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Castor oil polymer induces bone formation with high matrix metalloproteinase-2 expression

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Received 3 January 2013; accepted 12 February 2013
Published online 14 May 2013 in Wiley Online Library (wileyonlinelibrary.com). 10.1002/jbm.a.34696

Abstract: The aim of this study was to evaluate the modulation of matrix metalloproteinase-2 (MMP-2) and −9 (MMP-9) expression in newly formed bone tissue at the interface between implants derived from castor oil (*Ricinus communis*) polymer and the tibia medullary canal. Forty-four rabbits were assigned to either Group 1 ($n = 12$; control) or Group 2 ($n = 30$), which had the tibial medullary canals reamed bilaterally and filled with polymer. CT scans showed no space between the material surface and the bone at the implant/bone marrow interface, and the density of the tissues at this interface was similar to the density measured of other regions of the bone. At 90 days postimplantation, the interface with the polymer presented a thick layer of newly formed bone tissue rich in osteocytes. This tissue exhibited ongoing maturation at 120 and 150 days postimplantation. Overall, bone remodeling process was accompanied by positive modulation of MMP-2 and low MMP-9 expression. Differently, in control group, the internal surface close to the medullary canal was lined by osteoblasts, followed by a bone tissue zone with few lacunae filled with osteocytes. Maturation of the tissue of the medullary internal surface occurred in the inner region, with the bone being nonlamellar. © 2013 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 102A: 324–331, 2014.

Key Words: castor oil (*Ricinus communis*) polyurethane, biomaterials, computed tomography, matrix proteins, biomineralization, mechanisms

How to cite this article: Saran WR, Chierice GO, da Silva RAB, de Queiroz AM, Paula-Silva FWG, da Silva LAB. 2014. Castor oil polymer induces bone formation with high matrix metalloproteinase-2 expression. J Biomed Mater Res Part A 2014:102A:324–331.

INTRODUCTION

Bone loss caused by trauma, infections, tumors or congenital malformations is often treated with autogenous bone grafts, often yielding satisfactory results.[1,2] However, the complex repair of various deformities for which location, size and need for a donor site are determining factors[3,4] has led researchers to seek alloplastic materials or biomaterials that are suitable for bone grafting; these materials include polyethylenes, ceramics (hydroxylapatite, calcium aluminate and tricalcium phosphate), silicon, titanium, and polymethylmethacrylate.[5,6]

With regard to fracture fixation, an optimally resistant biomaterial that provides rigid fixation is currently being sought. In addition to fracture resistance, this biomaterial should be osteoconductive and should possess osteogenic properties. Among inert materials, a polymer based on castor oil has demonstrated promising properties, including osteoconduction and osteogenesis.[7]

The castor oil plant is a Brazilian tropical plant (*Ricinus communis*) of the class Dicotyledons, order *Geraneaces*, and family *Euphorbiaceae*.[8] Previous studies have shown that polymers prepared from castor oil plant are biocompatible when in contact with cultured cells and connective tissue and when used in the form of implants or grafts[9–11] in subperiosteal sites[12] or in bone tissue.[7,13–19] However, the mechanisms involved in the tissue remodeling process associated with the use of castor oil polyurethane are poorly understood.

Matrix metalloproteinases (MMPs), which are key enzymes of collagen metabolism that function under physiological conditions and during pathological changes, are involved in the tissue remodeling process occurring in bone tissue[20,21] that is in direct or indirect contact with biomaterials. MMPs are part of a key family of metal-dependent endopeptidases and are thought to be the primary enzymes involved in the remodeling of extracellular matrix components. MMPs are secreted in the form of inactive proenzymes called zymogens and are activated in the tissue through the cleavage of pro-peptides.[21,22] These proteinases

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Contract grant sponsor: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)

are expressed by resident connective tissue cells in response to specific stimuli as well as in cells that are recruited during the tissue remodeling process.[20,22–25]

The aim of this study was to analyze the phenomena of osteogenesis that occurs in response to plant polymer implants and to determine the effects of such implants on the expression of matrix metalloproteinases 2 and 9 through immunohistochemistry, histological analysis by light microscopy and computed tomography.

MATERIALS AND METHODS

Animals and experimental protocol

Following the approval of the research project by the Ethics Committee on Animal Use of the Animal Facility of the University of São Paulo (06.1.343.53.3), 44 male albino rabbits, *Oryctolagus cuniculus*, of the New Zealand lineage that were 8–10-weeks-old and weighed approximately 2.5 kg were selected. The selected animals were assigned into two groups. Group 1 consisted of 12 control animals whose tibial medullary canals were reamed bilaterally but were not filled, while Group 2 consisted of 30 animals whose tibial medullary canals were reamed and then filled with cylinders derived from castor oil polyurethane. The animals in Groups 1 and 2 were randomly divided into three experimental subgroups according to the scheduling of euthanasia (90, 120, or 150 days after surgery.

General anesthesia was performed intravenously with Nembutal sodium at a dose of 25 mg/kg body weight; supplemental anesthesia was performed with the same drug when needed. The animal was placed in a supine position on the operating table, trichotomy of the right and left knees was performed and antisepsis was performed with iodized alcohol. The surgical field in both knees had a fenestration in the areas of operation. Every instrument used was previously autoclaved at 120°C for 20 min and placed on sterile surgical drapes.

For anesthesia, the marginal ear vein was punctured using a siliconized needle with ultra-thin walls and a short 21-gauge butterfly with three-sided bevelled edges. The tibial medullary canal was selected for implant placement because it is straighter than the femur and provides technically less invasive access.

A cylinder-shaped polymer derived from the castor oil plant was introduced in the knee extensor surface at a dissection plane medial to the patellar tendon. Tibial access was performed through a superficial incision of approximately 1.5 cm in the ventral and medial side of the knee next to the patellar tendon insertion using a number 15 scalpel blade, followed by exposure of the tendon and its separation laterally to expose the medial side of the extra-articular proximal tibia.

Tibial perforation was initiated using a 2-mm dental bur with an electric motor to access the medullary canal. The dental bur was subsequently introduced throughout the tibial medullary canal from the proximal metaphysis to the distal epiphysis of the rabbit's tibia with the aid of a T-handle wrench with mandrel.

To prevent heat-induced cell damage caused by the high-speed dental drill, the drill motor was not used. After reaming the tibial medullary canal, a cylinder of polyurethane with calcium carbonate sterilized with ethylene oxide was introduced into the canal without exceeding the tibial distal epiphysis. The cylinders of polyurethane with calcium carbonate, which were 2-mm thick and thus filled the entire length of the medullary canal, were provided by the Institute of Chemistry of São Carlos, University of São Paulo (Instituto de Química de São Carlos da Universidade de São Paulo). Each cylinder was cut according to the length of the medullary canal (the average canal length was 11 cm) and arranged so that it was precisely buried in the medullary canal at the inlet port. The incision was then sutured with 4–0 monofilament thread and cleaned with saline solution, leaving it uncovered and without any type of immobilization. The animals were examined weekly by palpation of the surgical sites and manipulation of the knee joints to evaluate the status of surgical wounds and to identify signs of inflammation or dehiscence of the suture, recession of implanted materials and other restrictions.

Following the experimental period, the animals were euthanized using the same anesthetic used during surgery. The central portion of the tibial diaphysis was chosen as the study area.

Tomographic evaluation

Tomographic examinations were performed using Sytec GE Medical Systems Synergy 1999 computed tomography equipment with software version 3.5 A. The standardized method adopted in this study used 120 kv and 60 mA with a FOV (field of view) of 23.4 cm. Following euthanasia, 1-mm thick axial tomographic sections with 1-mm spacing between sections were prepared. For each tibia, the following images were acquired: two contiguous images of the proximal metaphysis, four contiguous sections from the middle third of the diaphysis and two contiguous sections from the distal metaphysis.

Histological processing

The pieces were fixed in buffered 10% formalin for 72 h at room temperature and demineralized in an EDTA-based solution activated in a microwave oven (Sharp Carousel; São Paulo, SP, Brazil). After demineralization, the pieces were neutralized in 5% sodium sulfate (JT Baker, Xalostoc, Mexico), washed in running water for 24 h, dehydrated in an ascending series of ethanol, cleared in xylol, and embedded in paraffin according to standard methods of histological processing.

The blocks were serially sectioned, and 5-μm thick longitudinal sections were stained with hematoxylin and eosin (HE), Mallory's trichrome and Brown and Brenn staining for histological analysis under optical microscopy by one blind examiner using a Zeiss Axio Imager M1 binocular light microscope (Carl Zeiss AG Light Microscopy). The presence or absence of inflammation and the biological mechanisms involved in bone tissue repair were assessed. The biological mechanism of bone tissue repair was based on the presence

of the following: (1) bone callus; (2) new vascular formation; (3) new bone formation (with the identification of the cellular elements involved and formation of osteon); (4) immature bone tissue; (5) mature bone tissue; (6) reversal lines in bone.

Immunohistochemistry

The Goat ImmunoCruz™ system (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used for immunohistochemical experiments. Briefly, tissue sections were quenched in peroxidase buffer for 5 min, and antigen retrieval was performed by incubation with 0.05% Proteinase K from *Engyodontium album* (Sigma-Aldrich, St. Louis, MO) at 37°C for 15 min. Nonspecific binding was blocked by treating sections with donkey serum blocker for 30 min. The sections were then incubated for 1 h with primary polyclonal goat antibodies to MMP-2 (5.0 μg/mL; Santa Cruz Biotechnology, sc-8835) and MMP-9 (5.0 μg/mL; Santa Cruz Biotechnology, sc-6840). The sections were incubated with anti-goat secondary antibody for 30 min followed by horseradish peroxidase-streptavidin for 20 min and the enzyme substrate 3,3′-diaminobenzidine for 5 min. Tissues were counterstained with Harris's hematoxylin and mounted using standard protocols. Negative controls were included in which the primary antibody was replaced with goat immunoglobulin G (IgG). The number of positively labeled cells was estimated for each antibody in three representative fields of view at 63× magnification. The data were analyzed using ANOVA followed by Tukey's test ($\alpha = 0.05$).

RESULTS

Tomographic analysis

Tomographic images of the implanted tibiae showed the polymer implant in intramedullary position. The implant was clearly identified in the images; it could be recognized in each of the sections as a small circular homogeneous structure denser than bone marrow and less dense than cortical bone. In the groups of animals euthanized at 90, 120, and 150 days after polymer implant surgery, the tomographic images showed no significant changes in the implant/bone marrow/bone interface. The density of tissues measured in this interface was similar to the density of other bone marrow portions. The boundaries between the implanted polymer and the marrow cavity are poorly defined in the computed tomography images, indicating the incorporation of material into newly formed tissue.

The polymer/medullary canal interface revealed filling of space with bone tissue. No signs of bone resorption around the implant were noted in the computed tomography images of animals in the 90, 120, or 150 days postimplantation groups (Fig. 1).

Microscopic analysis

At 90 days, the inner bone wall of the medullary canal and the interface with the polymer showed a thick layer of newly formed bone tissue that was rich in osteocytes and creating a new external fundamental system, as well as another fundamental system attached to the old bone and forming a reversal line of growth. This tissue was in the process of maturation, as evidenced by the fact that the lamellae of collagen fibers were organized in a circular way so as to form a Haversian system within which soft tissue and blood vessels were present [Fig. 2(A)].

The presence of a reversal line separating new bone from the old bone was evident. This strongly stained winding line delimited the newly formed bone along the inner surface of the medullary canal. Within a narrow strip, portions of the newly formed bone contained numerous osteocytes surrounded by a matrix whose collagen fibers ran parallel to the forming primary bone. There was a

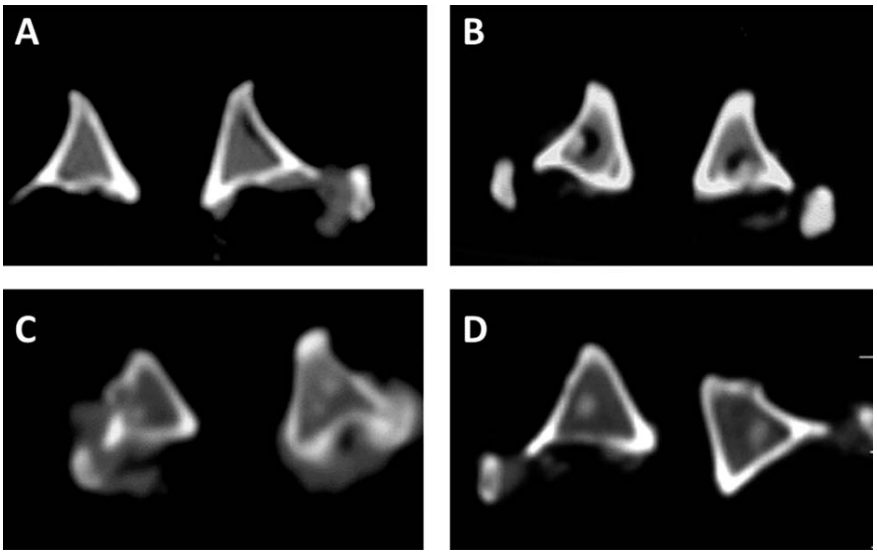

FIGURE 1. Computed tomography of the medullary canal of tibial metaphysis. Control without reaming (A), and 90 (B), 120 (C) and 150 (D) days after polymer implantation.

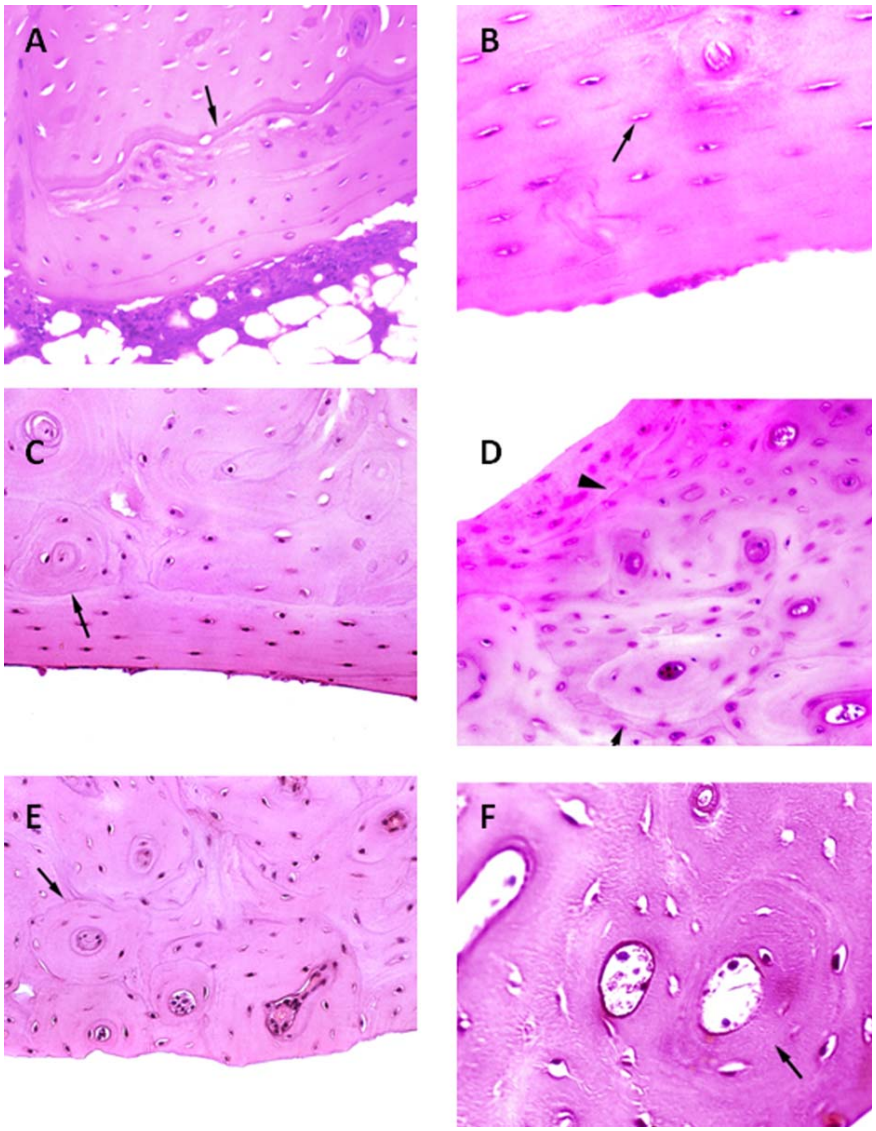

FIGURE 2. Histological sections of the medullary canal of tibial bone 90 (A), 120 (C), and 150 (E) days after reaming and castor oil polymer implantation. Controls included reaming without polymer implantation at 90 (B), 120, (D) and 150 (F) days. Reversal line (arrow) can be seen in A, B, C, and D. Lamellae concentric to the Haversian canal (arrow) indicate bone maturation (E, F). Hematoxilin and Eosin ×875. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

concentration of mononuclear cells in some areas of the bone surface of the medullary canal that often filled the recesses of the bone tissue. Volkmann's canals joining the newly formed bone with the bone from the receptor site were observed. Secondary mature bone was present at isolated sites in the medullary canal. There was also new bone formation on the inner wall of the medullary canal.

In the control group, the newly formed bone lining the wall of the medullary channel was noticeably rich in osteoblasts; in tissues from these animals, newly formed nonlamellar (immature) bone can be seen near the medullary canal. In addition, the newly formed tissue near the medullary surface showed a lower degree of maturation of collagen fibers than the bone tissue located at a distance [Fig. 2(B)].

At 120 days after implantation, the inner surface of the newly formed bone was found to be undergoing a maturation process; there were few osteocytes within the matrix, and collagen fibers were organized in concentric lamellae near the Haversian canals. Newly formed bone was undergoing an outright maturation process. The inner bone surface was lined with compact inner bone showing parallel fibers that form the internal fundamental system. The presence of a reverse line of growth was not detected. Well-organized concentric lamellae with osteocytes located around the Haversian canal and the presence of Volkmann's canals were observed [Fig. 2(C)].

In control animals, the inner surface near the medullary canal was lined with osteoblasts, which lay next to a strip

of bone tissue with few lacunae filled by osteocytes; a reverse line of growth of homogeneous diameter, delimiting the Haversian lamellar bone, was present. The Haversian canals were filled with blood vessels. The degree of tissue maturation progressing from the medullary inner surface to the inside is evident, and the inner surface of the bone is nonlamellar, consisting of collagen fibers that are less mature than the lamellar bone of the innermost bone portion [Fig. 2(D)].

At 150 days following implantation, there was a decrease in the number of osteocytes in the internal fundamental system; these were distant from each other and situated in parallel formation, demonstrating bone maturity [Fig. 2(E)]. The remaining bone was formed by well-organized concentric lamellae typical of mature bone. Hematoxylin and eosin staining revealed few osteocytes in the internal fundamental system, which filled the lacuna as a whole.

In control animals, lamellar bone was present from the inner portion near the medullary canal to the body of the tibia. The lamellae were concentric to the Haversian canals, which were of homogeneous diameter and filled with blood vessels. Osteocytes were numerous and surrounded collagen fibers forming lamellae concentric to the Haversian canals [Fig. 2(F)].

Matrix metalloproteinase-2 (MMP-2) expression

MMP-2-positive staining was found in osteoblasts and osteocytes in the initial period of evaluation and 90, 120, and 150 days after reaming of the medullary canals with or without insertion of the castor oil polymer.

An increase of approximately 20% in the percentage of osteocytes positively labeled for MMP-2 compared with the initial period prior to reaming ($p < 0.05$) was noted at day 90 in the group of animals subjected to reaming and polymer insertion. That increase was sustained up to 150 days after polymer implantation (Fig. 3).

No MMP-2 expression modulation was found at 90, 120, and 150 days after surgery in the animals subjected to reaming without polymer implantation; in these animals, the percentage of osteocytes positive for MMP-2 was similar to that found prior to implantation ($p > 0.05$). Slides were incubated with immunoglobulin G without primary antibody as a control to detect the presence of nonspecific labeling; in the controls, no intracellular, or extracellular labeling was found.

Matrix metalloproteinase-9 (MMP-9) expression

MMP-9-positive labeling was found in osteoblasts and osteocytes in the initial experimental period and 90, 120, and 150 days after reaming of the medullary canal with or without implantation of the castor oil polymer (Fig. 4). The percentage of osteocytes positively labeled for MMP-9 at day 90 in the group subjected to reaming and insertion of the castor oil polymer was similar to that found in the initial period prior to reaming ($p > 0.05$). A small percentage of labeled cells were present 120 and 150 days after implantation of the castor oil polymer, with no statistically significant difference between periods ($p > 0.05$). In the group of animals subjected to reaming without polymer implantation, no modulation of MMP-9 expression was found 90, 120, or 150 days after surgery; the percentage of MMP-9-positive osteocytes at each of these time points was similar to that found prior to implantation ($p > 0.05$).

Slides were incubated with immunoglobulin G without primary antibody as a control for the presence of nonspecific labeling; these slides showed no intracellular or extracellular labeling.

DISCUSSION

Based on their biological properties and chemical stability, polymers have emerged as materials for biological implants in orthopedic surgery, neurosurgery, otolaryngology, and dentistry. The biocompatibility of castor oil polymer has been demonstrated in numerous studies in which it was used as graft/bone implant material or implanted in animal alveoli after tooth extraction,[13,14] although in a tibial model it has not been demonstrated.

The polyurethane evaluated in this study was derived from a polyester synthesized from diphenylmethane diisocyanate and castor oil. The mixture from which it was synthesized included calcium carbonate to increase the mixture volume and enable the formation of pores, prepolymer rich in free NCO (isocyanate) and derived from diphenylmethane diisocyanate and polyester polyol, and polyol derived from the castor oil polyurethane polymer. The addition of calcium carbonate provides bone tissue with improved strength and elasticity.[8] Moreover, calcium carbonate supplies calcium ions, which facilitate ion exchange at the bone/polymer contact interface and form deposits in the collagen matrix.[7] In this study, the use of polyurethane rods containing calcium carbonate was chosen given the ease of insertion of these materials into the tibial medullary canals of rabbits. This polymer has an exothermic reaction from 42° to 45° C. Although such a reaction would not be expected to cause thermal cell damage, polymerized material was used in this study to avoid the possibility of heat generation.[8]

The results obtained in the experimental animals in which the castor oil polymer was used over periods of 90, 120, and 150 days showed an evolutionary process of tissue repair that involved interaction between the castor oil polymer and the medullary canal. At 90 days after polymer insertion, there was a thick layer of bone tissue rich in osteocytes, bone tissue undergoing a maturation process and the presence of organized collagen fibers with a clear reversal line delimiting the newly formed bone. At 120 days, rapid maturation of the bone tissue and organization of collagen fibers was evident, and complete repair was observed at 150 days, with no foreign body reaction indicated by the presence of giant cells, as demonstrated previously.[13] Areas of fibroblast proliferation were replaced by bone lamellae within which the polymer rod was associated with the presence of newly formed bone at the polymer/medullary canal interface. During the final period of the study, we noted that the polymer underwent structural and functional osseointegration with the surrounding bone tissue.

This type of osseointegration that occurs through direct contact between the implanted material and the bone tissue

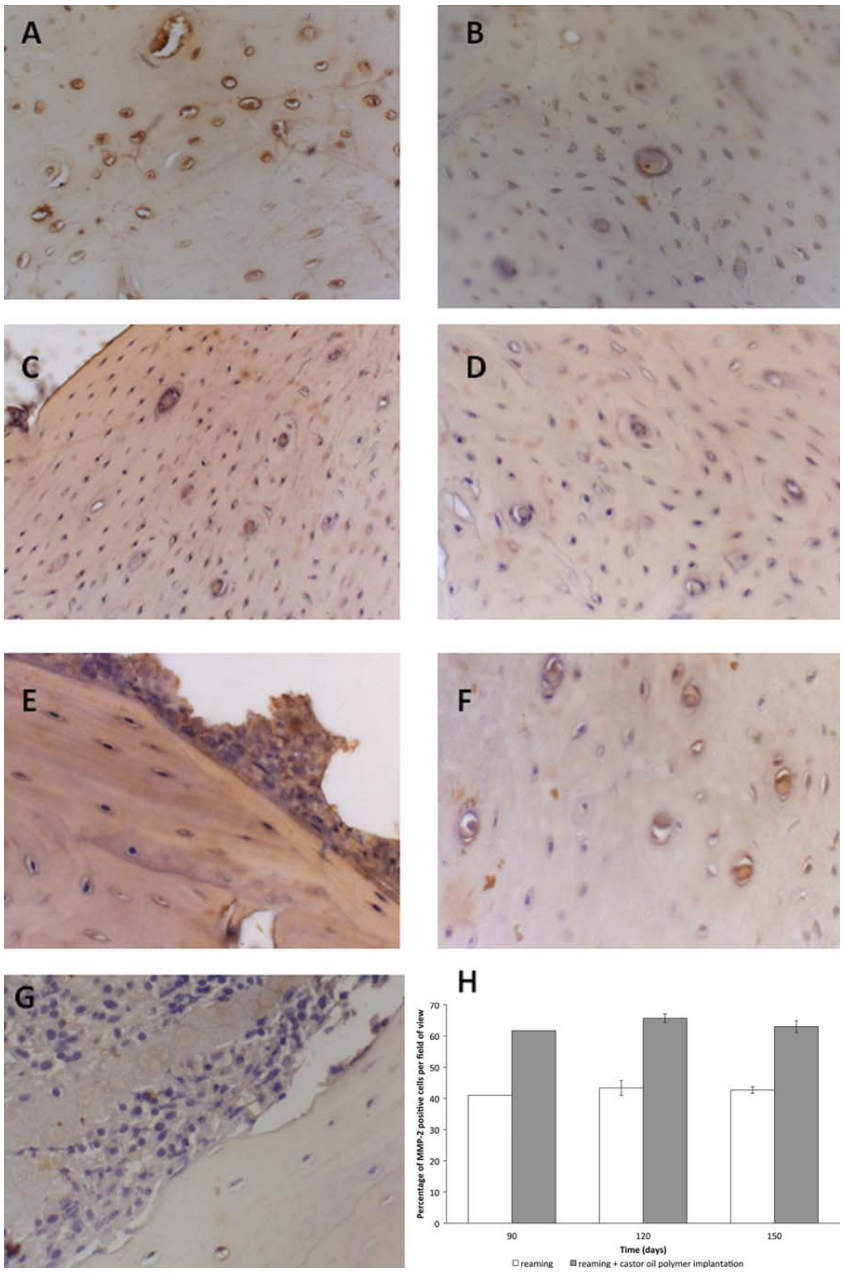

FIGURE 3. MMP-2 expression in bone surrounding the tibial medullary canal 90 (A), 120 (C), and 150 (E) days after reaming and castor oil polymer implantation. Controls included reaming without polymer implantation at 90 (B), 120, (D) and 150 (F) days. G represents a slide incubated with immunoglobulin G but without primary antibody. Percentage of cells positive for MMP-2 was quantified *per* field of view (H). A, D, E (×20); B, C, F, G (×40). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

is ideal. Indirect or fibro-osseous integration is acceptable only in the early stages of implantation and is unfavorable to the fixing of the implant material and the long-term stability of the implant.[26]

The increased expression of matrix metalloproteinase-2 during the postimplantation period indicates that the bone remodeling process develops rapidly.[27,28] We found that MMP-2 expression was higher in animals subjected to reaming and polymer implantation than in animals subjected to reaming without implantation of the polymer material. This expression remained high until 150 days postimplantation. MMP-2 is an endopeptidase that is expressed under physiological conditions and is critical for removing organic bone content during the process of bone maturation.

The expression of matrix metalloproteinase-9 in bone marrow tissue was lower than that of MMP-2. Reaming, whether followed by polymer implantation or not, did not result in increased MMP-9 expression, according to the

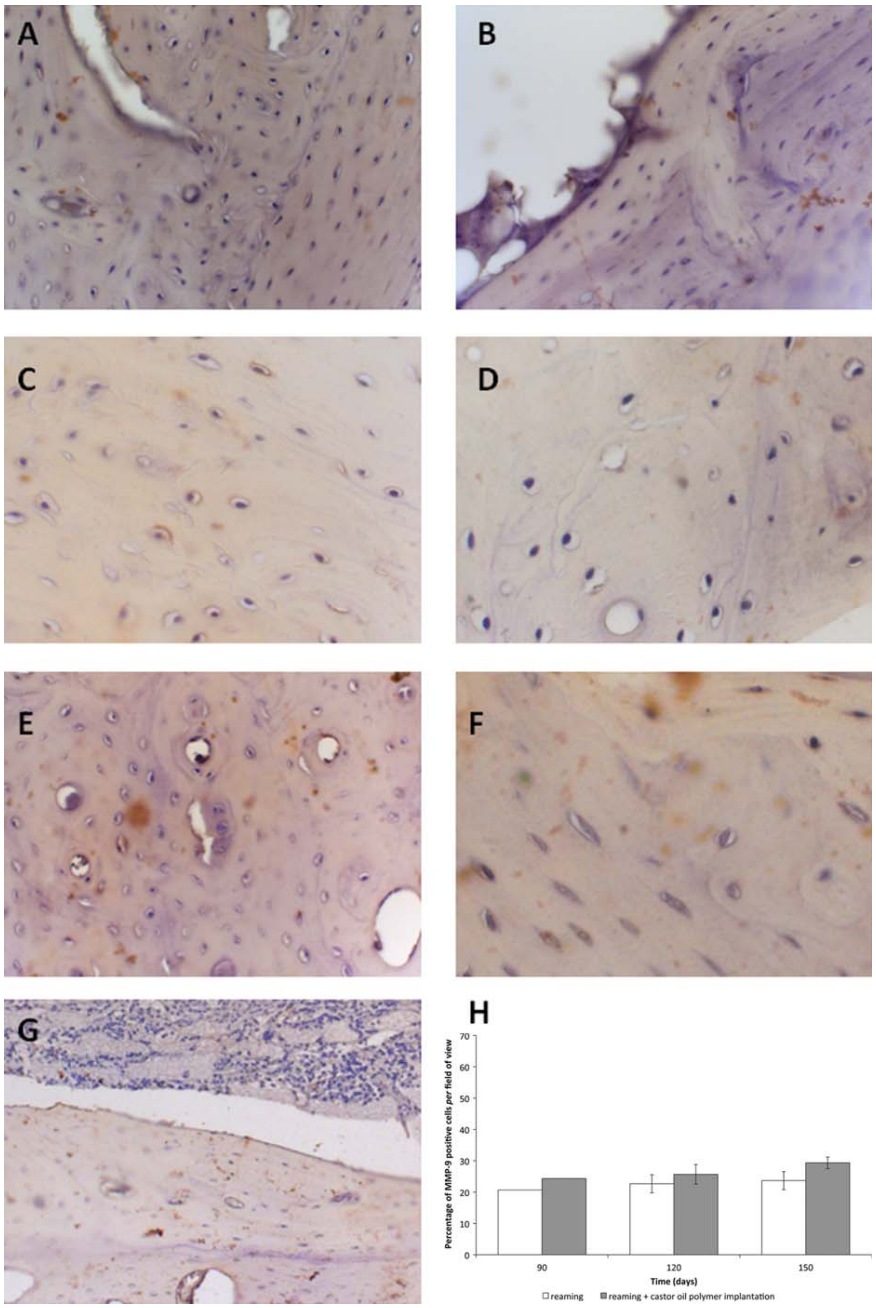

FIGURE 4. MMP-9 expression in bone surrounding the tibial medullary canal 90 (A), 120 (C), and 150 (E) days after reaming and castor oil polymer implantation. Controls included reaming without polymer implantation at 90 (B), 120, (D) and 150 (F) days. G represents a slide incubated with immunoglobulin G but without primary antibody. Percentage of cells positive for MMP-9 was quantified *per* field of view (H). A, B, D, F (×20); C, E (×40); G (×20). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

results of our histological analysis. This is most likely because MMP-9 is primarily expressed by osteoclasts during active bone resorption.[25,29]

Our results indicate that matrix metalloproteinase-2 modulation is critical for the bone maturation process observed after implantation of castor oil polymer because specimens in which reaming of the medullary canal was performed without polymer implantation showed no modulation of the expression of this enzyme.

CONCLUSIONS

Implantation of castor oil polymer after reaming of the tibial medullary canal resulted in the formation of new bone in contact with the implanted material as evaluated through computed tomography, conventional light microscopy and immunohistochemistry. At 90 days postimplantation, there was deposition of immature bone tissue that showed increasing maturity over time (at 120 and 150 days postimplantation), acquiring a lamellar aspect. The histologically

observed bone remodeling process was followed by the up-regulation of matrix metalloproteinase-2 during the evaluation period, with low expression of matrix metalloproteinase-9.

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