Estimation of drug absorption in antibiotic soaked bone grafts

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ABSTRACT

Introduction: There is paucity of literature about antibiotic uptake in bone grafts soaked in antibiotic solutions at room temperature in the operation theatre. We hypothesized that if bone grafts are dipped in different strengths of antibiotic solutions for sufficient period, their utilization at the target site helps in localized release of antibiotics in adequate inhibitory concentration to achieve the bacterial regression. The purpose of the study was to find out: (1) Optimum duration, strength, and volume of antibiotic solution required for dipping bone grafts at room temperature prior to the use. (2) What could be the clinical implications of the results obtained?

Materials and Methods: Bone shavings from total knee replacements were processed, frozen and transported to bio-analytical laboratory. The bone fragments were then impregnated with different volume and different strength of gentamicin and vancomycin over different time periods. The soaked bone samples underwent further processing for analysis on liquid chromatography tandem mass spectrometry (LC-MS/MS) system.

Results: After series of bio-analytical estimation for the soaked drug concentration among bone fragments; the optimal estimation was found with 0.2 mL of 2% strength of gentamicin and vancomycin, the optimal time was found with soakage up to 30 min. These estimated values of soaked antibiotics were five 5 times higher than required minimal inhibitory concentration (MIC) values for bacterial regression.

Conclusion: Use of antibiotic soaked bone allografts at target sites as potential drug carrier can be a hassle-free yet cost-effective and safe process for achieving maximum bacterial regression.

Key words: Antibiotic loaded bone graft (AbBGF), local antibiotic delivery, liquid chromatography tandem mass spectrometry, osteomyelitis

MeSH terms: Antibiotics, grafting, bone, chromatography, spectrometry, mass

INTRODUCTION

Local antibiotic delivery has become the mainstay for long term antibiotic management in orthopedics.¹ In infected total joint replacements, two stage surgical procedures with antibiotic cement spacer followed by revision surgery with antibiotic cement is the standard protocol.² In compound trauma, antibiotic cement spacer with a soft tissue procedure is a patient friendly technique.³ In preventive management, many surgeons are inclined towards antibiotic cement use during primary replacements to achieve excellent results.⁴ In arthroscopy, antibiotic wraps of harvested tendon grafts for anterior cruciate ligament (ACL) reconstruction is also documented in literature.⁵

Whenever conventional bone grafting with autograft or allograft is performed, we normally place “dead” bone at the operation site. Quite often, when bone grafting is done, immediately after removal of antibiotic cement spacers, or in acute compound fracture defects, the chances of infection remain high. We hypothesized that if bone graft fragments

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are dipped in sufficient strength of antibiotics for a sufficient period of time, their application at the target site could be done with the aim to have localized release of antibacterial agents in adequate inhibitory concentration to achieve the bacterial regression. We searched the literature but there is paucity of literature on antibiotic uptake on soaking bone grafts in antibiotic solutions at room temperature in the operation theatre. We could not find any literature on antibiotic uptake in fresh frozen cancellous bone.

The objective of this study was to answer following questions. (1) What should be the optimum time duration, strength, and volume of the antibiotic solution required for dipping bone grafts at room temperature in the operation theatre? (2) What could be the clinical relevance of the results obtained?

**MATERIALS AND METHODS**

Written consent was taken obtained from patients to utilize the discarded bone from shavings of total knee replacement (TKR) for further research. All the patients were in the age group of sixty to seventy (60-70) years, and were suffering from age related osteoarthritis. At the time of undergoing total knee replacement (TKR), the bone shavings are routinely discarded as biomedical waste. These shavings were cleared off articular cartilage. They were morcellized into small pieces (0.5 cm × 0.5 cm) and cleaned by a sterile pulse lavage of normal saline on the instrument trolley in the operating room [Figure 1(a-c)]. They were kept in sterile container made up of aluminum and frozen to –80°C and transported to bio-analytical laboratory for further testing.

The bone fragments were then impregnated with antibiotic concentration of gentamicin (Abbott Healthcare Pvt., Ltd., Mumbai, India) and vancomycin (Neon Laboratories Limited, Mumbai, India) of different volumes and different strengths over different periods of time for estimation of soaked drug concentration at bio-analytical laboratory.

For extraction of the drug from the bone graft, the soaked bone samples were collected on Whatman filter paper in the Petri dish to drain off the excess antibiotic [Figure 2]. They were then, dried in the oven at 60°C temperature for 30 min to evaporate excess amount of liquid in the bone fragments [Figure 3]. About 0.5 mL of 50% solution of hydrogen peroxide was added to dried bone fragments to extract the antibiotic [Figure 4]. The samples were then centrifuged at 3345 rcf for 5 min [Figure 5]. The supernatant was transferred into auto sampler vial [Figure 6] and subjected to analysis on liquid chromatography mass spectrometry tandem mass spectrometry (LC-MS/MS) system [Figure 7].

To achieve our objective of answering the two questions for the three variables: Time, strength and volume, we used six sets of experiments with only one variable at a time as shown in the Table 1.

**RESULTS**

There was no overlapping observed for peaks of both gentamicin and vancomycin concentration. Hence, the tests were not repeated and no statistical analysis was necessary.

**Concentration estimation for bone dipped in 2% drug solution over different time intervals**

The bone samples were dipped in 0.2 mL of 2% (20 mg/mL) drug solutions for different time intervals, i.e., 5, 10, 15, 30, 60 and 120 min [Table 2 and Chart 1].

Gradual increase in concentration for gentamicin and vancomycin was observed up to 120 and 30 min, respectively. There was a slope of slight decline at 30 min for gentamicin [Figure 8].

**Estimation of gentamicin concentration in bone fragments dipped in 5% drug solution for different time periods**

Each bone sample weighing 100 mg was dipped in 0.2 mL of 5% (50 mg/mL) drug solution for different time periods, i.e., 10, 20, 30, 45, 60, 75, 90, 120, 150, 180 and 240 min [Table 3 and Chart 2].

**Figure 1:** Peroperative photographs showing (a) View of the bone mill from side on instrument trolley, (b) View of the bone mill from above on instrument trolley, (c) Bone shavings from TKR morcellised on instrument trolley
Gradual increase in concentration estimation curve was observed up to the first 20–30 min time with almost consistent values till 75 min. There was slight decline noted between 90 and 125 min with further gradual increase in concentration for another 15 min reaching peak at 150 min.
Table 1: Experiments

| No   | Time | Strength | Volume          |
|------|------|----------|-----------------|
| 1    | Variable | 2% solution | 0.2 mL/100 mg |
| 2    | Variable | 5% solution | 0.2 mL/100 mg |
| 3    | 30 min | 2% solution | Variable        |
| 4    | 30 min | 5% solution | Variable        |
| 5    | 30 min | Variable up to 2% | 0.2 mL/100 mg |
| 6    | 30 min | Variable up to 5% | 0.2 mL/100 mg |

Table 2: Concentration estimation for bone dipped in 2% drug solution over different time intervals

| Time (min) | Obtained concentration for gentamicin in bone (mg/100 mg of bone) | Obtained concentration for vancomycin in bone (mg/100 mg of bone) |
|------------|-----------------------------------------------------------------|----------------------------------------------------------------|
| 5          | 3.463                                                           | 1.693                                                           |
| 10         | 3.703                                                           | 2.510                                                           |
| 15         | 3.935                                                           | 3.107                                                           |
| 30         | 3.730                                                           | 3.428                                                           |
| 60         | 4.030                                                           | 3.638                                                           |
| 120        | 4.446                                                           | 3.688                                                           |

Table 3: Estimation of gentamicin concentration in bone fragments dipped in 5% drug solution for different time periods

| Time (min) | Obtained concentration for gentamicin in bone (mg/100 mg of bone) |
|------------|-------------------------------------------------------------------|
| 10         | 3.63                                                              |
| 20         | 4.45                                                              |
| 30         | 4.45                                                              |
| 45         | 4.37                                                              |
| 60         | 4.33                                                              |
| 75         | 4.50                                                              |
| 90         | 4.80                                                              |
| 120        | 4.62                                                              |
| 150        | 5.33                                                              |
| 180        | 4.97                                                              |
| 240        | 4.81                                                              |

Concentration estimation for bone dipped in different volumes of 2% drug solution

The bone samples were dipped into different volumes of 2% (20 mg/mL) drug solution, i.e., 0.1 mL, 0.2 mL, 0.5 mL, 1.0 mL and 2 mL for 30-min time period [Table 4 and Chart 3].

Continuous increase in concentration value was observed for gentamicin and vancomycin up to 2.0 mL and 0.5 mL volume, respectively.

Estimation of gentamicin concentration in bone fragments dipped in different volumes of 5% drug solution

Each bone samples weighing 100 mg was dipped for 30 min in different volumes of 5% drug solution, i.e. 0.1 mL, 0.2 mL, 0.3 mL, 0.5 mL, 0.75 mL, 1 mL, 1.5 mL, 2 mL, 2.5 mL, 3 mL, 4 mL and 5 mL [Table 5 and Chart 4].
An increase in concentration estimation was observed for both vancomycin and gentamicin with higher concentration strengths of the antibiotics.

**Estimation of gentamicin concentration in bone fragments dipped in 0.2 mL solution of different strengths extended up to 5%**

Each bone sample weighing 100 mg was dipped in gentamicin solutions of different strengths, i.e. 0.25%, 0.5%, 2.5%, 3.76%, 4.5%, and 5% for 30 min [Table 7 and Chart 6].

Gradual increase in gentamicin concentration estimation was observed with an exponential increase in the value reaching peak with 10% strength.

**Discussion**

Previously, antibacterial concentrations were determined by bioassay, and efficient homogenization of the bone samples was often not done. As more advanced techniques for sample preparation and analysis are readily available, measurement of bone concentrations can be performed.
with better sensitivity, specificity and precision, and potentially with less bias due to incomplete recovery or drug degradation.\textsuperscript{7,8} While studies from some decades ago often reported that concentrations could not be detected in some of the samples,\textsuperscript{8} this problem is a minor issue with the advanced techniques now available.\textsuperscript{8} Probably this is the first study measuring concentration of antibiotics in the bone grafts dipped in different concentrations of antibiotic solutions.

Bone samples often contain a large amount of blood in excess of the intravascular portion due to intra operative

### Table 7: Estimation of gentamicin concentration in bone fragments dipped in 0.2 mL solution of different strengths extended up to 5%

| Concentration of gentamicin solution (%) | Obtained concentration for gentamicin immersed in 100 mg bone |
|------------------------------------------|---------------------------------------------------------------|
| 0.25                                     | 0.496                                                         |
| 0.75                                     | 1.549                                                         |
| 2.5                                      | 4.878                                                         |
| 3.76                                     | 6.963                                                         |
| 4.5                                      | 9.047                                                         |
| 5.0                                      | 10.972                                                        |

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soaking of the sample with blood. If the agent shows low tissue concentrations and high plasma concentrations, this can result in artificially high bone concentrations unless the excess blood is removed. Separation of cortical and cancellous bone is often reported, as antibacterial bone penetration may depend on the type of bone tissue that is investigated. Cancellous bone (the inner part of the long bones) contains a higher proportion of extra vascular fluid and a lower percentage of inorganic matter than cortical bone. Therefore, it is important to specify whether the analyzed specimens consist of cancellous or cortical bone, or both. Also, whether the bone specimens were properly cleaned and lavaged is an important indicator of the drug concentrations achieved.\textsuperscript{9} In this experiment, cancellous bone specimens were properly lavaged and cleaned to prevent false readings.

For determination of concentrations in the extraction fluid, microbiological methods have been frequently used especially in older studies. Usually, the supernatant after extraction of the bone tissue is analyzed, which has been found to yield more accurate results than direct application of tissue to agar plates.\textsuperscript{10} This method of adding hydrogen peroxide, centrifugation and collection of supernatant fluid is well documented and followed. This is the method which has been followed in this study. Liquid chromatography with mass spectrometry has been used for determination of gentamicin and vancomycin.\textsuperscript{11} Recent studies have usually used high-performance liquid chromatography (HPLC) with ultraviolet UV or fluorescence detection, or have not reported the method of detection.\textsuperscript{11} Böttcher et al. compared high-performance liquid chromatography (HPLC) with fluorescence detection with a microbiological assay for the determination of levofloxacin in several tissues, including bone.\textsuperscript{12} The authors concluded that HPLC was superior to a microbiological assay, although under optimized conditions the microbiological assay can yield comparable results. Most recently, liquid chromatography - mass spectrometry tandem mass spectrometry (LC-MS/MS) has been used for the analysis of garenoxacin, tigecycline, amoxicillin, and clavulanic acid in bone.\textsuperscript{12}

In summary, appropriate sample preparation and biomedical methods are vital to obtain valid results for bone concentrations. Current state-of-the-art methods include separation of cortical and cancellous bone homogenization by a cryogenic mill, extraction conditions that ensure stability of the drug and achievement of the extraction equilibrium, use of internal calibration standards prepared with blank bone tissue, and detection by high performance liquid chromatography (HPLC) or liquid chromatography tandem mass spectrometry (LC-MS/MS)
methods. Irrespective of the chosen method, the recovery, bias, and precision should be reported in detail in bone penetration studies.

Clinical relevance
Bone grafting is performed to fill bone defects and to provide biological stimulus to a healing bone. While antibacterials play key role in the treatment of bone infections and appropriate surgical prophylaxis,\textsuperscript{13} in areas of poor vascularity, by either trauma or healed infection, the addition of ‘dead’ bone for purposes of grafting can rekindle an infection.

We have been able to show for the first time from our experiments that dipping fresh cancellous bone from the lower end of femur and proximal tibia in antibiotic solutions helps achieve sufficient concentrations to ward off recrudescence of infection. The antibiotic concentrations achieved in carriers like cement or calcium sulphate granules are at least ten to hundred (10-100) times higher than what is achieved by the dipping the bone in antibiotic solutions.

The elution of antibiotics from these carriers also is well documented\textsuperscript{1} while elution of antibiotic from bone grafts is yet to be studied. So, the clinical applications for the use of antibiotic loaded cement or calcium sulphate granules are definitely different. But, this method of dipping the graft for 30 minutes would help load the graft to five 5 times the minimal inhibitory concentration (MIC) values to retard infection.

It is beyond the scope of this study to evaluate the effects of antibiotic on the osteoinductive or osteoconductive or osteogenetic properties of the bone graft. But, the amount of antibiotic attached to the graft is far lower than other antibiotic depot methods. Hence, we hypothesize that it is unlikely to affect the bone regeneration potential of the graft. Further research in the clinical setting would help to define how effective this method would be to ward off recrudescence as well as define how adversely it would affect bone healing. But, this clinical research would not be possible before establishing these parameters, as we have done with our series of experiments.

Conclusion
Bone grafts (autografts/allografts) could be dipped in antibiotic solution for achieving bacterial regression. For gentamicin, 0.2 mL of 2% solution (20 mg/mL)/100 g of bone is the optimal solution and the optimal duration of dipping could be 30 min to achieve concentration of 5 times higher the MIC values. Higher concentrations of the drug in the bone can be achieved with higher concentrations of the drug solution.

Similarly for vancomycin, 0.2 mL of 2% solution (20 mg/mL)/100 gms of bone is the optimal solution and the optimal duration of dipping could be also 30 min to achieve concentration of 5 times higher the MIC values. Higher concentrations of the drug in the bone can be achieved with higher concentrations of the drug solution.

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Conflicts of interest
There are no conflicts of interest.

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