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Controlled release of Clarithromycin from PLGA microspheres enhances bone regeneration in rabbit calvaria defects

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Abstract

Purpose: To evaluate the controlled release effect of Clarithromycin loaded in PLGA microspheres in a rabbit calvaria defect model.

Methods: Clarithromycin-loaded PLGA microspheres (MSPs) were formulated by modified O/W single emulsion/solvent evaporation method. After characterization, *in vivo* animal experiment was conducted. Four critical size bone defects were created in the calvaria of New Zealand White rabbits (n=21, n=7/time point). The bone defects were randomly designated to 4 groups: Group 1: No augmentation (sham), Group 2: beta-Tricalcium phosphate (β -TCP), Group 3: beta-Tricalcium phosphate (β -TCP) with 0.12 μ g clarithromycin, and Group 4: beta-Tricalcium phosphate (β -TCP) with 6.12 μ g PLGA microspheres (loaded with 0.12 μ g clarithromycin). After 2, 4 and 12 weeks of healing, the levels of bone regeneration were evaluated using micro- computed tomography and histology.

Results: The average size of the PLGA microspheres was 26.38 μ m that showed 94% encapsulation efficacy with clarithromycin. Clarithromycin release from PLGA microspheres revealed sustained release for around 4 weeks with approximately 50% release of clarithromycin during the first week. In the histological analysis, new bone formation was evident at 2 and 4 weeks of healing in all groups and bone formation increased as a function of healing time *in vivo*. At 12 weeks, Group 4 showed significantly higher amount of newly formed bone compared to Group 1 (p=0,002).

Moreover, during the micro CT exam, Group 4 expressed significantly higher bone formation compared to Group 1 at all time points tested ($p=0.00$, 0.014 , and 0.002 in 2, 4, and 12 weeks, respectively).

Conclusions: PLGA microspheres demonstrated initial burst release of clarithromycin followed by a sustained release profile. The *in vivo* findings showed that β -TCP with clarithromycin-loaded microspheres can enhance bone formation in bone defects.

1. Introduction

The loss of alveolar bone volume caused by tooth loss, trauma or tumor leads to the difficulty of the occlusal reconstruction, and decline in the quality of life [1]. In such cases, bone reconstruction becomes necessary prior to the restoration of the lost dentition. In clinical situations, some techniques and materials for bone augmentation were introduced, however, no complete reconstruction methods exist. The degree of bone healing depends on numerous factors, especially on the morphology and the type of defect [2]. In many cases, bone substitutes have been widely accepted for enhancing bone regeneration. These materials should be integrated in the body and sustain the natural healing capacity of bone, without disturbing new bone formation [3].

To enhance new bone formation, biomaterials doped with substances known to be osteoconductive were proposed and tested as filling materials for bone defects [4-6]. These biomaterials can tackle two important purposes: they are scaffolds that guide the colonization of defects by osteoprogenitor cells and provide an interface for cells to attach, proliferate and differentiate and they act as drug delivery systems. Substances as antibiotics, growth factors, and proteins have been loaded to the bone substitutes to favor bone healing in different ways[6-8]. In many studies, the early stage of bone healing was examined, however the results at longer healing times were overlooked, since a rapid burst release of the loaded substances was expected. This is presumably due to the fact that the main method for loading was dipping of the biomaterials in a medium containing the desired substance. Further, as certain substances could be more effective at later stages of the regeneration pathways, it would be desirable that they were released by bone substitutes in a sustained and controlled fashion, to influence healing at the most beneficial moment.

One interesting strategy to release the substances in a controlled manner is to utilize bioresorbable polymeric materials. In previous *in vivo* study, Poly (lactic-co-glycolic) acid (PLGA) microsphere loaded with simvastatin was incorporated within synthetic bone cement in a critical sized defect[9]. The microspheres demonstrated slow release of simvastatin for more than 4 weeks. Subsequently, higher bone formation was found in the defects. From these findings, we speculated that microspheres could become a material of choice for drug delivery, which can be load with different types of substances for sustained release.

In this study, we proposed Clarithromycin as the substance to be loaded in bone substitutes. This drug belongs to the Macrolides group and it is known to exhibit antibacterial properties against a broad-spectrum of Gram-positive bacteria and some Gram-negative bacteria[10]. It has been reported that Clarithromycin is effective in treating alveolar bone infections such as osteomyelitis[11]. Another report has shown that the use of Clarithromycin diminished the degree of mucositis[12] and presented promising outcomes in terms of suppressing infections in the oral cavity[13]. Moreover, as an additional effect, Clarithromycin was reported to exhibit anti-inflammatory properties, which may be effective for the treatment of chronic inflammation [14-16]. It is known that excessive inflammatory process can disturb new bone formation. It can be suggested that the use of Clarithromycin has the possibility to accelerate bone remodeling through antibacterial and anti-inflammatory properties[17, 18].

The purpose of this study is to confirm the sustained release of clarithromycin from PLGA microspheres within beta-TCP *in vitro* and to examine the efficacy of this materials as the bone substitute materials in rabbit calvaria defect model.

2. Materials and methods

2.1 Preparation of clarithromycin loaded PLGA microspheres (MSPs)

Poly(lactic-co-glycolic acid) (PLGA), Polyvinyl alcohol (PVA) and all organic solvents (dichloromethane (DCM), acetonitrile, ethanol and acetone) were prepared for fabricating MSPs (Wako Pure Chemical Industries, Osaka, Japan). Clarithromycin was kindly provided by Taisho Pharmaceutical Co., Ltd. (Tokyo, Japan).

Clarithromycin-loaded PLGA microspheres (MSPs) were formulated by modified O/W single emulsion/solvent evaporation method[19]. Briefly, 10 mg clarithromycin and 500 mg PLGA were dissolved in 3 mL of DCM. This mixture was dropped into 300 mL of 0.25% (w/v) PVA solution and homogenized at 10,000 rpm by PT 3100 (Polytron, Kinematica AG, Luzern, Switzerland) under room temperature for 3 min. The formed O/W emulsion was then stirred for 6 hours to remove DCM and allow evaporation of the organic solvent. Later, The MSPs suspension was centrifuged at 6,000 rpm for 5 min followed by vigorous washing for the MSPs in distilled water. MSPs were then frozen to -80°C for 30 min. then, they were freeze-dried for 24 hours using freeze dryer (FD-1; EYELA, Tokyo Rikakikai Co., Ltd., Tokyo, Japan).

2.2. Characterization of MSPs

For the determination of the optimal formulation parameters to prepare the MSPs, surface morphology, particle size and encapsulation efficiency were determined. The morphology of MSPs was visualized by scanning electron microscopy (SEM; S-4300, Hitachi High-Technologies Corporation, Tokyo, Japan). Calculation MSPs particle size was performed by measuring the feret diameter. To determine the

encapsulation efficiency, the percentage ratio of the amount of clarithromycin loaded into the MSPs to the initial amount. In brief, analysis was performed at 261 nm using a UV spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

2.3. *In vitro* release profile of MSPs

The amount of clarithromycin released from the MSPs in phosphate-buffered saline (PBS) was measured by UV spectrophotometer as the same way mentioned above. The concentration was calculated at 261 nm wavelength with reference to calibration curve determined. Thus, PBS solution containing the MSPs was placed in a dialysis tube (Spectra/Pore membranes MWCO 12-14,000) with 3 mL of PBS suspended in 500 mL of same medium under gentle stirring at 37°C. The dissolution medium outside the dialysis tube was collected, filtered and the amount of clarithromycin was determined at 1, 2, 3, 5, 7, 14, 21 and 28 days. Meanwhile, The dissolution medium outside the dialysis tubing was then replaced with fresh PBS at each time period.

2.4. *In vivo* experiment

The animal experiment conducted in this study were approved by the Ethical Committee for Animal Research at the École Nationale Vétérinaire d'Alfort (Maisons-Alfort, Val-de-Marne, France). Twenty-one New Zealand Rabbits were used in total. All surgical procedures were performed under general anaesthesia using ketamine chlorate. After shaving the head and disinfection procedures by iodine solution, a flap

was raised and four critical size bi-cortical bone defects (5mm diameter) were created in calvaria bone of each rabbits. The defects were randomly designated to the following experimental groups:

Group 1: No augmentation (sham)

Group 2: beta-Tricalcium phosphate (β -TCP)

Group 3: β -TCP with 0.12 μ g clarithromycin

Group 4: β -TCP with 6.12 μ g PLGA microspheres (0.12 μ g clarithromycin)

After 2, 4 and 12 weeks of healing, the animals were euthanized with anesthesia overdose (Seven rabbits for each time period). The specimens were removed en bloc and were thereafter soaked in 4 % formaldehyde for 24 h. After fixation, the samples were subjected to dehydration in a series of ethanol concentrations (70-100 %) and infiltration in resin under constant vacuuming. After complete infiltration, the samples were embedded in light curing-resin (Technovit 7200 VLC; Heraeus Kulzer Wehrheim, Germany).

The levels of bone regeneration in the samples were evaluated using micro-computed tomography (SkyScan 1176, Bruker Micro-CT, Kontich, Belgium). Newly formed bone volume in the defect area was measured using image software (Data Viewer, CT-Analyser, Bruker Micro-CT, Kontich, Belgium). Briefly, the image files were opened in the software and a calibration was set to have all the datasets at the same magnification. The whole defect area was selected, and then all the pixels within this area with grey value intensities over a decided threshold that corresponded to mineralized bone were counted and quantified as “newly bone formation volume”. The total defect volume was also calculated from the μ CTs.. The percentage of newly formed bone in each defect area was calculated, following the formula: The percentage

of newly formed bone (%) = newly bone formation volume (μm^3)/total defect volume (μm^3), and expressed as mean values $\pm$ standard error.

After taking micro CT images, the embedded resin blocks were subjected to non-decalcified cut and ground sectioning to a final thickness of 20 μm followed by staining with toluidine blue. Histological analyses then were performed using a light microscope (Eclipse ME600; Nikon, Japan). Analysis of the histomorphometrical data was performed using image analysis software (Image J v. 1.43u; National Institute of Health). Calculation of the total amount of bone and graft material was within the total area of the defect using a $\times 10$ magnification objective.

2.5. Statistical analysis

Friedman rank test using computer software SPSS was selected to evaluate the differences between all groups during the 3 time points. The test was followed by post hoc test for multiple comparisons. The significance level was set at $p=0.05$. All Data were plotted as mean $\pm$ standard error.

3. Results

3.1. Characterization of MSPs

In high-magnification SEM figures, microspheres (MSPs) form of PLGA at different sizes were observed (figure 1). The average size of the PLGA MSPs was around $26.4 \pm 3.1 \mu\text{m}$. The encapsulation efficiency was $94.3 \pm 2.8\%$ (table 1).

Figure 1, Scanning electron microscopy images showing clarithromycin encapsulated PLGA (A), b-TCP (B), b-TCP with clarithromycin (C), and b-TCP with Microspheres (D), the arrows indicate PLGA microspheres.

Table 1, Characterization of Microspheres

Clarithromycin: PLGA (weight ratio)	1:50
Encapsulation efficiency (%)	94.3 ± 0.4
Average particle size	$26.38 \mu\text{m}$

3.2. In vitro release profile of MSPs

Evaluation of release profile of Clarithromycin from MSPs walls showed sustained release profile for 4 weeks. MSPs released approximately 50% of clarithromycin during the first week. Furthermore, 31% of clarithromycin was released in the first 3 days, which can be considered as an initial burst release. The following 3 weeks demonstrated slower release (figure 2).

Figure 2, In vitro release profile of clarithromycin-loaded PLGA microsphere (msp/CPC).

3.3. Histologic evaluation of new bone formation

All groups showed bone formation that was localized mostly at the periphery of the defects after 2 weeks of healing (figure 3). After 4 weeks, more bone was seen within the defects that reached around 30% of total defects area. No significant differences were observed between Defects treated with β -TCP compared with sham at 2 and 4 weeks of healing time. Measurements of new bone formation were summarizes in figure 4.

At 12 weeks, β -TCP mixed with MSPs loaded with clarithromycin managed to express significantly higher amount of newly formed bone compared with sham ($p=0,002$). Furthermore, defects treated with β -TCP without MSPs also showed more bone formation compared to untreated defects ($p = 0,058$).

Figure 3, Descriptive histological micrographs after 2, 4, and 12 weeks of healing for all groups tested; (a) sham (group 1), (b) β -TCP (group 2), (c) β -TCP 1clarithromycin (group 3), (d) β -TCP 1PLGA with clarithromycin (group 4). Scale bars represent 500 μ m.

Figure 4, Bone area % within the defect.

3.4. Evaluation of bone formation using micro CT scan.

Micro Computed topography provided a 3D visualization of bone formation within the bone defects (figure 5). During all healing times, significantly more bone formation was found in the defects treated with β -TCP combined with clarithromycin-loaded MSPs than in the sham group ($p=0.00$, 0.014 , and 0.002 in 2, 4, and 12 weeks, respectively). Significant difference was also found between β -TCP with clarithromycin and sham after 2 weeks ($p=0.009$). Figure 6 summarise the values of bone formation calculated from micro CT images. It was possible to see that the presence of PLGA MSPs loaded with clarithromycin revealed the highest amount of bone formation compared with the other test groups.

Figure 5, 3D reconstructed images using mCT after 2,4,and 12 weeks for: (1) sham (group 1), (2) b-TCP (group 2), (3) b-TCP 1clarithromycin (group 3), (4) b-TCP 1PLGA with clarithromycin (group 4).

Figure 6, CT scan: Bone volume (Im^3)/Tissue volume (Im^3) 5 (%).

4. Discussion

This study investigated the controlled release of Clarithromycin using Poly (lactic-co-glycolic) acid (PLGA) MSPs as a carrier in a calvaria bone defect model. The results suggested that filling the defect with bone substitutes (β -TCP) + a MSPs eluting Clarithromycin in a sustained manner increased significantly the bone regeneration within the defect compared to non-treated areas, as observed in micro CT analysis. This difference was evident also on the histological analysis, but only at 12 weeks, probably because the micro CT captures the entire biologic phenomenon whereas the histological micrographs present a single cut in the middle of the defect, which may not be descriptive of the entire grafted area.

It was notable that the addition of Clarithromycin within the bone substitutes further increased the bone regeneration. Although at 4 weeks the effect of Clarithromycin positively influenced bone regeneration regardless of the use of MSPs, the group possessing Clarithromycin loaded in MSPs presented continued bone formation even at 12 weeks *in vivo*. Presumably this is the effect of the controlled release of the drug from the MSPs, which consents a longer availability of the drug in the site.

This is an indication that the release kinetics of the MSPs tested *in vitro* was also similar *in vivo*, where the drug release took place in critical sized defect.

In the current study, the MSPs were made of poly (lactic-co-glycolic) acid (PLGA). PLGA is a synthetic polymer with biodegradable properties widely used in fabricating 3D scaffolds for tissue engineering[20]. PLGA is known to be biocompatible and shows favourable biodegradable behaviour, which allows it to be used as a delivery vehicle for different drugs[21]. In addition, PLGA can easily be tailored with different physical and chemical properties to suit the drug delivery needs[22]. For instance, microspheres or nanospheres can be fabricated from the PLGA that allow sustain drug release [23],

and this can be controlled by different parameters such as the thickness of the sphere wall or the size of the spheres, or by adjusting the monomer ratio[24].

Drug release mechanism from microspheres can involve surface diffusion of drug from MSPs surface that is associated with the initial burst release. The release then continues as bulk diffusion and erosion from the MSPs as the degradation process of MSPs take place[25].

In previous studies, it was reported that the incorporation of bone substitutes with MSPs demonstrated initial burst release in the first days, which was followed by slower release kinetics as the MSPs biodegraded [9, 26]. In the current study, the release profile presented that the MSPs released around 50% of Clarithromycin during the first week. Thereafter, slower and sustained release profile was observed as it reached around 100% cumulative release 3 week later.

Among numerous factors, drug concentration play an important role in burst release and release rate. Its been reported that higher drug loading in MSPs will reveal larger burst release[27]. The mechanism of drug release from MSPs also depends on drug type, which could influence not only the release rate but also the degradation mechanism of MSPs [25, 28]. However, in the present study, it can be said that the degradation properties of MSPs was the major factor in the sustain release of clarithromycin.

The results of the current study suggested that Clarithromycin could be successfully loaded and could be released sustainedly by MSPs and this release kinetics was associated with the highest regeneration of new bone at extended healing periods. The

finding of the sustain release property of MSPs can open the doors for more experiments using numerous drugs.

In this study, Clarithromycin was selected for its known anti-inflammatory effects [29, 30]. Furthermore, it was reported that Clarithromycin could interfere with the biofilm formation from many aerobic and anaerobic bacteria strains[31]. These factors may positively contribute to bone regeneration since excessive inflammation and infection leads to failure of the bone augmentation.

Another possible reason for the successful bone regeneration observed in this study is that the bone substitutes (β -TCP) used and PLGA exhibited a favourable degradation process that may enhance bone formation[32, 33]. It is of utmost importance that these so-called bioresorbable biomaterials are replaced by newly formed bone, because bone ingrowth can take place within such materials at the site of resorption[34]. For these reasons, using the fast resorbing β -TCP, which can stimulate bone regeneration without interfering with the long-term health of bone in the site, may be a good approach for the filling of critical size bone defects and the addition of Clarithromycin can be further beneficial for the healing processes.

Future works, focusing on improving the sustained drug release of MSPs, to prevent the spontaneous release and to minimise the burst release and to obtain prolonged drug availability in the healing area, are of great interest in the fields of biomaterial and tissue engineering.

In addition, it is important to propose and further investigate combinations of drug releasing systems and scaffolding materials that yield the best performances for tissue regeneration and achieve clinical success in patients.

5. Conclusion

In summary, PLGA MSPs loaded with clarithromycin was suggested as drug delivery system for sustained drug release to enhance bone regeneration in the calvaria defect model used in the current study. Our data showed that MSPs can exhibit initial burst release of clarithromycin that will be followed with slower release profile. In addition, our animal experiment revealed that incorporation of clarithromycin-loaded MSPs within β -TCP cement can stimulate bone formation in bone defects.

6. References

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