of GEN (Freeman et al., 1997). Previous experimental and clinical studies also reported similar longer elution of GEN from CaS/HA biomaterial (Sebastian et al., 2022b; Stravinskis et al., 2016). Considering this prolonged GEN elution from CaS/HA, it could be argued that the ZOI shown by four of the six pellets from systemic RIF injection group may be from antibacterial effects conferred by remaining GEN in the pellets rather than accretion of RIF. Since more animals with RIF injection showed antibacterial effects than those without RIF injection, and comparing the biomodulation observed with CaS/HA and CaS/HA-VAN pellets, we are confident about the CaS/HA-GEN pellets RIF biomodulation. Nevertheless, it would be interesting to have further studies where systemic RIF administration could be performed after a longer postoperative period allowing the complete elution of GEN from the CaS/HA pellets.

Some limitations to the present study are noteworthy. We have tested only one HA-seeking antibiotics (RIF) in this study. However, future studies are planned to create a library of HA-seeking antibiotics and thus increase the availability of different classes of antibiotics for biomodulation. It needs to be acknowledged that this study does not provide in vivo data about the efficacy of biomodulation in infection prevention or treatment. However, being a proof-of-concept study, we believe our findings are valuable for future in vivo experiments. It should be noted that for the observed differences in RIF affinity between the pure n-/m-HA particles and biphasic CaS/HA, we could only speculate that protein passivation may have played a role, but it calls for a separate study. Further studies are needed to explore the local apatite accretion threshold (both the total amount and the duration) for different systemically administered antibiotics.

5 Conclusions

This proof-of-concept study provided valuable information on the systemic biomodulation of locally implanted HA carriers. It is important to highlight that our findings are obtained with a clinically mimicking dose of RIF. Before clinical application, relevant in vivo bone infection models should verify these findings. Future studies should also aim to identify more HA-seeking antibiotics to cover a broader bacterial spectrum.

Appendix A

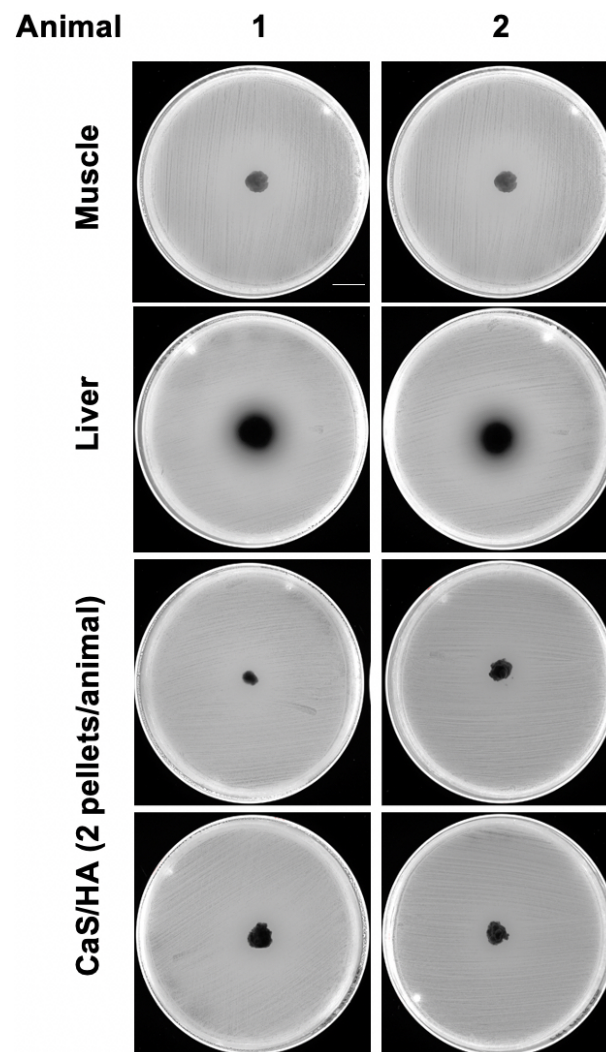


Figure A1. Muller Hinton agar plates inoculated with *S. aureus* ATCC 25923 showing no zone of inhibition (ZOI) by muscle ($n = 2$), liver ($n = 2$), and CaS/HA ($n = 4$) samples collected from group 2 control animals ($n = 2$). Scale bar is 2.1 cm.

Data availability. Data are available upon request.

Author contributions. SS, JH, and YL led the investigation. SS and YL developed the methodology. SS, JH, and YL curated the data. SS conducted the formal analysis. SS and LL acquired the funding. SS wrote the original draft. JH, YL, MC, MT, DBR, and LL reviewed and edited the paper. MC, MT, and LL supervised and collected the resources and validated the findings with DBR. DBR conceptualized the paper with LL and also administered the project.

Competing interests. Yang Liu, Deepak Bushan Raina, Magnus Tägil, and Lars Lidgren, are stockholders in Moroxite AB, Sweden. Lars Lidgren is a board member of BoneSupport AB, Sweden, and OrthoCell, Australia. All other authors declare that they have no conflict of interest.

Ethical statement. All animal experiments were conducted with prior consent from the Swedish Board of Agriculture (Jordbruksverket) following the protocol number 15288/2019. All personnel working with animals had received prior training and certification as per the FELASA guidelines. Animals were housed in pairs in ventilated and temperature-controlled rooms with 12 h day and night cycles and free access to food and water.

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