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# PLGA microspheres for oral osteopenia treatment: preliminary "in vitro"/"in vivo" evaluation

P. Perugini [a], I. Genta [a], B. Conti [a], T. Modena [a], D. Cocchi [b], D. Zaffe [c], F. Pavanetto [a,*]

[a] *Department of Pharmaceutical Chemistry, University of Pavia, V.le Taramelli 12, Pavia 27100, Italy*
[b] *Department of Biomedical Sciences and Biotechnologies, University of Brescia, Brescia, Italy*
[c] *Department of Anatomy and Histology, University of Modena and Reggio Emilia, Italy*

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**Abstract**

The aim of this work was to prepare and to evaluate "in vitro"/"in vivo" microspheres based on poly(D,L-lactide-co-glycolide) copolymers containing ipriflavone, for the local treatment of oral bone loss.

The first objective was the preparation and "in vitro" characterization of ipriflavone loaded microspheres, by emulsion/solvent evaporation method. Process parameters such as drug:polymer weight ratio, and molecular weight of copolymers, were also investigated.

The second objective was to elaborate a suitable animal model of mandibular osteoporosis, to evaluate the efficacy of these microparticulate drug delivery systems. "In vivo" experiments were carried out on female rats, in which oral osteopenia was induced by gonadectomy and molar avulsion. Morphometric analysis of mandibular segment were carried out to quantify the development of oral osteopenia and the efficacy of drug loaded microspheres.

Results showed that ipriflavone loaded PLGA microspheres can be successfully obtained with good "in vitro" characteristics, utilizing the emulsification/solvent evaporation method.

"In vivo" experiments revealed that local administration of microspheres produced only mild inflammation on the injection site. Morphometric analyses showed, at the level of the third molar, a slight increase in spongy and total bone mass on rat jaw treated with microspheres with respect to control. Control animals exhibited a scarce degree of osteopenia demonstrating that this animal model is not suitable for this purpose.


*Keywords:* PLGA; Ipriflavone; Bone loss; Microspheres

## 1. Introduction

Bone loss in the oral cavity can have many causes. Some are related to the presence of systemic illnesses, such as Down syndrome, HIV infection, neutropenia, diabetes mellitus and osteoporosis (Jeffcoat, 1993; Hildebolt, 1997). This occurs more frequently as a consequence of local infections, such as periodontal diseases or as result of dental loss (Wactawski-Wende et al., 1996).

The atrophy of the alveolar bone begins as soon as teeth are lost. This process can diminish the stability of artificial dentures. The solution to this problem could be that of an oral surgical operation which could

* Corresponding author. Tel.: +39-0382-507377; fax: +39-0382-422975.
*E-mail address:* franca.pavanetto@unipv.it (F. Pavanetto).

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enlarge the alveolar ridge, or the use of dental implants instead of dentures.

Within the sphere of dentistry, the relation between systemic osteoporosis and the bone resorption after teeth extraction, are of great clinical importance. In fact, this pathological resorption can lead to the incapacity to stabilize dentures and subsequently to the impossibility of their use. In the worst cases mandibular nerve exposure is observed. Nowadays, the residual ridge resorption (RRR) is statistically significant, especially in women affected by post-menopausal osteoporosis.

Since oral bone loss is associated to an alteration of the balance between bone resorption and bone formation, therapeutic agents promoting bone formation, such as ipriflavone, could be advantageously employed.

Ipriflavone is a synthetic flavonoid derivative that improves osteoblast cell activity inhibiting bone resorption (Civitelli, 1997). This compound is usually employed in the systemic treatment of post-menopausal and senile osteoporosis through oral administration (Brandi, 1993; Head, 1999).

Local administration of this drug may be useful in dentistry for the treatment of RRR that occurs in edentoulus osteoporotic patients, avoiding possible systemic side effects such as gastrointestinal complaints, depression and tachycardia.

Recently, some authors investigated the potential beneficial effect of local application of ipriflavone on perialveolar bone formation in experimentally injured rats (Martini et al., 1998).

In the local therapy of alveolar bone loss, the use of polymeric drug delivery systems for the veicolation of ipriflavone should sustain drug concentration in the target site, prolonging its pharmacological activity.

The aim of this research was to prepare microparticulate systems based on polylactide-co-glycolide (PLGA), for the local release of ipriflavone, in order to reduce oral bone loss.

Microparticulate drug delivery systems based on biodegradable polymers are suitable for administration of ipriflavone in the alveolus, allowing to achieve a suitable drug dosage form and a prolonged drug release.

Among biodegradable polymers, PLGA copolymers were selected for this study because of their biodegradability and biocompatibility properties. Moreover, these polymers have been recently used as constituents of biodegradable implant material in dentistry and orthopedics (Gogolewski and Mainil-Varlet, 1997).

PLGA are thermoplastic aliphatic poly(esters) whose properties vary depending on polymer molecular weight and composition (Jain et al., 1998). In this study, PLGA copolymers with 50:50 molar composition, and with two different molecular weights, were selected.

Research was divided into two phases: the first inherent to the preparation by emulsion/solvent evaporation technique and characterization of ipriflavone loaded microparticulate systems. The second phase of this study concerned the elaboration of an animal model of oral bone loss and the "in vivo" evaluation of microsphere formulation effectiveness.

The skeleton anatomy and physiology of lower species, usually employed as animal model for oral bone loss such as rodents (i.e. rats and rabbits) have the disadvantage of being substantially different from humans. However, they are easier to house with respect to other more suitable species such as dogs, pigs and sheep.

As in man, gonadectomy induces systemic osteoporosis in rats and this model has been widely used to evaluate pathogenesis and the treatment of post-menopausal bone loss (Sones et al., 1986).

Rats in normal conditions are not a useful model for oral bone loss. In fact, in rats a lot of variables produce a continuous and considerable stress on the mandible and maxilla, resulting in an increase of bone formation instead of a bone resorption (Zaffe et al., 1999).

In this work oral osteopenia was induced by applying a protocol that associated a gonadectomy with the extraction of the molars, to create such a degree of mechanical unloading to enhance the alveolar bone resorption. That animal model was used to evaluate the tolerability and efficacy of ipriflavone loaded PLGA microspheres.

## 2. Materials and methods

### 2.1. Materials

D,L-Lactide-co-glycolide polymers 50:50 molar ratio (Resomer RG 502, $M_r$ 12,000 and Resomer

RG 503 $M_r$ 34,000) were purchased from Boerhinger Ingelheim (Ingelheim, Germany).

Ipriflavone, $M_r$ 280.31, was granted by Chiesi Farmaceutici (Chiesi Farmaceutici S.p.A., Italy); polyvinyl alcohol (PVA), $M_r$ 85,000–146,000, was purchased by Sigma-Aldrich (Milano, Italy); all reagents were of analytical grade.

### 2.2. Microsphere preparation

Ipriflavone loaded PLGA microspheres were prepared by O/W emulsion/solvent evaporation method (Conti et al., 1995).

Ipriflavone and polymer were dissolved in methylene chloride; this solution (8 g) was dropped into 160 ml of 1% (w/v) PVA solution at 15 °C under mixing using a Vibromixer E1 (Chemap AG, Volketswil, Switzerland) at 60 vibrations/s. The O/W emulsion was then brought to 40 °C and stirred for 3 h to allow solvent evaporation. The microsphere suspension was centrifugated at 4000 rpm for 20 min and the microspheres washed twice with water; an additional ethanol washing step was sometimes employed. Microspheres were then collected on a Millipore 0.8 μm membrane, and dried under vacuum.

Certain parameters in phase composition were evaluated to investigate microsphere morphology and drug loading.

- Molecular weight of the copolymer used.
- Drug/polymer weight ratio (1/5; 1/10; 1/20).
- Presence of an emulsifier agent (Span 20) in the polymeric solution.

Table 1 shows the composition of all batches of ipriflavone loaded microspheres prepared.

Table 1
Composition of ipriflavone loaded PLGA microspheres

| Batch no. | Polymeric composition | Drug/polymer weight ratio |
|---|---|---|
| 1 | RG 502 | 1/5 |
| 2 | RG 502 | 1/10 |
| 3 | RG 502 | 1/20 |
| 4[a] | RG 502 | 1/10 |
| 5[b] | RG 503 | 1/5 |
| 6[b] | RG 503 | 1/10 |

[a] Batch no. 4 was prepared using Span 20 (1% (w/v)) in the polymeric solution.
[b] Batch nos. 5 and 6 were prepared washing microspheres with ethanol.

### 2.3. Microsphere characterization

#### 2.3.1. Scanning electron microscopy

Microsphere shape and surface structure were evaluated by scanning electron microscopy (SEM) using a Jeol JX 840-A Jeol Ltd., Tokyo, Japan.

Samples for SEM analysis were prepared by gold-sputtering the microspheres in an argon atmosphere and visualized at 10 kV.

#### 2.3.2. Particle size analysis

Granulometric analyses of all batches of microspheres were performed utilizing a light blockage method, which consists in the determination of particle size by obscuration of a white light beam. Particles were measured as a function of the two-dimensional cross-section area. Microsphere samples were suspended in distilled filtered water, containing an emulsifier agent and analyzed by an HIAC/ROYCO, equipped with HC120HR detector (model 3000, AM Instruments, Desio, Italy). The analyses were performed in a size range between 2 and 120 μm. The results are the average of five determinations for each sample.

#### 2.3.3. Drug content

The drug content was performed by ultraviolet spectrophotometry using a UV-Vis spectrophotometer Beckman (model DU 7500, Beckman Fullerton, CA, USA). In order to eliminate the UV absorption of PLGA polymer, the following procedure was applied to prepare samples.

Samples were dissolved in methylene chloride.
After ethanol (ethanol:methylene chloride 3:1 (v/v)) addition, methylene chloride was removed by a rotary evaporator (IKA Laboratory Technology, Staufen, Germany) under vacuum.
The concentration of ipriflavone in ethanol was determined at 298 nm using a calibration curve between 4 and 40 μg/ml of ipriflavone ($R^2 = 0.999$).

Analyses were carried out in triplicate on each batch of drug loaded microspheres, on “blank” microspheres, and on physical mixture ipriflavone:PLGA, to establish the drug recovery percentage of the method.

#### 2.3.4. “In vitro” ipriflavone release

A dialysis method was used to perform the “in vitro” release test of all batches of ipriflavone loaded

microparticulate systems. Amounts of microspheres were placed in a dialysis tubing (Spectra/Por membranes MWCO 12-14,000) with 300 μl of dissolution medium, suspended in 10 ml of the same medium at 37 °C.

At scheduled time intervals, the dissolution medium outside the dialysis tubing was collected, filtered and the amount of ipriflavone was spectrophotometrically determined at 298 nm. The dissolution medium outside the dialysis tubing was then replaced with fresh dissolution medium.

Two different dissolution media were investigated: the ethanol:water mixture (60:40 (v/v)) and saline phosphate buffer pH 7.4 (PBS), containing sodium dodecyl sulfate 0.15% (w/v) and sodium azide 0.01% (w/v).

All dissolution tests were run in triplicate and mean values were reported.

### 2.4. "In vivo" experiments

#### 2.4.1. Experimental procedure

Sprague–Dawley female rats (Charles River, Calco, Italy), 10 months old at the beginning of the study, were used. All rats were kept individually at 22 °C, $U = 65\%$ with 12/12 h light/dark cycle. They received a soft pellet diet and water ad libitum.

In order to induce systemic osteoporosis, 12 animals were subjected to gonadectomy under general anesthesia (ketamine, 58 mg/kg and xylyzine, 12 mg/kg).

At 12 months of age, gonadectomized rats underwent molar avulsion on the left side (upper and lower) and were randomly divided into three groups. One group of four rats, subjected to molar avulsion, served as controls. The other two groups were subjected to microsphere administration in the left side through two procedures.

(a) The first group of four animals (A) were injected by means of a syringe (with a 10 gauge needle) containing ipriflavone loaded microspheres (containing 20 mg of ipriflavone) (batch no. 6) into the soft tissues around the mandible corner, passing through the buccal plica around the incisors. After 20 days the animals received a second dose of microspheres, with the same procedure.
(b) The second group of four animals (B) were treated with chitosan gel extemporaneously prepared containing ipriflavone loaded microspheres (batch no. 6); this gel formulation was placed directly into the alveolus. After 20 days the animals received a second administration, with the same procedure.

Twenty days after the second treatment, a single dose of tetracycline (50 mg/kg s.c.) was administered to all animals in order to mark the newly formed bone. Seventy two hours after tetracycline administration, rats were killed by decapitation.

#### 2.4.2. Analysis of jaws

Rat jaws were fixed, for 4 h in paraformaldehyde at 4%, in 0.1 M phosphate buffer, pH 7.2. Specimens were dehydrated through ethanol series and embedded in methyl methacrylate (PMMA). The embedded jaws were sectioned with a diamond saw microtome to obtain 200 μm thick serial sections.

Microradiographs of sections taken at the jaw level of the first and the third molar were carried out at 6 kV and 2.1 mA using a microradiographic device (Italstructures, Italy). Each microradiograph was analyzed using an image analyzer (VIDAS Zeiss, Germany) connected to a CCTV camera. The calcified tissue amounts, i.e. the absolute and relative amount of incisor, molar, cortical and spongy bone tissues, by means of an adequate software program implemented the image analyzer, were calculated. Fig. 1 shows a schematic digital image of mandibular section microradiograph.

## 3. Results and discussion

Oil in water emulsion/solvent evaporation process is the main method used to encapsulate lipophilic drugs into PLGA copolymers (Jain et al., 1998). Drug encapsulation is affected by several parameters, such as the solubility of drug in the polymer and in water and its molecular weight. The physical chemical characteristics of ipriflavone, such as low molecular weight and very low water solubility, affected the microsphere characteristics in terms of drug content and homogeneous dispersion throughout microsphere matrice. In fact, the drug precipitates at the organic solvent/water interface during solvent evaporation step. Thus, the presence of drug crystals

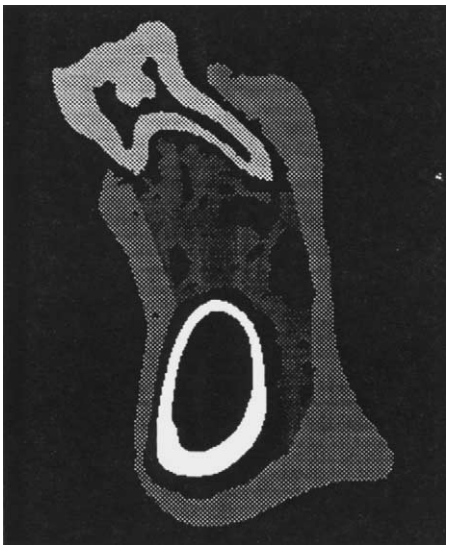

Fig. 1. Schematic digital image of a microradiograph of a transverse section of rat jaw taken at the level of the first molar (blank: incisor tooth; pale grey: molar tooth; medium grey: cortical bone; dark grey: spongy bone).

on the microsphere surface was evident, as shown in the photomicrograph of batch no. 3 (Fig. 2).

Different drug polymer weight ratios, obtained by reducing the amount of drug used during microsphere preparation, were employed in order to reduce this effect, as shown in the Table 1. Drug precipitation is evident for both polymers, also when a very low drug/polymer ratio was used (batch no. 3), or in spite

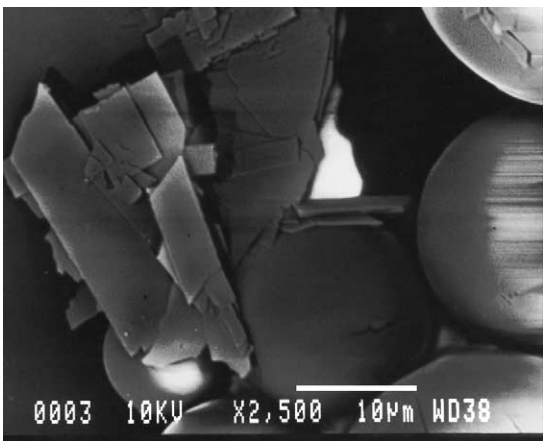


Fig. 2. Scanning electron microscope photomicrograph of batch no. 3.

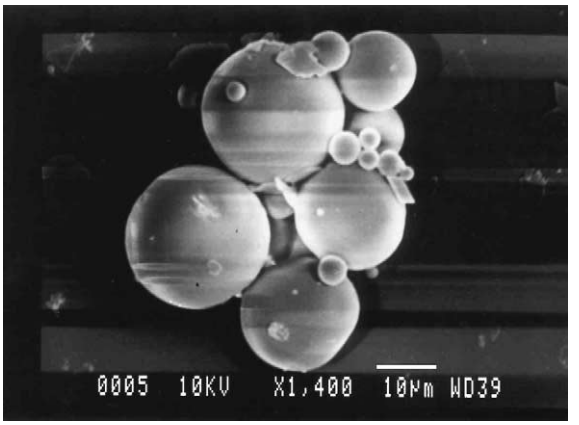


Fig. 3. Scanning electron microscope photomicrograph of batch no. 6.

of the addition of an emulsifying agent to the internal phase (batch no. 4). In this preliminary work, the drug/polymer weight ratio was strictly bound to the amount of microspheres to be administered "in vivo", that must be between 10 and 20 mg. Drug/polymer (w/w) ratio 1/10 was designed to achieve the objective of administering a suitable dose to rats.

An ethanol washing step was employed to eliminate drug crystals from microparticle surface. This step is suitable for RG 503 polymer, and gives microspheres with good morphology without drug crystals on the surface (Fig. 3), whereas RG 502 polymer (when in contact with ethanol), softened, loosing its physical integrity.

Table 2 shows production yields, the actual drug contents and encapsulation efficiencies of all micro-

Table 2
Production yields, actual drug content and encapsulation efficiencies of ipriflavone loaded PLGA microspheres ($n = 3$; S.D. $< 5\%$)

| Batch no. | Yield of production (%) | Actual drug content (%) | Encapsulation efficiency (%) |
|---|---|---|---|
| 1 | 43.0 | 15.9 | 95.8 |
| 2 | 62.5 | 8.5 | 93.5 |
| 3 | 65.4 | 4.5 | 94.5 |
| 4[a] | 60.3 | 8.3 | 91.3 |
| 5[b] | 45.0 | 5.8 | 35.1 |
| 6[b] | 64.8 | 5.5 | 60.5 |

[a] Batch no. 4 was prepared using Span 20 (1% (w/v)) in the polymeric solution.
[b] Batch nos. 5 and 6 were prepared washing microspheres with ethanol.

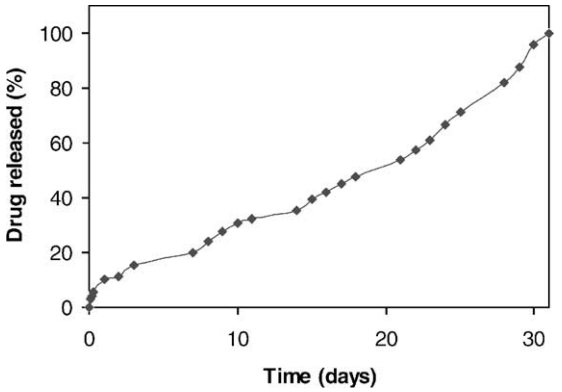


Fig. 4. "In vitro" release profile of ipriflavone from batch no. 6, using PBS as dissolution medium ($n = 3$, S.D. < 5%).

sphere batches. The ethanol washing step enabled an encapsulation efficiency of 60% in batch no. 6.

Particle size analyses were only performed on batch nos. 5 and 6, as the presence of drug crystals made particle size analyses of no value for all other batches. Slight differences were highlighted between the two batches (batch no. 5 $d_{50} = 21.29\,\mu m$, $d_{90} = 43.58\,\mu m$; batch no. 6 $d_{50} = 24.42\,\mu m$, $d_{90} = 53.10\,\mu m$ being $d_{50}$ and $d_{90}$ the diameters corresponding to 50 and 90% of particles, respectively).

Further characterization (i.e. "in vitro" dissolution test, "in vivo" experiments) could not be performed on batches 1–4 because of the presence of ipriflavone crystals on microsphere surface. Due to its good encapsulation efficiency and absence of drug crystals, batch no. 6 was chosen for "in vitro" dissolution tests and "in vivo" experiments.

In literature, dissolution testing on ipriflavone tablets was carried out using a hydroalcoholic mixture as dissolution medium (Plaizier-Vercammen et al., 1994; Boonkerd et al., 1992); ethanol:water 60:40 (v/v) as medium was used in our "in vitro" study. Moreover, in order to verify the physical integrity of PLGA during prolonged contact with ethanol, dissolution testing was also carried out in saline phosphate buffer.

Fig. 4 shows drug release profile from batch no. 6 using PBS as dissolution medium. Release was completed in approximately 1 month. This result is consistent with PLGA physical characteristics. Drug release is completed in 24 h with dissolution test made in an ethanol:water mixture (data not reported). This result can be related to the influence of ethanol on structure and solubility of RG 503, therefore leading to the conclusion that ethanol:water mixture is unsuitable for dissolution tests on microparticulate systems made of PLGA.

"In vivo" experiments revealed that the administration of microspheres directly into the alveolus became complicated due to the small volume of the alveolus cavity and the rapid elimination of the dosage form through biological fluids. Since this route of administration did not appear to be effective for a long period of time, it was neglected.

The local administration of microspheres into the soft tissues around the mandible corner only produced a temporary, mild inflammation on the injection site.

Results from morphometric analyses in control rats are reported in Fig. 5 as mean values of the four calcified tissues and the total bone, calculated in microradiographs of tranverse sections, on the right (R) and left (L) jaw.

The comparison between left and right jaw was necessary in order to identify the influence of molar avulsion on oral osteopenia development.

At the level of the third molar, a bone mass increase was evident on the left side, indicating a continuous and considerable stress on the jaw after molar avulsion. Given that in rats the lower incisor is formed in correspondence to the masseteric tubercle growing anteri-

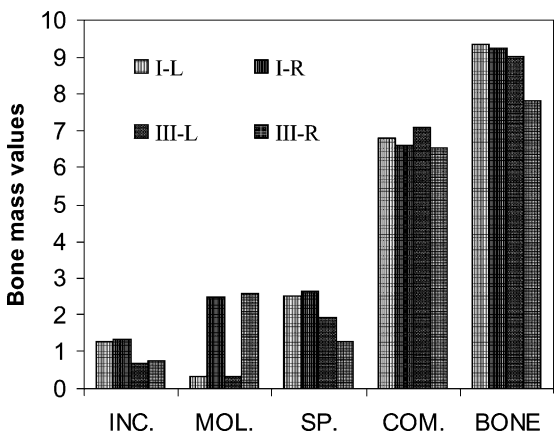


Fig. 5. Graph showing the calcified tissue contents expressed as $mm^2$ (INC: incisor tooth, MOL: molar tooth, SP: spongy bone, COM: cortical bone, BONE: total bone) of microradiographs of transverse sections, taken at the level of the first and the third molar, of the right (R) and the left (L) jaw of control rats.

Table 3
Calcified tissue amounts, expressed as $mm^2$, calculated on microradiographs of jaw transverse sections of rats treated with ipriflavone loaded microspheres (Student's *t*-test $P < 0.01$).

| | Right | | | | | *P* | Left | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | INC | MOL | SP | COM | BONE | | INC | MOL | SP | COM | BONE |
| I molar | | | | | | | | | | | |
| *m* | 1.22 | 2.34 | 2.88 | 6.85 | 9.73 | 0.06 | 1.23 | 0.16 | 2.37 | 6.61 | 8.98 |
| S.D. | 0.43 | 0.28 | 0.34 | 0.32 | 0.50 | | 0.38 | 0.26 | 0.40 | 0.63 | 0.89 |
| S.E. | 0.19 | 0.12 | 0.15 | 0.14 | 0.22 | | 0.17 | 0.11 | 0.18 | 0.28 | 0.40 |
| III molar | | | | | | | | | | | |
| *m* | 0.62 | 2.24 | 1.62 | 7.43 | 9.05 | 0.74 | 0.75 | 0.02 | 2.21 | 7.06 | 9.27 |
| S.D. | 0.29 | 0.42 | 0.53 | 0.23 | 0.53 | | 0.30 | 0.04 | 0.66 | 0.93 | 0.92 |
| S.E. | 0.13 | 0.19 | 0.24 | 0.11 | 0.24 | | 0.13 | 0.02 | 0.29 | 0.42 | 0.41 |

INC: incisor tooth; MOL: molar tooth; SP: spongy bone; COM: compact bone; BONE: total bone.

orly along the entire jaw, it is therefore inevitable that the stress on the incisors may spread through the whole jaw and the alveolar bone surrounding the molars.

Table 3 reports data from morphometric analyses in rats treated with ipriflavone loaded microspheres. A slight increase in spongy and total bone mass result at the level of the third molar. Nevertheless, the detected bone increase is not statistically significant, due to a simultaneous reduction of the cortical bone.

## 4. Conclusions

Results from this preliminary work showed that ipriflavone loaded PLGA microspheres can be successfully obtained through emulsion/solvent evaporation technique, with good morphological characteristics and high encapsulation efficiency.

The administration of ipriflavone loaded microparticles to animals revealed good tolerability.

The animal model proposal in this study did not result as being suitable to correctly evaluate the efficacy of microparticles produced. In order to induce severe oral osteopenia in rats, it is necessary to produce a still more notable alteration in the mechanical stresses.

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## References

Boonkerd, S., de Nève, R.E., Michotte, Y., Plaizier-Vercammen, J.A., 1992. Controlled-release ipriflavone tablet formulations containing polyvinyl chloride. S.T.P. Pharma. Sci. 2, 488–493.

Brandi, M.L., 1993. New treatment strategies: ipriflavone, strontium, Vitamin D metabolites and analogs. Am. J. Med. 95, 69S–74S.

Civitelli, R., 1997. In vitro and in vivo effects of ipriflavone on bone formation and bone biomechanics. Calcif. Tissue Int. 61, S12–S14.

Conti, B., Genta, I., Modena, T., Pavanetto, F., 1995. Investigation on process parameters involved in polylactide-co-glycolide microspheres preparation. Drug Dev. Ind. Pharmacy 21, 615–622.

Gogolewski, S., Mainil-Varlet, P., 1997. Effect of thermal treatment on sterility, molecular and mechanical properties of various polylactides. Biomaterials 18, 251–255.

Head, K.A., 1999. Ipriflavone: an important bone-building isoflavone. Altern. Med. Rev. 4, 10–22.

Hildebolt, C.F., 1997. Osteoporosis and oral bone loss. Dentomaxillofacial Radiol. 26, 3–15.

Jain, R., Shah, N.H., Malick, A.W., Rhodes, C.T., 1998. Controlled drug delivery by biodegradable poly(ester) devices: different preparative approaches. Drug Dev. Ind. Pharmacy 24, 703–727.

Jeffcoat, M.K., 1993. Bone loss in the oral cavity. J. Bone Miner. Res. 8, 467–473.

Martini, M., Formigli, L., Tonelli, P., Giannelli, M., Alunni, F., Naldi, D., Brandi, M.L., Zecchi Orlandini, S., Orlandini, G.E., 1998. Effects of ipriflavone on perialveolar bone formation. Calcif. Tissue Int. 63, 312–319.

Plaizier-Vercammen, J.A., Boons, E., Callaerts, A., 1994. Controlled release of ipriflavone in lipophilic matrices. In: Proceedings of the 21st International Symposium on Control. Rel. Bioact. Mater., pp. 818–819.

Sones, A.D., Wolinsky, L.E., Kratochvil, F.J., 1986. Osteoporosis and mandibular bone resorption in the Sprague–Dawley rat. Calcif. Tissue Int. 39, 267–270.

Wactawski-Wende, J., Grossi, S.G., Trevisan, M., Genco, R.J., Tezal, M., Dunford, R.G., Ho, A.W., Hausmann, E., Hreshchyshyn, M.M., 1996. The role of osteopenia in oral bone loss and periodontal disease. J. Periodont. 67, 1076–1084.

Zaffe, D., Paganelli, C., Cocchi, D., 1999. Induction and pharmacological treatment of oral osteopenia in rats. Minerva Stomatologica 48, 45–62.