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A novel methodology for imaging new bone formation around bioceramic bone substitutes

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Abstract: In this study, Frost's bulk-staining in combination with confocal laser scanning microscopy (CLSM) was used to image and characterize ground sections of undecalcified rat bone tissue with *in situ* bioceramic implants. This was addressed by bulk staining specimens in alcohol-soluble basic fuchsin dye. The ground sections were imaged using CLSM in the confocal fluorescence mode. Confocal images revealed that the newly formed bone could be clearly distinguished from bone marrow and cortical bone, as well as from the implant material. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 81A: 443–445, 2007

Key words: confocal laser scanning microscopy; bone; bioceramics

INTRODUCTION

An appropriate histological stain and an accurate quantitative analysis of the bone healing process by standardized histomorphometric methods are essential to validate therapeutic efficacy and address questions about cellular and tissue level response during bone repair.[1]

Frost's bulk-staining technique, based on *en bloc* staining specimens in alcohol-soluble basic fuchsin dye, is widely used to characterize bone tissue.[2–6] This procedure allows the basic fuchsin to permeate into all bone spaces, including the canaliculi and lacunae. Since basic fuchsin is both a diachrome, as it appears colored under transmitted light, and a fluorochrome,[4] basic fuchsin staining in combination with bright-field microscopy, fluorescence (UV light) microscopy, and confocal laser scanning microscopy (CLSM) has been used to evaluate undecalcified bone tissue sections from human and experimental animals.[2–6]

The aim of the present study was to image and characterize ground sections of undecalcified rat bone tissue with *in situ* bioceramic implants using Frost's bulk-staining in combination with CLSM.

MATERIALS AND METHODS

Animals

Male Wistar rats ($n = 10$) (International Laboratory Code Registry: Hsd:Wi-ffyb) weighing 100 ± 20 g were used throughout. The animals were not given a special diet. They were fed rat chow and given water *ad libitum,* housed in steel-cages, and maintained on a 12:12 h light dark cycle. The guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH Publication No. 85–23, Rev. 1985) were observed. The protocol was examined and approved by the institutional ethics committee of the School of Dentistry, University of Buenos Aires.

Surgical procedure

All the animals were anesthetized by intraperitoneal administration of a 4:1 solution of ketamine/xylazine, i.e. ketamine chlorhydrate, 50 mg/mL (Ketamina 50® Holliday-Scott, Buenos Aires, Argentina), and xylazine, 20 mg/mL (Rompun® Bayer, Buenos Aires, Argentina) at a dose of 0.15 mL/100 g body weight. The skin was disinfected and

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shaved. A longitudinal 1.5 cm incision was made along the frontal aspect of both tibiae. The subcutaneous tissue, muscles, and ligaments were dissected to expose the external surface of the tibiae in the area of the diaphyseal bone. An end-cutting bur (1.5 mm in diameter) was used to drill a hole reaching the bone marrow. Overheating and additional bone damage were prevented by using manual rotating impulsion.[7]

Boron-modified melt-derived 45S5 bioactive glass (BG) particles (300–350 μm)[8,9] were placed inside the medullary compartment of the tibiae. The wounds were carefully sutured with an absorbable polyglactin 910 suture (Vicryl, Ethicon).

The animals were killed 30 days after implantation by an intraperitoneally administered overdose of sodium pentobarbital. The tibiae were resected, fixed in 20% formalin solution, cleaned of soft tissue, and radiographed.

Preparation of specimens

The tibiae were stained following Frost's bulk-staining technique.[2,3] In brief, the bones were immersed in 20 mL of 1% basic fuchsin in absolute ethanol. The basic fuchsin solution was changed after 8 h to eliminate the water from the specimen. Twelve hours after this, the bones were placed in the basic fuchsin solution in a watch glass and allowed to evaporate until dry (about 48 h). The specimens were then rehydrated for 4 days in deionized water.

Histologic processing

The undecalcified tibiae were processed for embedding in methyl–methacrylate resin. The samples were then sectioned using a diamond saw (Exakt Apparatebau, Germany) and three slices were cut at ~500 μm, perpendicular to the major axis of the tibiae, i.e., in the middle of the implant bed and two points equidistant from the middle. The cross sections were ground using a grinding machine (Exakt Apparatebau, Germany) and finished manually with sandpaper to obtain sections about 100 μm thick, which were mounted on glass slides and coverslipped for histologic analysis.

Imaging procedure

A confocal laser scanning Zeiss Pascal LSM 5 equipped with a HeNe laser and a Zeiss Axioplan 2 imaging microscope (Carl Zeiss, Germany) was used to image specimens in the confocal fluorescence mode. Ground sections were examined using 543-nm wavelength excitation and a long-pass 560-nm emission filter. The laser was fixed to an output of 30%. A line average of 4 was applied while collecting images to reduce noise. Sequential optical sections, at an interval of 10 μm/section, were collected using a 10× (AN0.30) objective to build a 50 μm thick stack image. In addition, sequential images taken at 1 μm/section were acquired using a 40× (NA1.2) objective so that each final image had an effective thickness of 10 μm.

Images from the sample were collected uniformly at a resolution of 512 by 512 pixels. Images were acquired at an 8-bit resolution (0–256 gray levels) and were projected as a single image using Zeiss LSM 5 Image Analyser software (Version 31099).

RESULTS

Confocal images revealed that a large proportion of BG particles was surrounded by newly formed medullary bone. Bone tissue bridges between BG particles were observed [Fig. 1(A)]. A close bone-to-implant contact was detected at the interface [Fig. 1(B)]. It is noteworthy that newly formed bone could be clearly distinguished from bone marrow and cortical bone, as well as from the implant material. The methodology described herein could prove useful to perform histomorphometric analyses of the bone tissue surrounding implant materials, providing optimally thin optical sections. However, this imaging technique does not provide enough details at the cellular level, which occurs during bone healing.

DISCUSSION

This is the first study to use Frost's bulk-staining in combination with CLSM to image and characterize ground sections of undecalcified rat bone tissue with *in situ* bioceramic implants.

Bulk staining specimens in alcohol-soluble basic fuchsin dye improves and enhances visualization of the newly formed bone by distinguishing it from different histologically contiguous tissues (bone marrow and cortical bone), as well as from the implant material at the interface, as detected by CLSM. It might, however, be problematic to evaluate differences in cellular events and between woven bone, parallel-fibered bone, and lamellar bone.

Ground sections of undecalcified bone tissue with implants *in situ* may be performed with various equipments, and depending on the utilized technique the quality of the sections may vary, which may be of importance while performing histomorphometrical analysis of the bone tissue surrounding implants.[10]

Our results show that CLSM allowed rapid nondestructive optical serial sectioning of thick ground sections of undecalcified bone tissue presenting *in situ* bioceramic implants. In addition to CLSM, there are alternative methods utilized for nondestructive two-dimensional and three-dimensional images of bone ingrowth after implantation of bone substitutes.[11–13] For example, micro-computed tomography (μCT) and synchrotron X-ray μCT gave results that were highly correlated to those obtained

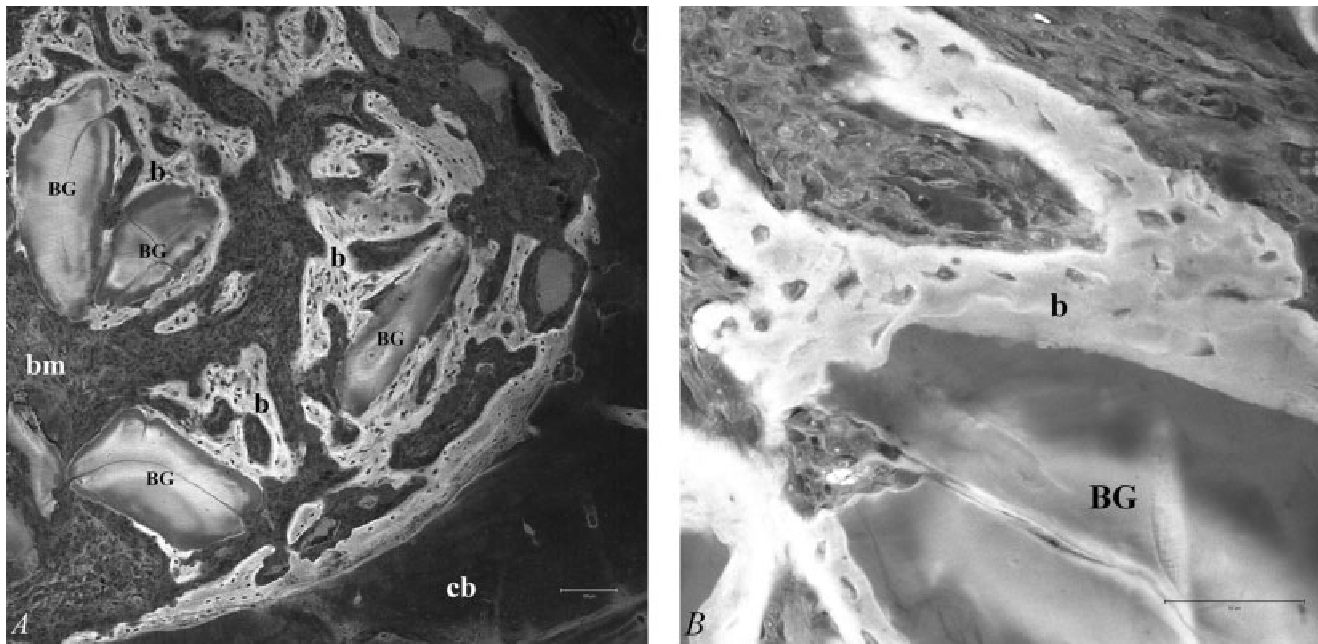

Figure 1. A: Stack of images, 50 μm thickness. B: Stack of images, 10 μm thickness. BG: bioactive glass; b: newly formed bone; bm: bone marrow; cb: cortical bone.

with scanning electron microscopy and surface staining in the cut-and-grind method.[11–13]

CONCLUSION

Herein we present a simple methodology that allows for easy visualization of new bone formation around bioceramic bone substitutes.

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