BASIC RESEARCH

The use of vancomycin-loaded poly-l-lactic acid and poly-ethylene oxide microspheres for bone repair: An in vivo study

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OBJECTIVE: The aim of this study was to investigate bone repair after the implantation of vancomycin-loaded poly-L-lactic acid/poly-ethylene oxide microspheres compared with vancomycin-unloaded poly-L-lactic acid/poly-ethylene oxide microspheres.

METHODS: Poly-L-lactic acid/poly-ethylene oxide microspheres were implanted in rat tibiae and evaluated for periods of 2, 4, 8, and 12 days and 4, 8, 16, and 32 weeks. The groups implanted with vancomycin-loaded and vancomycin-unloaded microspheres were compared. Histopathologic (semi-quantitative) and histomorphometric analyses were performed to evaluate the bone formation process.

RESULTS: During the first period (second day), fibrin and hemorrhaging areas were observed to be replaced by granulation tissue around the microspheres. Woven bone formation with progressive maturation was observed. All of the histopathological findings, evaluated by a semi-quantitative assay and a quantitative analysis (percentage of bone formation), were similar between the two groups.

CONCLUSION: Vancomycin-loaded poly-L-lactic acid/poly-ethylene oxide microspheres are a good bone substitute candidate for bone repair. Local antibiotic therapy using vancomycin-loaded poly-L-lactic acid/poly-ethylene oxide microspheres should be considered after the microbiological evaluation of its efficacy.

KEYWORDS: Poly-L-lactic acid; Poly-ethylene oxide; Vancomycin; Bone grafting.

Coraça-Huber DC, Duek EAR, Etchebehere M, Magna LA, Amstalden EMI. The use of vancomycin-loaded poly-l-lactic acid and poly-ethylene oxide microspheres for bone repair: An in vivo study. Clinics. 2012;67(7):793-798.

Received for publication on November 4, 2011; **First review completed on** January 16, 2012; **Accepted for publication on** March 12, 2012

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INTRODUCTION

Due to the limitations of bone grafts (1,2), synthetic materials for orthopedic use have become a focus of scientific studies. These products can replace or aid bone grafting for bone healing. Thus, many polymers have been studied. Polymethylmethacrylate (PMMA) is one of the most commonly used polymers as a bone cement. It is also sometimes applied as a substitute for bone grafts; however, this type of polymer is mechanically weak and requires high temperatures for synthesis, which can therefore cause tissue necrosis (2).

Bioabsorbable polymeric materials are potential candidates for replacing metallic, ceramic, and polymeric non-bioabsorbable materials. Additionally, when manufactured together with those materials, they can enhance the osteo-integration processes (3-10). Poly-L-lactic acid (PLLA) is a bioabsorbable polymer that is widely investigated for its use in drug delivery systems, surgical sutures, and bone fracture fixation materials (11). However, due to a very slow degradation rate, this polymer may be undesirable for some applications (12). Thus, polymeric blends, such as the blend of PLLA with poly-ethylene oxide (PEO), have been used to control the PLLA degradation rate. PEO is a biomaterial known for its biocompatibility and low toxicity (4). Coraça et al. (13) observed the crack formation in PLLA/PEO pins after implantation in rat tibia, which allowed tissue growth into the implant and improved the bone interaction compared with pure PLLA implants. PLLA is a Food and Drug Administration (FDA)-approved material that can be implanted or administered by injection (14,15).

No potential conflict of interest was reported.

In addition to the positive results with PLLA and PEO, infections associated with metal and polymeric surfaces or dead tissues, such as bone grafts, can still be fatal for the patient and represent a major financial burden for the economy. The adhering bacteria in these cases can evade host defenses by forming biofilms. Therefore, the prevention of bacterial colonization is of great interest. Injury to the surrounding tissue is unavoidable during implantation, resulting in impaired blood circulation in this area and triggering an inflammatory reaction. Bacteria are more apt to colonize the surface of the implant, where the host's defense is weakened and the blood supply is insufficient. When antibiotics are administered systemically, they do not reach a high therapeutic level in the area directly surrounding the implant, rendering long-term high doses of antibiotics unsuccessful in most cases (16). When examining the relationship among the implant, the host and bacteria, competition for the colonization of the implant surface can be observed. If the body's own cells are the first to colonize the surface, they form a cell ''lawn'' of connective tissue, which makes adhering to the surface difficult for the bacteria and thus effectively protects the implant from infections. If, however, the bacteria colonize the surface first, they can, similar to the body's own cells, form a biofilm that protects itself from host immune cells. This phenomenon is called the ''race for surface'' (17).

The aim of this study was to investigate bone repair using implants of vancomycin-loaded and vancomycin-unloaded PLLA/PEO microspheres as a bone graft substitute and possible carriers of antibiotics for local therapy. The microspheres containing vancomycin should not impair the process of bone healing.

MATERIALS AND METHODS

Animals

A total of 80 adult (eight weeks old) male Wistar rats were divided into two groups as follows: 40 animals were implanted with vancomycin-loaded PLLA/PEO microspheres and 40 animals were implanted with vancomycin-unloaded PLLA/PEO microspheres as a control. Although the objective of this study was the evaluation of bone healing in the presence of vancomycin-loaded implants, no tests for spontaneous bone healing were performed. During the experimental period, the animals remained in plastic cages under controlled light and ventilation, receiving solid food (Nuvilab CR-1 from Nuvital) and water *ad libitum*. The study was carried out with the approval of the Ethical Committee of Animal Research (CEEA/Unicamp Protocol n. 339-1) of the Campinas State University, São Paulo, Brazil.

Production of the microspheres

Pure PLLA and pure PEO granulates (Sigma Aldrich) were mixed in a ratio of 80:20 and melted in a Mini Max Molder LMM-2017 injector (Custom Scientific Instruments, Inc., Whippany, NJ). The polymer was heated inside a mold and stirred for gravity collection. The resulting stick blend was cooled at room temperature. Half a gram of the 80:20 PLLA/PEO blend was dissolved in 10 ml of dichloromethane (DCM). Vancomycin was added to the polymeric solution (0.75 ml) and mixed to create an emulsion. To gather the microspheres, the emulsion was placed in a 2% isopropanol solution. The solution was vortexed until total DCM evaporation occurred, and the microspheres were collected from the supernatant and vacuum dried. The microspheres, sized 100-200 μm in diameter, were compressed to facilitate their use during surgery.

Surgical procedures

The animals were anesthetized before the surgical procedure to create the defects. The anesthesia was induced interperitoneally with 0.1 mg/100 mg body weight ketamine (Ketalar®, Pfizer, Guarulhos, Brazil) and 0.08 mg/100 mg body weight xylazine (Rompun®, Bayer, Sao Paulo, Brazil). Under anesthesia, a longitudinal incision was made in the skin above the proximal portion of the left tibia. The muscle tissue was retracted, and the bone was exposed. An odontological drill was used to create a cavity (3-mm diameter) in the tibia. During this process, the area was continuously irrigated with a saline solution to avoid overheating. The cavity was filled with the microspheres, and the muscle and skin were sutured. In each group, 40 animals were divided into subgroups of five animals for each experimental period (2, 4, 8, and 12 days and 4, 8, 16, and 32 weeks). No animals died during these periods. At the end of each period, the animals belonging to each subgroup were euthanized by an overdose of anesthesia, and the implanted tibia was removed for histological evaluation.

Table 1 - Estimated histopathological features used in the semi-quantitative analysis.

Items	Intensity			
	Absence (0)	Light (1)	Moderate (2)	Intense (3)
Granulation Tissue (HE 10×)	-	Occupying up to 1/3 of the microscopic field	Occupying up to 2/3 of the microscopic field	Occupying from 2/3 to all of the microscopic field
Acute Inflammation (HE 40×)	-	Occupying up to 1/3 of the microscopic field	Occupying up to 2/3 of the microscopic field	Occupying from 2/3 to all of the microscopic field
Bone Formation	-	Around the microspheres	Around and partially filling the center of the microspheres	Around and completely filling the center of the microspheres
Item	**Intensity**			
	Level 1	**Level 2**	**Level 3**	**Level 4**
Connective Septae	Loose connective tissue	Dense connective tissue	Connective tissue with partial ossification	Total ossification

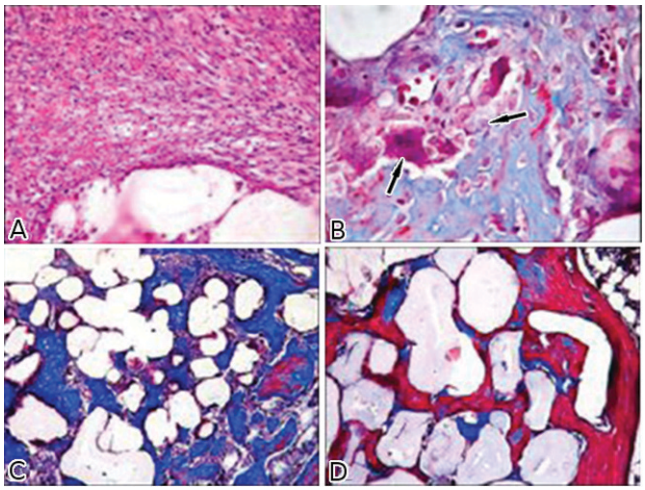

Figure 1 - Histology of a tibia implanted with vancomycin-loaded microspheres composed of PLLA/PEO: **A)** implant on the 4th day, characterized by exuberant granulation tissue around the microspheres (Original H&E 20×); **B)** implant on the 12th day, showing remodeling into woven bone characterized by osteoblastic (arrow right) and osteoclastic (arrow left) activity (Original TM 40×); and **C and D)** implants on the 8th day, characterized by woven bone surrounding the microspheres that becomes mature when composed of lamellar bone at 32 weeks (Original TM 10×).

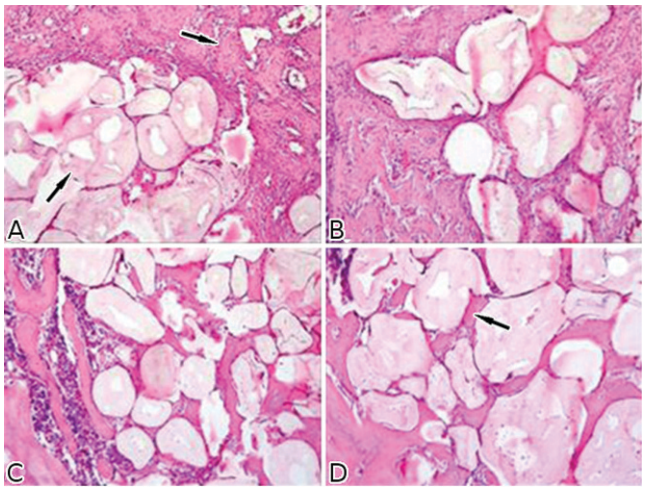

Figure 2 - Histology of tibias implanted with PLLA/PEO that were **A)** vancomycin-loaded or **B)** vancomycin-unloaded on the 4th day, showing evident granulation tissue around the microspheres (arrow) with the initial replacement by bone formation (arrow). This figure also shows the histology of the tibias of animals implanted after 12 days with PLLA/PEO that were **C)** vancomycin-loaded or **D)** vancomycin-unloaded, showing trabecular bone encasing the microspheres (Original H&E 10×).

Microscopic evaluation and morphometric analysis

The removed tibiae were fixed in 37% formalin, decalcified in ethylenediaminetetraacetic acid (EDTA) solution and submitted to histological analysis. The sections were stained with hematoxylin/eosin and Masson's trichrome stain. Conventional light microscopy was used for the histopathological evaluation. Qualitative (descriptive) and semi-quantitative analyses were used according to the following criteria (Table 1).

For the histomorphometric analysis, the Masson's trichrome-stained sections were photographed, and the images were analyzed with the software Image Lab® (ImageLab, Sao Paulo, Brazil). The results were used to measure the new bone formed (%) around the implant (microspheres).

Statistical analysis

The Mantel-Haenszel test was used to compare the different levels of intensity obtained by the morphometric (semi-quantitative) assessments. The co-variance analysis and Mann-Whitney test were used to compare the differences between the percentages of bone formation around the microspheres during the experimental periods, as obtained by the histomorphometric analysis. *p*-values of 0.05 or lower were considered statistically significant. SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis. The software GraphPad® Prism 5.01 (GraphPad Software Inc., La Jolla, CA, USA) was used to create the graphs.

RESULTS

Microscopic findings

The microscopic findings were similar in both groups throughout the experimental periods. The vancomycin-loaded and vancomycin-unloaded PLLA/PEO microspheres were easily visible inside the medullary cavity in both groups during all evaluated periods (Figures 1, 2, and 3). After two days, fibrin and hemorrhaging were visibly close to the implant in the medullary cavity and were replaced by granulation tissue on approximately the fourth day (Figure 1A).

In addition, a loose interstitial connective mesh was observed wrapped around the microspheres. Septa of a connective-like tissue were observed invading the spaces between the microspheres. Those septa were comprised of fibroblast-like mesenchymal elements with osteoblast differentiation and endothelial-like cells that created vascular lumens. Discrete inflammatory cell foci with neutrophilic predomination were observed only during the initial period (two and four days). On the fourth day, the proliferation of initial immature bone formation (woven bone) was observed around the cluster of microspheres,

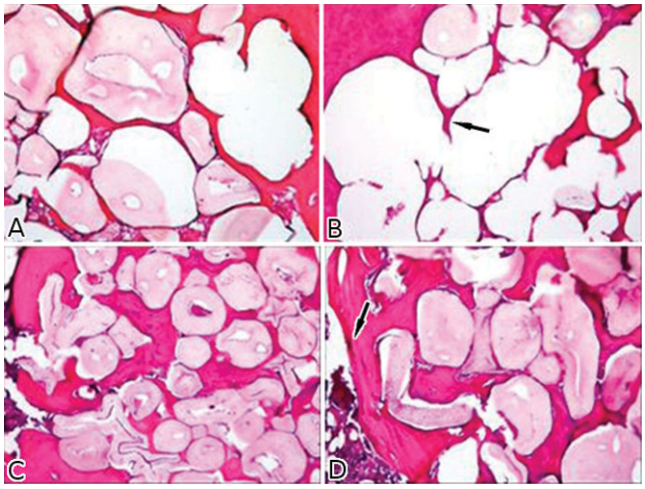

Figure 3 - Histology of tibias implanted with PLLA/PEO that were **A)** vancomycin-loaded or **B)** vancomycin-unloaded after 4 weeks, showing thin trabecular bone (arrow); and PLLA/PEO that were **C)** vancomycin-loaded or **D)** vancomycin-unloaded during a 32-week period, showing massive bone formation surrounding the microspheres with evident maturation and the presence of lamellar bone (arrow) (Original H&E 10×).

gradually replacing the granulation tissue and the interstitial connective septa (Figures 2A and 2B) and becoming abundant in the eight-week implants (Figure 1C). On approximately the 12th day, obvious trabecular bone was observed around the microspheres (Figures 2C and 2D). This period was also characterized by bone remodeling with intense osteoblast and osteoclast activity (Figure 1B). Thin trabecular bone was observed in the fourth week (Figures 3A and 3B). After this event, gradual bone maturation was observed with lamellar-like bone centripetally in the microsphere group, which had the thickness of trabecular bone. In the final periods, a completely mature ossification of the septum inside the implant and around each microsphere was observed (Figures 3C and 3D). This ossification formed a capsule encasing the implant. Tissue rejection signals, such as thick fibrous capsules or necrosis, were not observed.

Histopathological (semi-quantitative) analysis

The level of intensity for each item evaluated by the histopathological (semi-quantitative) analysis (Figure 4 and

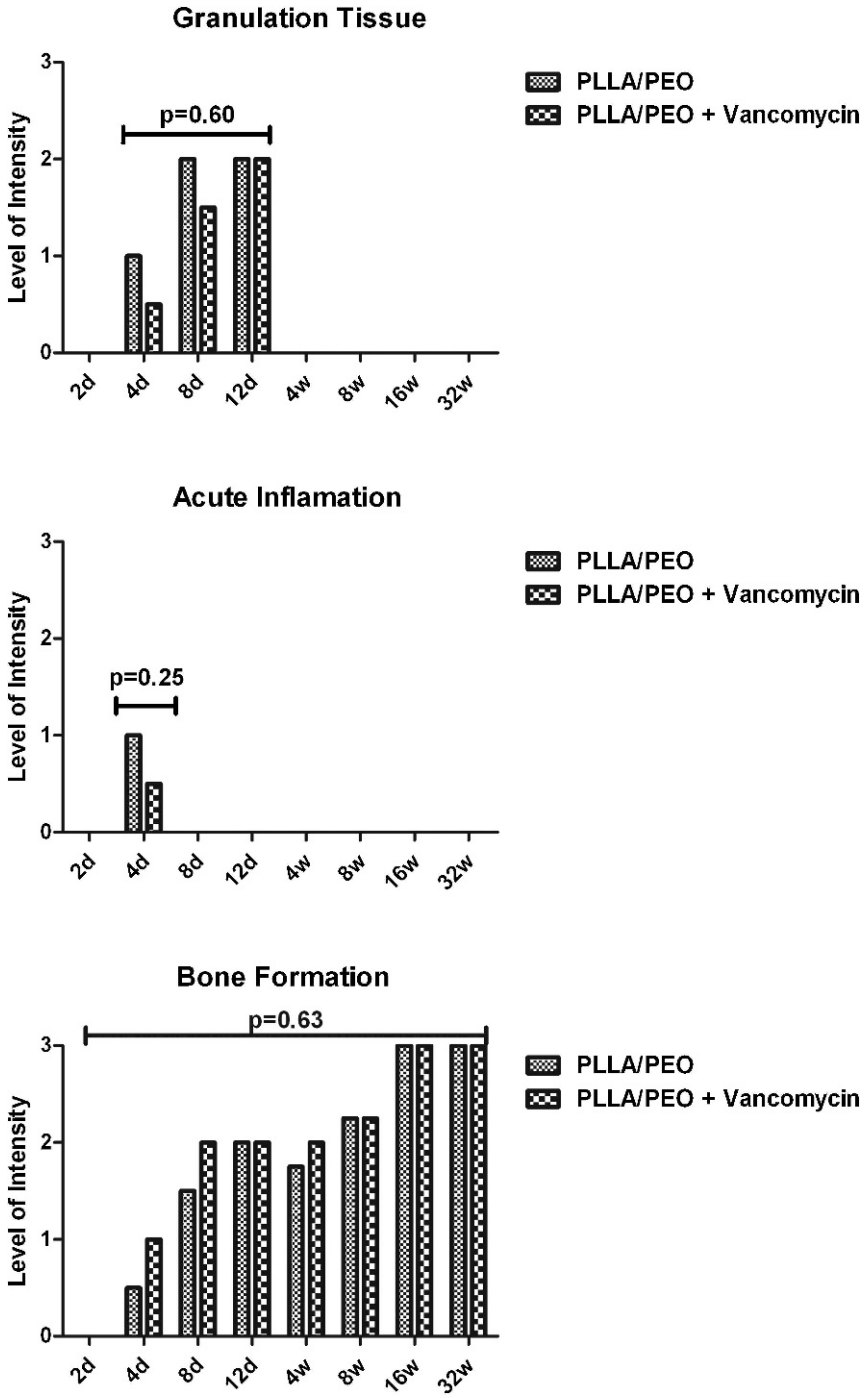

Figure 4 - Histopathological (semi-quantitative) analysis: 0 = absent, 1 = light, 2 = moderate and 3 = intense. The periods that showed no values are equivalent to 0 = absent.

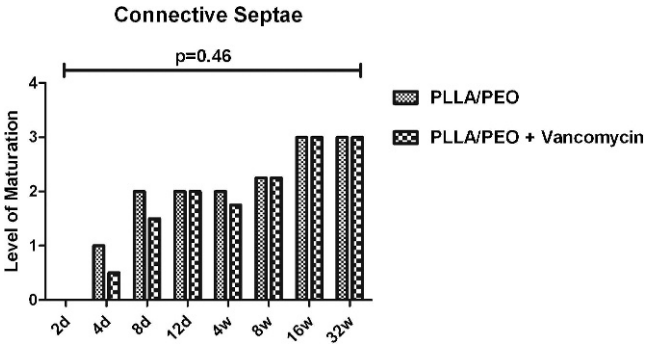

Figure 5 - Level of maturation of the connective septae formed around the microspheres (1 = loose connective tissue, 2 = dense connective tissue, 3 = connective tissue with partial ossification and 4 = total ossification). The periods that showed no values are equivalent to 0 = absent.

5) did not exhibit any significant differences between the vancomycin-loaded and vancomycin-unloaded PLLA/PEO microsphere groups.

Histomorphometric analysis

The percentage of bone formation was similar between the vancomycin-loaded and vancomycin-unloaded PLLA/PEO microsphere groups. However, a significant difference in the bone formation was noted over the course of the entire experimental period ($p \leq 0.05$). The bone formation increased gradually during the four earliest experimental periods and stabilized in approximately the fourth week. Subsequently, the bone formation process underwent a maturation phase that continued into the last period (Figure 6).

DISCUSSION

The PLLA/PEO blend was selected for this study due to its well-known biocompatibility (5,13). Vancomycin was added to the PLLA/PEO blend because of its common use as a local therapy for bone infection (18).

After implantation, the microspheres remained inside the medullar cavity in all animals. This result may be explained first by the connective and bone tissue proliferation encasing the microspheres and, second, by the long-term degradation of the PLLA (12). The inflammatory process was mild and present only during the initial periods (from 2 to 4 days) with neutrophilic predominance, a characteristic process of tissue repair.

A mild inflammatory reaction with giant cells was observed without edema, and, therefore, the bone regeneration was not affected. Beginning on the fourth day after the implantation, the areas of fibrin and hemorrhage, organized in the granulation and fibrous connective tissue, were gradually replaced by bone. The bone grew between the individual microspheres, encasing the implant. This finding demonstrated the characteristic osteointegration capacity of this material. Ueng et al. (20) detected this reaction in rabbits loaded with vancomycin-loaded poly (L-lysine) (PLL) beads, which were encased by bone after eight weeks.

The osteointegration process in the present study was better for PLLA/PEO microspheres compared with PLLA/PEO molded as pins (13). This result can likely be attributed to the sphere morphology, which permits more tissue interaction with the bone formation that occurs not only around but also inside the implant and in between the microspheres. Another advantage of the microsphere morphology is that it facilitates the use of beads in surgical bone cavities. Furthermore, the microspheres do not need to be cast in advance or be polymerized under high temperatures during surgery. Ueng et al. (20) described this advantage for bone repair after tumor removal or infectious material curettage, such as in osteomyelitis. In addition to the morphology, the microsphere-shaped implants, in association with antibiotic drugs, could be candidates for local antibiotic therapy to prevent and treat bone infections.

In this study, the gradual bone formation was morphologically and morphometrically assessed. The observed bone was of a ''membranous'' type formed independent of cartilage. Arrotéia et al. (21) have confirmed that a local vessel supply and granulation tissue formed by hemorrhagic clots and fibrin nets are basic requisites for the formation of bone of this type. The bone healing process involves a series of highly regulated steps that depend on the interactions of two cell lineages: the mesenchymal osteoblasts and hematopoietic osteoclasts. During this process, the bone matrix is deposited in an irregular manner and with a definitive form characterized by a trabecular bone structure that is sculptured or remodeled by the reabsorptive action of the osteoclasts. This process is followed by the deposition of new bone via the action of osteoblastic cells during the following periods until reaching the thickness of the trabeculae of the local pre-existing bone bars (21,22). This study demonstrated similar aspects in the bone healing process. Gradual bone growth was morphologically and morphometrically proven during the experimental

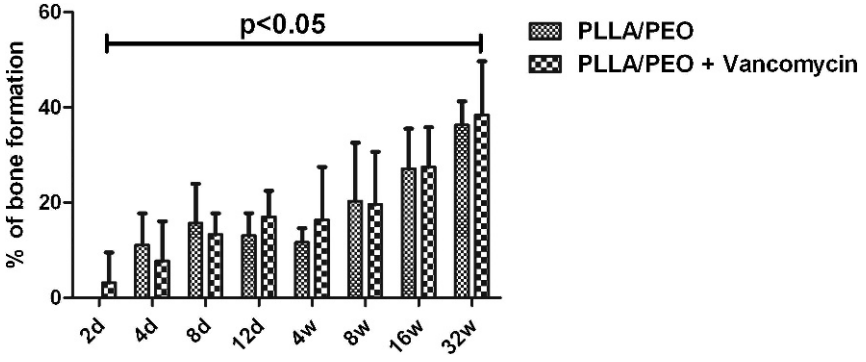

Figure 6 - Percentage of bone formation during the different experimental periods for the vancomycin-loaded PLLA/PEO microspheres and vancomycin-unloaded PLLA/PEO. The periods that showed no values are equivalent to 0 = absent.

periods. Thin trabeculae were observed after four weeks, followed by a stabilization of the bone formation. The presence of thin trabeculae can be explained by the osteoclastic activity in the bone remodeling cycle. This phenomenon was clearly verified in the previous period analyzed (12 day), which was followed by osteoblastic activity that was responsible for the new bone deposition and the progressive trabecular thickening observed in the successive periods.

According to Raisz (22), after the reabsorption activity by the osteoclasts during the bone remodeling cycle, there is a subsequent reversal phase in which mononuclear cells are present. These cells may complete the reabsorption process and produce the signals that initiate the bone formation. Finally, successive waves of mesenchymal cells differentiate into functional osteoblasts, which lay down the matrix during the bone formation phase. In our study, this reversal phase was observed from the eight week until the last experimental periods in both groups. Complete bone maturation, characterized by lamination of the trabeculae and a return to its original thickness, was observed in the last period (32 weeks).

Ribeiro et al. (23) describe this bone architecture change during the evaluation of cavities obtained in dogs in an experimental study of spontaneous bone regeneration. In this case, from six to ten weeks after the bone formation, an osteoporotic appearance was observed in the trabecular bone compared with the appearance in the other periods. This phenomenon could be related to the osteoclastic activity. However, the trabecular thickness and distribution returned to the initial thickness and distribution after 12 weeks. The transition reported in both the study by Ribeiro et al. and our own study did not impair the lesion healing.

The statistical analyses showed progressive bone formation during all of the experimental periods. The new bone growth curve revealed the characteristics of the bone healing process. These findings were also observed by Ribeiro et al. (23), who described bone maturation and lamination with trabecular thickening on the implants after 12 weeks during spontaneous bone regeneration in dogs.

In conclusion, our results demonstrated efficient bone regeneration using PLLA/PEO blends mixed with vancomycin. In this case, the vancomycin did not impair the bone healing process when associated with PLLA/PEO microspheres. Further investigation is needed with respect to the delivery rate of vancomycin from the PLLA/PEO microspheres and its efficacy against microorganisms usually involved in implant infections. Overall, the vancomycin-loaded PLLA/PEO microspheres appear to be good candidates as a bone substitute or as an adjuvant for bone repair.

ACKNOWLEDGMENTS

This work was supported by the Coordination for the Improvement of Higher Level Personnel (CAPES) and the Foundation of Support to Education and Research (FAEP), Sao Paulo, Brazil. We thank Dennis Huber for his assistance with the statistics analysis and manuscript edition.

AUTHOR CONTRIBUTIONS

Coraça-Huber DC participated in the development of the study plan, performance of the laboratory experiments and animal surgery, analysis of the data and writing of the manuscript. Amstalden EMI participated in the development of the study plan, analysis of the data and writing of the manuscript. Duek EAR participated in the development of the study plan, performance of the laboratory experiments, production of the biomaterials and analysis of the data. Etchebehere M participated in the development of the study plan and analysis of the data. Magna LA participated in the performance of the statistical analysis, analysis of the data and writing of the manuscript.

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