Methotrexate Locally Released from Poly(ε-Caprolactone) Implants: Inhibition of the Inflammatory Angiogenesis Response in a Murine Sponge Model and the Absence of Systemic Toxicity

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ABSTRACT: In this study, the methotrexate (MTX) was incorporated into the poly(ε-caprolactone) (PCL) to design implants (MTX PCL implants) aiming the local treatment of inflammatory angiogenesis diseases without causing systemic side effects. Sponges were inserted into the subcutaneous tissue of mice as a framework for fibrovascular tissue growth. After 4 days, MTX PCL implants were also introduced, and anti-inflammatory, antiangiogenic, and antifibrogenic activities of the MTX were determined. MTX reduced the vascularization (hemoglobin content), the neutrophil, and monocyte/macrophage infiltration (MPO and NAG activities, respectively), and the collagen deposition in sponges. MTX reduced tumor necrosis factor-α and IL-6 levels, demonstrating its local antiangiogenic and anti-inflammatory effects. Furthermore, hepatotoxicity, nephrotoxicity, and myelotoxicity, which could be induced by the drug, were evaluated. However, MTX did not promote toxicity to these organs, as the levels of AST and ALT (hepatic markers) and creatinine and urea (renal markers) were not increased, and the complete blood count was not decreased. In conclusion, MTX PCL implants demonstrated to be effective in regulating the components of the inflammatory angiogenesis locally established, and presented an acceptable safety profile. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:3731–3742, 2015

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INTRODUCTION

Inflammation and angiogenesis are concomitant and synergistic events of several pathologies such as rheumatoid arthritis, psoriasis, and neoplasias. One of the hallmarks of the inflammation is an increase in vascular permeability, permitting plasma components, and inflammatory cells to exit the bloodstream. The leukocytic infiltration results in an acute inflammation, which induces an angiogenic response, producing a highly vascularized granulation tissue.[1] Neutrophils infiltrate quickly; and concomitantly, circulating monocytes enter the wound and differentiate into mature tissue macrophages. Subsequently, the number of mast cells in the wound progressively increases.[2,3] In the late inflammatory stage of tissue repair, the newly formed vasculature and the influx of inflammatory cells regress, resulting in the restoration of homeostatic control. However, if the resolution of the inflammatory response does not occur, the new vasculature and the inflammatory infiltrate establish a positive feedback, exacerbating the inflammatory response.[1,4]

Methotrexate (MTX) is the central drug in the management of rheumatoid arthritis and other immune-mediated chronic inflammatory diseases. The anti-inflammatory effects of the MTX involve the inhibition of polyamine synthesis in lymphocytes.[5] Besides, a number of other mechanisms are discussed that probable mediate the anti-inflammatory effects of this drug, involving the inhibition of cytokines and mediators of the inflammatory response[6] and the increase of the plasmatic adenosine concentration.[7] Despite the therapeutic efficacy of the MTX, the clinical application of this drug is limited by its toxic dose-related side effects. An alternative to overcome the toxicity of the MTX is the development of implantable devices capable of controlling the delivery of this drug directly at the site of inflammatory angiogenesis.

Implants are controlled drug delivery systems based on polymers. These implantable devices are designed to achieve prolonged therapeutic drug concentrations in the target tissues that are not readily accessible by conventional means while limiting the side effects from systemic drug exposure, as well as improving patient compliance.[6] A number of studies have demonstrated the efficacy of these drug delivery systems, derived from different polymers, in suppressing inflammation and angiogenesis in experimental animal models.[8–11]

Recently, we have been elaborated implants using poly(ε-caprolactone) (PCL) and MTX for the treatment of human cancer. The polymeric implants allowed the controlled release of the drug in the therapeutic range for a prolonged period, without peak-and-valley drug levels, and efficiently provided tumor growth inhibitory effect in a murine tumor model.[12] The MTX is a folate antimetabolite and interferes with the formation of DNA, RNA, and proteins.[13,14] Finally, the obtained implantable devices offer the advantage of being degraded in the body

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because of the hydrolysis of the ester bonds of this polymer. Therefore, the MTX-loaded PCL implants do not need to be surgically removed from the body after depletion of the drug, which could increase patient's compliance.

In this study, the efficacy of these MTX-loaded PCL implants in inhibiting the inflammatory response and the angiogenesis in a murine sponge model was investigated. This *in vivo* model of inflammatory angiogenesis allowed to explore the capability of the MTX controlled-leached from the PCL implants in decreasing the key components of these mutually codependent processes, represented by the recruitment and influx of inflammatory cells, the blood vessel formation, the cytokine release, and the extracellular matrix deposition. Additionally, this *in vivo* model permitted to investigate the inability of the MTX locally released from the polymeric implants in inducing severe systemic adverse effects, including nephrotoxicity, hepatotoxicity, and an adverse hematopoietic profile.

MATERIALS AND METHODS

Preparation of the Implants (MTX PCL Implants)

The implants were prepared by fully blending MTX (Sigma Chemical Company, St. Louis, Missouri) particles with melting PCL (PCL; MW ~14,000 g mol^{-1}, density = 1.145 g/mL at 25°C; Sigma Chemical Company) and then molding the blends into spherical implants using a metallic mold. Briefly, PCL was heated until it was completely melted. Afterward, MTX was added slowly into the melting PCL and mixed at approximately 70°C for 20 min at a screw speed of 50 rpm.[15] The resultant blend was collected and further molded into spherical implants (6 mm in diameter) using a metallic mold at approximately 70°C. The MTX-loaded PCL implants (MTX PCL implants) contained approximately 2 mg of the drug and 15 mg of the polymer. Implants without drug were also prepared (PCL implants).[12]

Preparation of the Sponge Discs

Nonbiocompatible polyester–polyurethane sponge discs of 5 mm in thickness, 8 mm in diameter, and approximately 4.6 mg in weight (Vitaform Ltd., Manchester, UK) were used as the matrix for fibrovascular tissue growth. The sponge discs were soaked overnight in a 70% (v/v) ethanol solution and sterilized by boiling in distilled water for 15 min before the implantation surgery.

Animals

Ninety male mice of the strain BALB/c aging 6–8 weeks and weighing approximately 30 g, from the Centre for Animal Science (CCA) from Federal University of Ouro Preto (UFOP), were maintained with water and food *ad libitum*. The light/dark cycle was 12/12 h with lights on at 7:00 AM and lights off at 7:00 PM. Experiments were approved by the Ethics Committee in Animal Experimentation at UFOP under protocol numbers 16/2012 and 54/2012. Additionally, experiments were in accordance with the guidelines of the National Council on Animal Experiments and Control from Brazil.

Implantation of the Sponge Discs and MTX PCL Implants

The animals were anesthetized with a mixture of xylazine (10 mg/kg) and ketamine hydrochloride (100 mg/kg) intraperitoneal. Their dorsal hair was shaved and the skin wiped with 70% (v/v) ethanol. The sponge disc was aseptically implanted into a subcutaneous pouch that had been made with curved artery forceps through a 1-cm long dorsal mid-line incision. Four days postoperatively, a new incision was made and the MTX PCL implant (treated group) or the PCL implant (control group) was inserted in the subcutaneous tissue adjacent to the sponge (~1 cm of distance between the sponge and implant). After 24, 48, and 96 h of the insertion of the PCL implants with or without MTX, animals were euthanized and the sponge was carefully collected in order to perform the analysis.

In Vivo Release of MTX from the PCL Implants

The MTX PCL implants were inserted into the subcutaneous tissue of mice as previously described. At predetermined intervals (1, 2, 4, 6, 8, and 10 days) after the insertion of the implant, the animals were euthanized and the polymeric system was carefully collected (n = five animals per day). Each implant was dissolved in 50 mL of 0.1 mol/L HCl. An aliquot of 1 mL was transferred to a 10-mL volumetric flask and dissolved using the same solvent. The content of MTX remaining in the removed implants was determined by measuring the absorbance of the solution at 307 nm.

Determination of Hemoglobin Levels

The extent of vascularization of the sponge discs was assessed by measuring the hemoglobin (Hb) content of the sponge discs using the method of Drabkin and Austin,[16] adapted as an index of neovascularization by Plunkett and Hailey,[17] which was considered an indirect index. At predetermined periods (24, 48, and 96 h after the insertion of the PCL implants with or without MTX), the sponge discs were dissected from adherent tissue of mice from all groups, weighed and homogenized in 2 mL of Drabkin's reagent (kit-Hb dosage; Labtest, Lagoa Santa, Minas Gerais, Brazil). The supernatant was centrifuged at 4°C at 13,000*g* for 20 min and filtered in 0.22-μm filter (Millipore). The Hb concentration of the samples was determined by spectrophotometric reading at 570 nm and was compared against a standard curve of Hb. The content of Hb in the sponge discs was expressed as micrograms of Hb per milligram of the wet tissue (μg Hb/mg tissue).

The extent of vascularization of the sponge discs was also assessed by counting the number of vessels using a morphometric analysis, which was considered a direct index. The sponge discs were fixed in 10% (v/v) buffered formaldehyde pH 7.4 for at least 48 h. Fragments of the sponge, measuring approximately 1 cm^2, were embedded in 60% (p/v) paraffin. The sections (4 mm) were stained by hematoxylin-eosin. The microscopic images of cross-sections were obtained with a planapochromatic objective (40×) in light microscopy. The images were digitized through a JVC TK-1270/JGB microcamera and transferred to an image analyzer (Kontron Electronics, Carl Zeiss-KS300 version 2). To quantify the fibrovascular area, 15 fields were obtained for each cross-section and they were morphometrically analyzed. The results were expressed as mean ± SEM of the total number of vessels/15 fields.

Determination of Myeloperoxidase and N-Acetyl-β-D-Glucosaminidase Activities

The extent of neutrophil accumulation in the sponge discs was measured by assaying myeloperoxidase (MPO) activity.[18,19] After processing the supernatant of the sponges for Hb determination, a part of the corresponding sponge was weighed,