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In vitro release of vancomycin from vancomycin-loaded blood coated demineralised bone

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Abstract In vitro and in vivo studies have demonstrated the possibility that cancellous bone could be used as a carrier of antibiotics for local delivery. However, the release of antibiotics from the loaded cancellous bone is too rapid and uncertain. We hypothesised that demineralisation of cancellous bone would increase the amount of antibiotic adsorbed, and coating of the freeze-dried antibiotic-impregnated cancellous bone with bio-compatible material would prolong antibiotic release. Bovine cancellous bone blocks of equal size were demineralised using a 0.5N HCl solution and loaded with vancomycin solution under vacuum. The loaded bone blocks were then freeze-dried. To obtain a bio-compatible coating, the vancomycin-impregnated bone blocks were soaked in fresh human venous blood for 3 h. The release of impregnated antibiotic from the bone blocks was evaluated in phosphate-buffered saline and foetal bovine serum. It was found that significantly larger amounts of vancomycin were adsorbed into the demineralised bone blocks than into the un-demineralised blocks. The blood coating was found to increase the duration of vancomycin release from the blocks. With demineralisation and blood coating, the blocks eluted vancomycin higher than therapeutic concentration for a 5-week period.

Résumé Les études in vivo et in vitro ont démontré la possibilité pour l'os spongieux d'être utilisé comme un porteur d'antibiotiques pour une distribution locale. Cependant, le relargage d'antibiotiques à partir de l'os spongieux chargé est trop rapide et incertain. Nous avons

supposé que la déminéralisation de l'os spongieux augmenterait la quantité d'antibiotiques adsorbée et que le revêtement avec un matériau bio-compatible de l'os spongieux lyophilisé et imprégné d'antibiotique prolongerait le relargage. Des blocs d'os spongieux bovin de même dimension ont été déminéralisés avec une solution d'HCL 0.5 N. Les blocs ont été chargés sous vide avec une solution de vancomycine puis lyophilisés. Pour obtenir le revêtement biologique les blocs osseux ont été trempés dans du sang veineux humain frais pendant trois heures. Le relargage de la vancomycine a été évalué dans du sérum salé tamponné au phosphate et du sérum bovin fœtal. Il a été trouvé qu'une beaucoup plus grande quantité de vancomycine était adsorbée dans les blocs déminéralisés que dans les blocs non-déminéralisés. Le revêtement par l'hématome a augmenté la durée du relargage de l'antibiotique. Avec la déminéralisation et le revêtement sanguin les blocs osseux ont délivré de la vancomycine à une concentration plus élevée que la dose thérapeutique pendant 5 semaines.

Introduction

Antibiotic-impregnated polymethylmethacrylate (PMMA) has been used widely for the prophylaxis and treatment of skeletal infections [1, 3, 4, 7, 10]. The disadvantages of PMMA include the fact that it can be used only with heat-stable antibiotics and that an additional surgical procedure is necessary for its removal.

Cancellous bone has been tested as a carrier for antibiotics [6, 8, 9, 12]. Because of its osteo-conductivity and osteo-inductivity, it acts as a good defect-filler and does not need to be removed. However, release of the drug from antibiotic-impregnated cancellous bone is too rapid and uncertain, because the antibiotic merely adheres to the surface of the trabeculae.

To obtain higher antibiotic concentration over a longer period, we demineralised the cancellous bone in order to increase the amount of antibiotic adsorbed [5] and coated the antibiotic-impregnated cancellous bone with blood as

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a biological barrier to control the drug release rate. Antibiotic release was evaluated by elution in phosphate-buffered saline (PBS) and foetal bovine serum (FBS).

Materials and methods

For this study, we used freeze-dried bovine cancellous bone blocks 5×5×5 mm (Unilab Surgibone Inc., Canada), commercially available vials of vancomycin hydrochloride (Donga Pharmaceutical, Seoul, Korea), and PBS (pH 7.4) and FBS (HyClone, Utah, USA) as elution media. Twenty-four bone blocks were randomly divided into four groups of six blocks for each elution medium (PBS and FBS). Bone-block weight for study in PBS was 70.3–88.1 (mean, 79.2±5.6) mg and for FBS 59.7–81.3 (mean, 66.9±4.9) mg. There was no significant difference in bone-block weight among the four groups ($P=0.698$ and 1.000).

For demineralisation, bone blocks in groups 1 and 2 were immersed in a 0.5N HCl solution for 24 h. Multiple washings with PBS terminated demineralisation until pH 7.4 was reached. The blocks were then dehydrated by immersion in 90% ethyl alcohol for 1 h and 100% ethyl alcohol for 30 min and an overnight air-drying.

Adsorption of vancomycin into all bone blocks was achieved by placing each block into a well of a 48-well culture plate filled with 800 µl of vancomycin solution (125 mg/ml) under vacuum conditions (100 mmHg) for 24 h. The loaded blocks were then freeze-dried, and groups 1 and 3 were soaked in fresh human venous blood for 3 h. Block weight was measured before and after demineralisation, and after vancomycin adsorption.

After these procedures, the characteristics of each group were defined as follows: Group 1, demineralised and coated with blood; group 2, demineralised without blood coating; group 3, un-demineralised and coated with blood; and group 4, un-demineralised without blood coating. Each block was placed in 1 ml of elution media and incubated at 37°C. The blocks were incubated for 6 weeks, being transferred to fresh aliquots of solutions every 24 h. The removed elution fluids were preserved at -20°C for later determination of vancomycin concentration, which was measured by a fluorescence polarisation immunoassay using a TDx machine (Abbott Laboratories Inc., Abbott Park, IL, USA.) that can detect a minimum concentration of 0.6 µg/ml. Eluted solutions at days 1, 3, 5, 7, 14, 21, 28, 35 and 42 were analysed.

Bone-block weight before and after demineralisation, and the amount of vancomycin adsorbed to the blocks, were analysed using the one-way ANOVA test. All data and results acquired from day 1 to day 28 were analysed statistically using the Mann-Whitney and Kruskal-Wallis tests.

Results

There was a significant increase in bone-block weight after vancomycin adsorption. The average increase was 18.0 (±3.1) mg for demineralised bone blocks (groups 1 and 2), and 9.4 (±4.9) mg for un-demineralised blocks (groups 3 and 4). This difference was statistically significant ($P=0.001$).

Vancomycin was released quite rapidly in the first week, regardless of whether the blocks were demineralised or coated with blood in both PBS and FBS. However, the rate of release over a 6-week period was quite different in each group. Vancomycin was released more slowly over the first 7 days in the groups coated with blood (groups 1 and 3) than in the uncoated groups (groups 2 and 4) ($P=0.001$) in both PBS and FBS. In the PBS groups, there was a significant difference in concentration between the demineralised and un-demineralised groups at days 7, 14, 21 and 28 ($P=0$, 0.002, 0.004 and 0.004 respectively). The difference between the blood-coated and uncoated groups was also significant at days 1, 5, 7, 14, 21 and 28 ($P=0.001$ for days 1, 7, 14, 21 and 28; $P=0.024$ for day 5). The same statistical trend was observed in the FBS groups. The most significant difference was observed between groups 1 and 2 (all P values were 0.002 throughout the study in both media); however, group 1 showed the longest period of release. In this group, the average concentration of vancomycin at day 42 was 22.9 µg/ml in PBS and 3.0 µg/ml in FBS (Tables 1 and 2).

Table 1 Average concentration of vancomycin (micrograms per millilitre) released in phosphate-buffered saline (PBS). *D* demineralized, *B* blood coating, *U* un-demineralised

Groups/day	1 ^b	5 ^{a,b}	7 ^{a,b}	14 ^{a,b}	21 ^{a,b}	28 ^{a,b}	35	42
DB	2005.2±799.6	812.9±128.3	590.6±47.8	301.2±64.0	179.8±28.5	66.7±28.9	37.5±24.6	22.9±16.0
D	10274.2±1379.3	629.0±68.4	477.8±84.2	78.1±28.2	7.8±5.1	0	0	0
UB	3910.0±2558.9	1036.6±186.2	439.7±82.7	83.0±42.0	15.5±16.8	2.1±4.3	0	0
U	7769.2±1715.0	21.4±5.9	9.4±15.0	0	0	0	0	0

^a Significant difference in concentration between demineralised (DB and D) and un-demineralised (UB and U) groups ($P<0.05$)

^b Significant difference in concentration between blood-coated (DB and UB) and non-coated (D and U) groups ($P<0.05$)

Table 2 Average concentration of vancomycin (micrograms per millilitre) released in foetal bovine serum (FBS). *D* demineralised, *B* blood coating, *U* un-demineralised

Groups/day	1 ^b	5 ^a	7 ^b	14 ^b	21 ^b	28 ^b	35	42
DB	2154.9±389.6	634.4±238.5	308.6±152.2	127.9±90.5	64.6±52.9	21.1±30.4	12.4±22.9	3.0±9.9
D	3029.3±751.4	207.2±110.7	54.5±44.3	0	0	0	0	0
UB	2194.3±492.9	581.6±180.0	245.7±95.6	73.9±36.5	24.5±22.4	2.3±5.6	0.8±2.0	0
U	5510.3±2084.1	421.4±327.9	34.3±55.5	0	0	0	0	0

^a Significant difference in concentration between demineralised (DB and D) and un-demineralised (UB and U) groups ($P<0.05$)

^b Significant difference in concentration between blood-coated (DB and UB) and non-coated (D and U) groups ($P<0.05$)

Discussion

In 1947, De Grood [2] reported his clinical experience of treating bone defects in patients with chronic osteomyelitis by grafting autogenous cancellous bone mixed with penicillin. After the report, several *in vitro* and *in vivo* studies of antibiotic-impregnated bone were published [6, 8, 9, 12]. Those studies demonstrated the possibility that cancellous bone could be used as a vehicle for local antibiotic delivery without losing its osteo-conductivity and osteo-inductivity. However, the release of antibiotics from the antibiotic-loaded cancellous bone was so rapid that an effective local concentration of the antibiotic could be maintained only for a limited period.

Adequate debridement for skeletal infections frequently brings about large bone defects that later require bone grafting. Therefore, if more gradual release of an antibiotic could be achieved, cancellous bone would be an ideal carrier for local delivery in the treatment of skeletal infections.

This study demonstrated that demineralisation could increase the amount of antibiotic adsorbed into the bone blocks. It also showed that blood coating could slow down the initial release of antibiotic from those blocks. With a combination of demineralisation and blood coating, the concentration of eluted vancomycin in PBS at day 42 was not only higher than that of the therapeutic level of vancomycin (5 µg/ml) but also higher than the minimal inhibitory concentration (MIC) of vancomycin-intermediate resistant *Staphylococcus aureus* (17 µg/ml). In FBS, the concentration of the eluted vancomycin was higher than that of the therapeutic level over a 5-week period.

We expected a slower release of vancomycin in FBS than in PBS because the solubility of vancomycin is lower in serum than in saline. In the present study, however, the release of vancomycin was more rapid in FBS than in PBS medium. The reason for the unexpected results is not clear. However, we believe one reason could be that the bone blocks used in the FBS groups were lighter than those used in the PBS groups. An additional experiment showed that vancomycin was released longer from the heavier bone blocks when volume was the same (data not shown). Secondly, the bone blocks used in FBS groups seemed to be demineralised to a lesser degree. The same procedures were used for decalcification in both groups, but the weight reduction of the blocks after decalcification was much less in the FBS groups than in the PBS groups.

Blood coating of the blocks was found to slow down the initial rate of vancomycin release significantly. In clinical situations, most drug carriers are soaked in the host blood naturally. Therefore, it is expected that the release of a drug from its carrier would be somewhat slow-

er *in vivo* than *in vitro*. We soaked the vancomycin-impregnated bone blocks in blood and waited 3 h for the formation of blood clots. During the operation, if the drug carriers are soaked in the patient's own blood from the beginning, there would be sufficient time for the formation of blood clots before the carriers are implanted.

With demineralisation and blood coating of vancomycin-impregnated cancellous bone, the duration of release of vancomycin was prolonged for the length of time usually recommended for antibiotic administration for the treatment of chronic osteomyelitis [11]. However, the release rate of the impregnated vancomycin was still very high in the early phase. In addition to *in vivo* studies, further research will be aimed at finding methods to decrease the release rate in the early stage of treatment.

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