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The effect of simvastatin-loaded polymeric microspheres in a critical size bone defect in the rabbit calvaria

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ABSTRACT

The present study describes the development of a microsphere capsule based on polylactide-co-glycolide (PLGA) loaded with simvastatin that was subsequently incorporated into synthetic bone cement. The osteogenic effect of simvastatin-loaded bone cement was in a critical sized defect *in vivo* to test the hypothesis the biologic response would be different depending on the dosage of simvastatin applied to bone cement. Our results showed that simvastatin loaded PLGA microspheres can be successfully obtained through O/W emulsion/solvent evaporation method with appropriate morphologic characteristics and high encapsulation efficiency for incorporation in bone cements. The biodegradable characteristic of the microspheres successfully presented a slow release and the duration of the release lasted for more than 1 month. The *in vivo* experiment revealed that the microspheres containing simvastatin significantly enhanced bone formation in the rabbit calvaria critical size defect.

1. Introduction

Dental implant therapy is an established treatment and has become one of the most reliable options to restore edentulism. The success of implant therapy depends partly on the status of the patients' bone, and it has been proven that placing implants in fully healed ridge provides the most predictable clinical outcome (Botticelli et al., 2006). However, patients with fully healed ridges do not necessarily represent the clinical reality and clinicians are often obliged to insert the implants in compromised areas, *i.e.* inadequate bone volume area due to numerous causes.

It has been suggested that small bone defects can spontaneously regenerate to the original anatomic configuration, however, regeneration of defects that exceed a certain size requires bone augmentation using bone substitutes to fill this gap (Schwartz-Arad and Chaushu, 1997; Zeren, 2006). For this purpose, the use of autologous bone still remains the gold standard. However, limited bone-harvesting sites and patient morbidity has always been an issue, and thereby allogeneic, xenogeneic or synthetic bone substitutes have been often selected by clinicians to treat large defect cases. These materials have shown successful bone regeneration (Tovar et al., 2013; Vandeweghe et al., 2013), although potential risk of transmitting unknown diseases with the use of allogeneic or xenogeneic substitutes cannot be excluded. Ideally, but not yet the case, synthetic materials that present comparable osteogenic properties to that of the autografts would be highly desirable.

In order to enhance the bone regeneration, many research groups have attempted to incorporate bioactive agents such as proteins, and different drugs into the bone substitutes (Boden et al., 1999; Ginebra et al., 2006). One of the drugs of interest from a bone regeneration point of view, are the statins. Statins are primarily used as cholesterol-lowering drugs (Golomb et al., 2004); however, studies have proven that as a side effect, the bone forming capabilities seemed to have increased (Ayukawa et al., 2004b, 2009). In particular, simvastatin, a liposoluble statin, has its ability to increase the expression of bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor (VEGF) (Chen et al., 2010; Maeda et al., 2003). Clinical studies have also shown that simvastatin can reduce the risk of bone fracture, increase bone

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mineral density, and promote biochemical markers within bone metabolism expression to serve as a tool for the diagnosis of osteoporosis (Montagnani et al., 2003; Rejnmark et al., 2004).

A problem that remains with such drug incorporation is the difficulty to control its release rate. It has been suggested that the use of polymeric drug delivery systems could sustain drug concentration in the target site, prolonging its pharmacological activity (Uhrich et al., 1999). Thus, in the present study, a microsphere capsule based on polylactide-co-glycolide (PLGA) was loaded with simvastatin and was incorporated into synthetic bone cement. The aim of the current study was to evaluate the osteogenic effect of simvastatin-loaded microspheres incorporated into bone cements in a critical sized defect *in vivo*. This defect size was chosen since it has been known that complete bone regeneration is impossible without the use of bone augmentation procedures (Hollinger and Kleinschmidt, 1990), and it was hypothesized that the biologic response would be different depending on the dosage of simvastatin applied to bone cement, where the higher dosage would present higher osteogenesis within the defect.

2. Materials and methods

2.1. Preparation of PLGA microspheres loaded simvastatin

PLGA which molecular weight of 66,000–105,000 was purchased from Sigma–Aldrich (St. Louis, MO, USA). Polyvinyl alcohol (PVA) and dichloromethane (DCM) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Simvastatin was obtained from Teva Pharma Japan Inc. (Nagoya, Japan).

Simvastatin-loaded PLGA microspheres were prepared by O/W emulsion/solvent evaporation method (Okada et al., 1988). In brief, 10 mg simvastatin and 500 mg PLGA were dissolved in 3 mL of DCM; this solution was dropped into 300 mL of 0.25% (w/v) PVA solution at room temperature under homogenized for 5 min using PT 3100 (Polytron, Switzerland) at 10,000 rpm. The O/W emulsion was then stirred for 6 h to allow DCM, organic solvent evaporation. The microsphere suspension was centrifugated at 6000 rpm for 15 min and the microspheres were washed 3 times with distilled water. The microspheres were then frozen to −80 °C, they were freeze dried for 24 h (Freeze Dryer FD-1, EYELA, Tokyo Rikakikai Co., Ltd., Tokyo, Japan).

2.2. Microsphere characterization

The impregnated condition and the surface structure of the microsphere were confirmed by scanning electron microscopy (SEM; S-4300, Hitachi. Tokyo, Japan). The particle size was determined by measuring the feret diameter and the size distribution was obtained.

The amount of simvastatin into the microspheres was determined by high performance liquid chromatography (HPLC; LC-20 series, Shimadzu, Japan).

A reverse phase Inertsil® ODS-80A column (GL Science, Tokyo, Japan) was used for dissolving 10 mg sample of microspheres in acetonitrile, and the solution was filtered by 0.45 μm membrane into a vial for HPLC detection of the simvastatin concentration. The mobile phase was acetonitrile, delivered at a flow rate of 0.20 mL/min with a pump (LC-20AD, Shimadzu, Japan). A 5 μL of the samples was injected with an auto sampler (SIL-20AC, Shimadzu, Japan). The column effluent was detected at 238 nm by a UV-VIS detector (SPD-20A, Shimadzu, Japan). The calibration curve for the quantification of simvastatin was at 0.1–50 μg/mL with a correlation coefficient of $R^2 = 0.972$. The encapsulation efficiency was calculated based on the percentage ratio of the amount of simvastatin loaded into microspheres to the initial amount used.

2.3. In vitro release profiles of simvastatin

The amount of simvastatin released from the microspheres in simulated body fluid (SBF, prepared according to the Kokubo's method (Kokubo et al., 1990)) solution was measured by HPLC (LC-20 series Shimadzu, Japan) as the same way mentioned above, at 238 nm wavelength and the concentration was calculated with reference to calibration curve determined. For this purpose, microspheres were placed in a dialysis tube (Spectra/Por membranes MWCO 12-14,000) with 3 mL of SBF, suspended in 50 mL of same medium under a stirring speed of 250 rpm at 37 °C. The dissolution medium outside the dialysis tube was collected, filtered and the amount of simvastatin was determined at 1, 2, 3, 5, 7, 14, 21 and 28 days. The dissolution medium outside the dialysis tubing was then replaced with fresh SBF. To determine the control release profile of simvastatin from the microspheres, the mixture with 1 mg microsphere and 50 mg calcium aluminate cement (ceramir®, Doxa Dental Inc., CA, USA) was examined in the same manner as above.

2.4. Surgical procedure

This study was performed on 7 New Zealand White Rabbits and was conducted following the Ethics Committee (#13-011) for Animal Research at the École Nationale Vétérinaire d'Alfort (Maisons-Alfort, Val-de-Marne, France). All surgical procedures were performed under general anesthesia using ketamine chlorate. Thereafter, the head of the rabbit was shaved and disinfected with iodine solution. After anesthetic and disinfection procedures, a flap was raised and four bone defects of 8 mm were created (Fig. 1A and B). The bone defects were randomly designated to the following groups (Fig. 1C):

Group 1: No augmentation (sham).
Group 2: Calcium aluminate cement (ceramir®, Doxa Dental Inc., CA, USA).
Group 3: Calcium aluminate cement with 0.4 μg microspheres (0.7 ng simvastatin).
Group 4: Calcium aluminate cement with 0.04 μg microspheres (0.07 ng simvastatin).

2.5. Micro computed tomography

After 6 weeks of healing, the animals were euthanized with anesthesia overdose and the specimens were removed *en bloc* and were thereafter soaked in 4% formaldehyde. After fixation, the 3-dimensional bone formation within the defect was examined using micro computed tomography MCT-CB 130F (Hitachi Medico, Tokyo, Japan) with a slice resolution of 20 μm. Two hundred μCT slices were imaged at an X-ray energy level of 60 kV, and a current of 100 μA. All data were exported in an image file format and were imported in ImageJ (v.1.45s, National Institutes of Health, USA) for three-dimensional (3D) reconstruction, where the bone cements were subtracted from the image to observe newly formed bone within the original defect.

2.6. Histologic processing and histomorphometry

After microCT scanning, the samples were dehydrated in a series of ethanol (70–100%) and infiltration in resin (30–100%) under constant vacuuming. After complete infiltration, the samples were embedded in light curing-resin (Technovit 7200 VLC; Heraeus Kulzer Wehrheim, Germany). The embedded resin blocks were subjected to non-decalcified cut and grind sectioning to a final thickness of 30 μm and were finally stained with both toluidine blue.