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

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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,

homogenized in 2 mL of buffer at pH 4.7 (0.1 mol/L NaCl, 0.02 mol/L NaH_2PO_4, 0.015 mol/L Na-EDTA), centrifuged at 12,000*g* for 10 min. The sponges were then resuspended in 0.05 mol/L sodium phosphate buffer (pH 5.4) containing 0.5% hexa-1,6-bis-decyltrimethylammonium bromide followed by three freeze–thaw cycles using liquid nitrogen. MPO activity in the supernatant samples was assayed by measuring the change in absorbance (optical density, OD) at 450 nm using tetramethylbenzidine (1.6 mM) and H_2O_2 (0.3 mM). The reaction was terminated by the addition of 50 μL of 4 mol/L H_2SO_4. Results were expressed as change in OD per milligram of wet tissue (implant). The infiltration of mononuclear cells into the sponge discs was quantified by measuring the levels of the lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG), which is present in high levels in activated macrophages.[18,19] A portion of the sponges that remained after the Hb measurement was kept for this assay. These sponges were weighed, homogenized in NaCl solution (0.9%, w/v) containing 0.1% (v/v) Triton X-100 (Promega), and centrifuged (3000*g*; 10 min at 4°C). Samples of the resulting supernatant (100 μL) were incubated for 10 min with 100 μL of *p*-nitrophenyl-*N*-acetyl-beta-D-glucosaminide (Sigma) prepared in citrate/sodium phosphate buffer (0.1 M citric acid, 0.1 mol/L Na_2HPO_4; pH 4.5) to yield a final concentration of 2.24 mM. The reaction was stopped by the addition of 100 μL of 0.2 mol/L glycine buffer (pH 10.6). Hydrolysis of the substrate was determined by measuring the absorption at 400 nm. NAG activity was expressed as change in OD per milligram wet tissue (OD/mg wet tissue).

Cytokine Measurement

The IL-6 and tumor necrosis factor-α (TNF-α) levels were determined using the Cytometric Bead Array (CBA, mouse Th1–Th2 cytokine kit; BD Bioscience, San Diego, California) method for cytokine measurement according to the manufacturer's manual. Fluorescence was measured using a BD flow cytometer FACS Calibur (BD Bioscence). The results were analyzed using BD CBA Analysis software and the graphics were obtained by using the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, California).

Collagen Measurement

Total soluble collagen was measured in the whole homogenated tissue by the Sirius Red reagent-based assay.[20,21] The sponges ($n = 5$ per group) were homogenized in 1 mL of phosphate-buffered saline and 50 μL of sample were mixed with 50 μL of Sirius Red reagent. Samples were mixed by gentle inversion. The collagen–dye complex was precipitated by centrifugation at 5000*g* for 10 min. The supernatants were drained off discarded, and the sponge was washed with 500 μL of ethanol (99% pure and methanol free). One milliliter of a 0.5–mol/L NaOH solution was added to the remaining sponge of collagen-bound dye. After solubilization, samples were transferred to a 96-well plate and read at 540 nm. The calibration curve was set up on the basis of a gelatin standard (Merck). The results are expressed as microgram of collagen per milligram of wet tissue (μg collagen per mg wet tissue).

Evaluation of the Hepatic and Renal Toxicities of MTX Released from the PCL Implants

Blood of all animals under experimentation was withdrawn by cardiac puncture and a portion of this blood was centrifuged at 164*g* for 15 min at room temperature. Each serum was processed by the fully automatic random access clinical analyzer Metrolab 2300 (Wiener, Belo Horizonte, Minas Gerais, Brazil) prior to load the respective calibrator and specific controls for Bioclin-Quibasa diagnostic kits (Calibrator—Biocal K072; normal control—Biocontrol NK073; and pathologic control—Biocontrol PK074). All used reagents were kindly provided by the Bioclin-Quibasa Company through its "Bioclin at the Scientific Laboratory" initiative. Kidney's performance was accessed through measurement of the creatinine and urea levels. The creatinine and urea levels obtained from the blood of the animals that received PCL implants with or without MTX (treated and control groups) were compared with referential levels of urea (45.1 ± 8.6 mg/dL) and creatinine (0.45 ± 0.07 mg/dL) produced by healthy BALB/C animals.[22] The hepatic function was evaluated through serum activities of aspartate (AST) and alanine aminotransferase (ALT). The AST and ALT levels obtained from the blood of experimental animals receiving polymeric implants was compared with referential levels of AST (125.6 ± 47.10 U/L) and ALT (110.8 ± 30.5 U/L) produced by healthy BALB/C animals.[23]

Evaluation of the Myelosuppression of the MTX Released from the PCL Implants

Another portion of the collected blood was transferred to BD Vacutainer™ tubes containing EDTA anticoagulant. The samples were homogenized and submitted to autoanalyzer MICROS Horiba® 60 for counting the differential leukocytes. For confirmation of the leukocyte counting and assessment of hematological profiles, as red series analysis, slides with blood smears were prepared and they were read in optical microscope Olympus BX50 (40×). The Hb, hematocrit, and red blood levels obtained from the blood of the animals that received PCL implants with or without MTX (treated and control groups) were compared with referential levels of Hb (13.9 ± 1.0) g/dL, hematocrit (42.3 ± 3.9%), red blood (9.0 ± 0.9) $\times 10^6$/mm^3 produced by healthy BALB/C animals.[23] The levels of neutrophils (16.9 ± 6.1%), monocytes (2.3 ± 1.2%), and lymphocytes (80.1 ± 9.2%) in the blood of healthy BALB/C animals were used as reference values.[23]

Histological Analysis

The sponge discs, liver, and kidney tissues of each mouse under experimentation were fixed in 10% (v/v) buffered formaldehyde pH 7.4 for at least 48 h. Fragments of the sponge and organs, measuring approximately 1 cm^2, were included in 60% (w/v) paraffin. The sections (4 mm) were obtained followed by hematoxylin-eosin staining. Images were digitalized through a microcamera (JVC-TK 1270/JGB) and transferred to an analyzer (Kontron Electronics, Carls Zeiss-KS300 version 2).

Statistical Analysis

To establish the number of animals per group, a statistical analysis was conducted to determine the sample size to a level of significance of $p < 0.05$. All data were subjected to normality test using the Kolmogorov–Smirnov test. The results were represented as mean ± SEM. The comparison among groups was performed using the tests: ANOVA and Student's *t*-test. All results were considered statistically significant at $p < 0.05$ using the Graph PadPrism software 5.0.

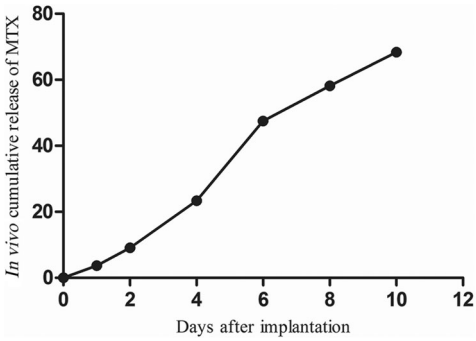

Figure 1. *In vivo* cumulative MTX (%) released from PCL implants inserted into the subcutaneous tissue of mice. Results represent mean ± standard deviation ($n = 5$ animals per day). The no displayed standard deviation indicated that it was lower than 0.5.

RESULTS

In Vivo Release of MTX from the PCL Implants

The profile of *in vivo* MTX release from the PCL implants was demonstrated in Figure 1. Approximately 68% of the drug was leached from the implantable devices in the subcutaneous tissue of mice during 10 days of the experiment. The mechanism of release of the MTX from the polymeric devices probable was represented by the diffusion of the drug to the aqueous environment.[12] Additionally, the MTX release rate from the systems was 0.14 mg per day.

Hb Levels

The effect of the MTX released from the PCL implants on the vascular proliferation was assessed by measuring the Hb content into the sponge discs. The applied method[19] was considered an indirect index of neovascularization. In the group treated with MTX PCL implants, the concentration of Hb was significantly lower than that obtained from the group that did not receive the MTX PCL implants (control group) at 48 and 96 h of implantation of the polymeric systems (Student's *t*-test, $p < 0.05$). At 24 h, the Hb content was not statistically different in both groups (Student's *t*-test, $p > 0.05$). Finally, the drug leached from the polymeric devices promoted a regression of 85% of the Hb level in the treated group compared with the control group during the experimentation period (Fig. 2a). The effect of the MTX released from the PCL implants on the formation of new vessels was also evaluated by counting the number of vessels present in the sponge discs. This morphometric analysis was considered as a direct index of the neovascularization; and it evidenced that the number of vessels in the treated group, which received MTX PCL implants, was significantly lower than that visualized for the control group at 48 and 96 h of insertion of the implantable devices (Student's *t*-test, $p < 0.05$). At 24 h, the number of vessels was not statistically different in both groups (Student's *t*-test, $p > 0.05$). Additionally, the MTX was capable of inducing a reduction of 90% of the counted vessels in the treated group as compared with the control group after 96 h of insertion of the implants (Fig. 2b).

MPO and NAG Activities

The extent of the enzyme MPO activity represents as an indirect index for analyzing the presence of neutrophils in the

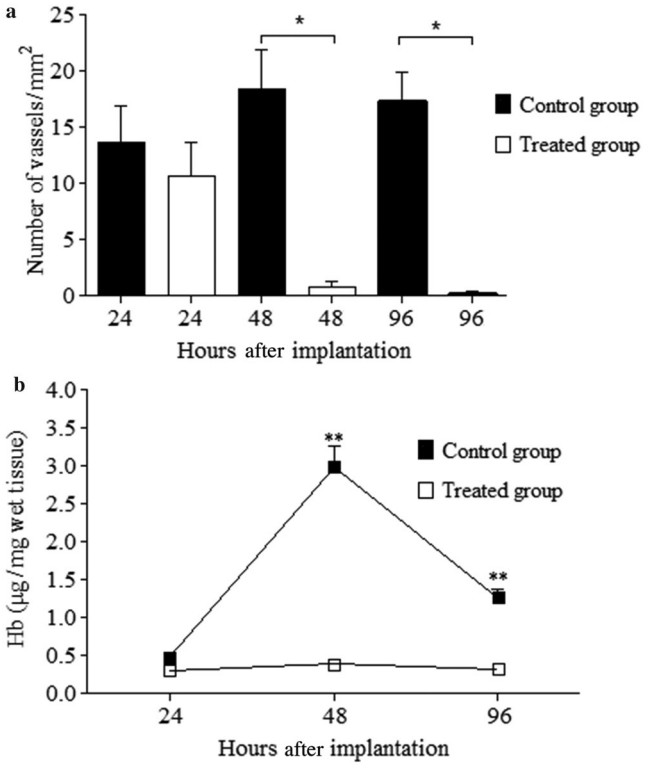

Figure 2. Levels of Hb measured using an indirect index of neovascularization (a) and measured using a morphometric analysis (b) in the sponge discs of animals of the treated group (receiving MTX PCL implants) and control group. Results represent mean ± standard deviation ($n = 8$–10 animals per each time point). The no displayed standard deviation indicated that it was lower than 0.5. Results were analyzed by using the Student's *t*-test (**$p > 0.05$).

inflammatory focus. The statistical analysis revealed that the concentration of the MPO in the sponge discs of the treated group, receiving MTX PCL implants, was lower than that obtained from the control group at 24 and 48 h after implantation (Student's *t*-test, $p < 0.05$) (Fig. 3a), indicating that the drug reduced the influx of neutrophils in the sponge discs at the first 48 h of experimentation. At 96 h, the MPO concentration was not statistically different in both groups (Student's *t*-test, $p > 0.05$).

The extent of the enzyme NAG activity represents as an indirect index for evaluating the accumulation of monocytes/macrophages in the inflammation. The statistical analysis demonstrated that the concentration of the NAG in the sponge discs of the treated group, in direct contact with the MTX leached from the PCL implants, was extremely lower than that obtained from the control group at 48 and 96 h after implantation (Student's *t*-test, $p < 0.05$) (Fig. 3b), suggesting that the MTX was capable of decreasing the monocytes/macrophages recruitment in the sponge discs in the later periods of experimentation. At 24 h, the NAG concentration was not statistically different in both groups (Student's *t*-test, $p > 0.05$).

Cytokine Measurement

The TNF-α and IL-6 levels decreased significantly in the treated group, receiving MTX PCL implants, compared with the levels of these cytokines in the control group (Student's

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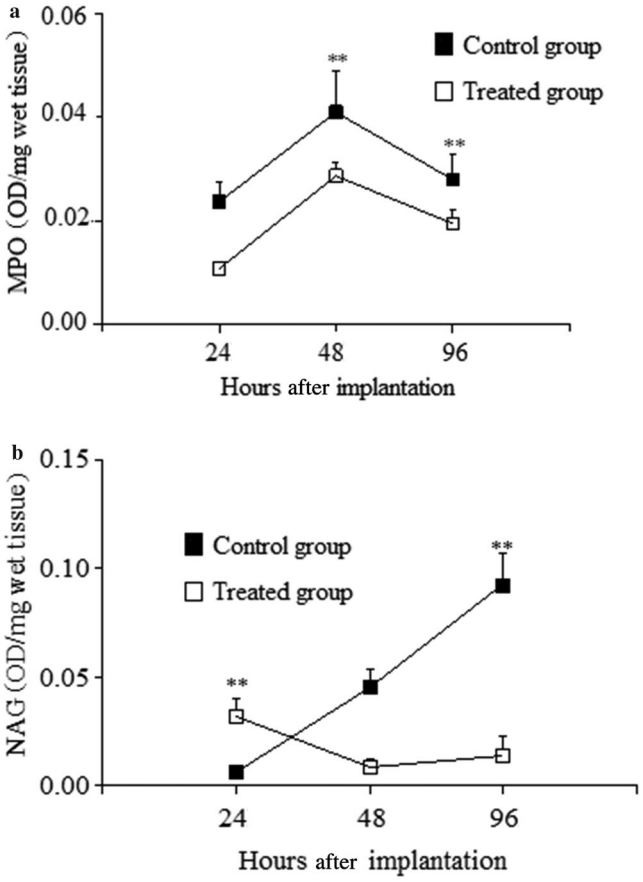

Figure 3. Kinetics of the influx of neutrophils (a) and monocytes/macrophages (b), determined by MPO and NAG activities, respectively, in the sponge discs of animals of the treated group (receiving MTX PCL implants) and control group. Results represent mean ± standard deviation (n = 8–10 animals per each time point). The no displayed standard deviation indicated that it was lower than 0.5. Results were analyzed by using Student's t-test (**$p > 0.05$).

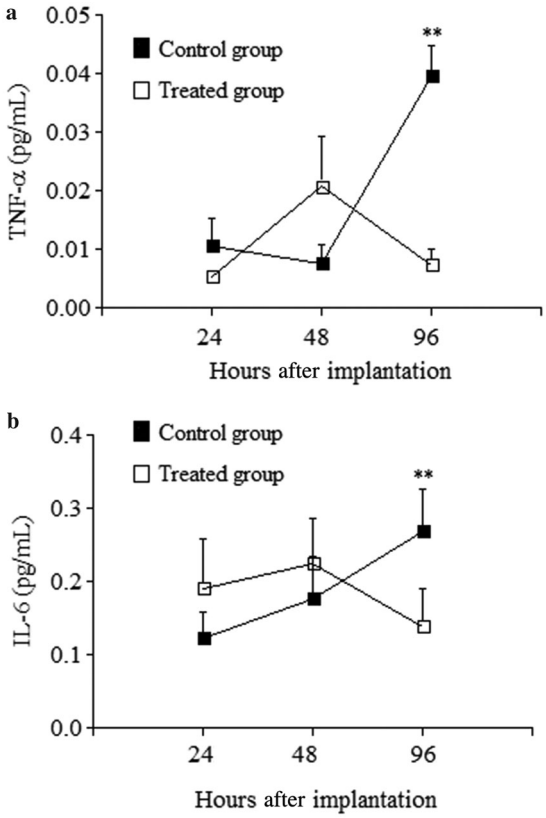

Figure 4. Levels of TNF-α (a) and IL-6 (b) measured in the animals of the treated group (receiving MTX PCL implants) and control group. Results represent mean ± standard deviation (n = 8–10 animals per each time point). The no displayed standard deviation indicated that it was lower than 0.5. Results were analyzed by using the Student's t-test (**$p > 0.05$).

t-test, $p < 0.05$) at 96 h of experimentation (Fig. 4), indicating the ability of the drug to modulate the production of these inflammatory mediators in the later stage of the inflammatory response.

Collagen Measurement

The collagen deposition in the sponge discs of the treated group with MTX PCL implants was lower than that obtained from the control group at 48 and 96 h after implantation (Student's t-test, $p < 0.05$) (Fig. 5), demonstrating the influence of the MTX released from the PCL implants in reducing the fibroproliferative phase in the later periods of experimentation. At 24 h, the collagen concentration was not statistically different in both groups (Student's t-test, $p > 0.05$).

Histological Analysis of the Sponge Discs

No sign of infection was visualized in the sponge compartment during the entire period of experimentation. Subcutaneous implantation of the sponge discs in mice of the control group induced an inflammatory angiogenesis response extremely superior to that obtained in the animals of the MTX-treated group. At 24 h after implantation, the number of inflammatory cells in the sponge disc of the untreated group was higher compared with the treated group, and neutrophils were the predominant cells with scarce macrophages and fibroblasts. At 48 and 96 h after implantation, the sponge discs in mice of the control group showed a diversified inflammatory infiltrate

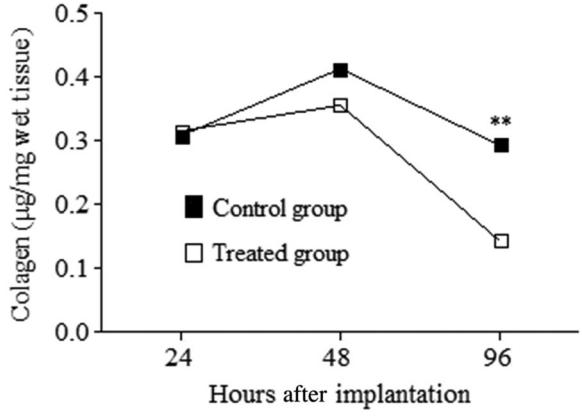

Figure 5. Levels of collagen in the sponge discs of animals of the treated group (receiving MTX PCL implants) and control group. Results represent mean ± standard deviation (n = 8–10 animals per each time point). The no displayed standard deviation indicated that it was lower than 0.5. Results were analyzed by using the Student's t-test (**$p > 0.05$).

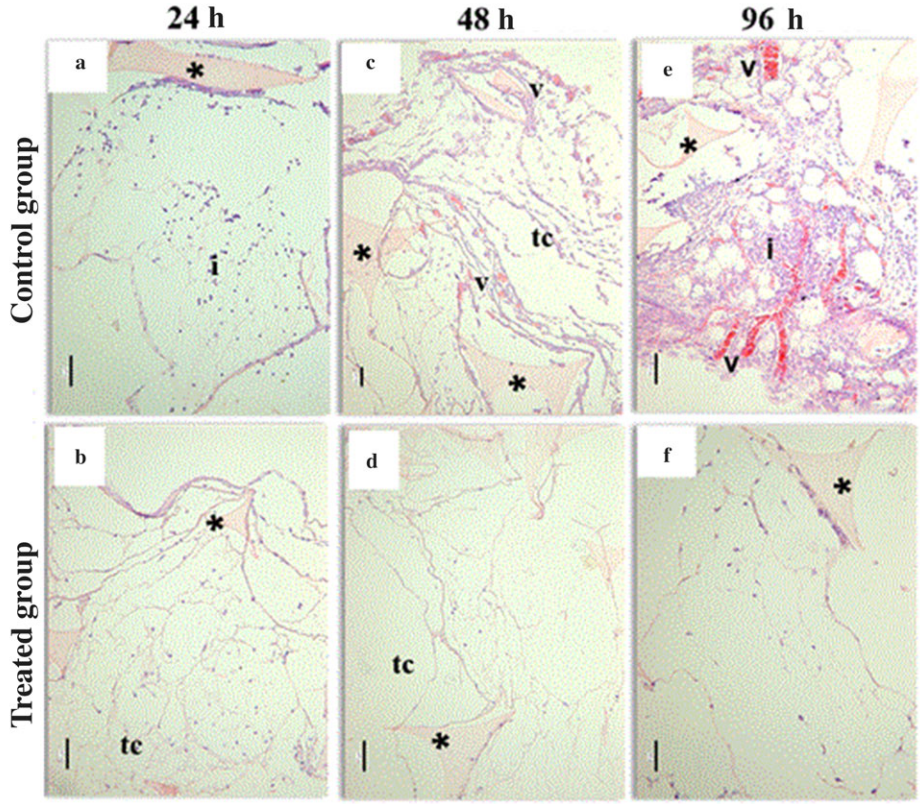

Figure 6. Representative histological sections (4 mm, stained with HE) of the sponge discs collected after 24, 48, and 96 h after implantation of the polymeric drug delivery systems. Photomicrographs in (a), (c), and € demonstrated a dense inflammatory infiltrate and angiogenesis in the sponge discs of animals of the control group (receiving PCL implants without drug). Photomicrographs in (b), (d), and (f) indicated the inhibition of the inflammatory angiogenesis response in the sponge discs of animals of the treated group, induced by the MTX controlled-released from the PCL implants (n = 8–10 animals per each time point). V, blood vessel; tc, connective tissue; i, inflammatory infiltrate; *, sponge. ×40 magnification. Bar = 50 μm.

represented by multinucleated giant cells, macrophages, and fibroblasts. Additionally, the sponge matrix was vascularized and filled with a fibrovascular stroma. In contrast, the MTX released from the PCL implants clearly reduced the inflammatory angiogenesis response in the sponge matrixes, indicating the effectiveness of these polymeric systems in promoting inhibition of the fibrovascular tissue and its cellular components (Fig. 6).

Hepatic and Renal Toxicities

Biochemical parameters from serum were measured to evaluate the possibility of hepatic and/or renal damages following the insertion of the PCL implants with and without MTX in the subcutaneous tissue of mice. It was intended to verify whether the MTX leached from the polymeric implants and the metabolites of the drug could induce toxicity in the liver and/or kidney of the animals. Accordingly, the AST and ALT assayed in the serum of animals of the treated and control groups did not present a difference statistically significant at 24, 48, and 96 h after implantation (Student's t-test, $p < 0.05$) (Fig. 7a). Additionally, the AST and ALT levels in both groups were similar to the reference concentrations of these hepatic parameters in healthy BALB/C animals.[23] The obtained results suggested that the MTX constantly released from the PCL implants did not promote hepatic injuries. The urea and creatinine measured in the serum of animals of the treated and control groups showed no statistical difference at 24, 48, and 96 h after implantation (Student's t-test, $p < 0.05$) (Fig. 7b). Moreover, the urea and creatinine levels in both groups were similar to the reference concentrations of these renal parameters in healthy BALB/C animals.[23] These results indicated that the leached drug did not interfere in the renal functionality.

Histological analysis of the liver of animals of both groups showed no morphological changes in this organ. There was no structural alteration in the organization of hepatocytes, ducts, vessels, arteries, or stellate cells. The hepatocytes exhibited a polyhedral shape and a homogeneous cytoplasm without any sign of degeneration. The nucleus showed evident nucleolus and picnotic formations were not visualized. The extracellular matrix did not present either signs of necrosis or connective modifications. Therefore, the liver architecture was completely preserved. Finally, hemorrhage and inflammatory infiltrate were not observed in the hepatic tissues (Fig. 8a). Histological analysis of the kidney of the animals of both groups revealed no morphological modifications in this organ. There were no signs of glomerulonephritis and intratubular hyaline casts were not also visualized, representing the absence of nephrotoxicity (Fig. 8b).

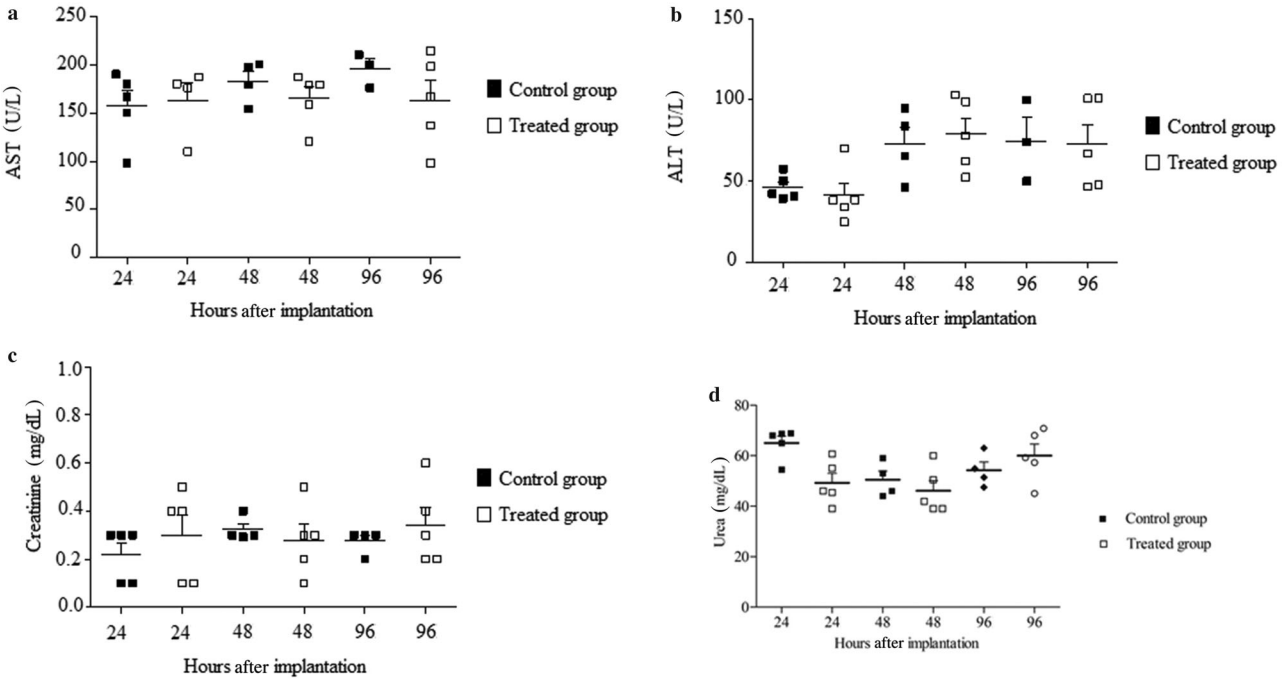

Figure 7. Levels of AST (a), ALT (b), creatinine (c), and urea (D) in the serum of animals of the treated group (receiving MTX PCL implants) and control group. Results represent mean ± standard deviation (n = 4–5 animals per each time point). Results were analyzed by using the Student's t-test ($p < 0.05$).

Myelosuppression Evaluation

The blood of the animals from both groups was collected to assay the hematological parameters. Accordingly, the level of hematocrit, Hb, and red blood in the MTX-treated group was not statistically different from the level of the same cells assayed in the animals of the untreated group at 24, 48, and 96 h of experimentation (Student's t-test, $p < 0.05$) (Fig. 9). Moreover, the level of these cells of the red blood series in animals of both groups was similar to the referential concentration of those cells obtained in healthy BALB/C animals.[23] The same analysis was carried out to evaluate the possible effects of the MTX and the polymeric implants under white blood series. The level of neutrophils, monocytes, and lymphocytes in the MTX-treated group showed no statistical difference at 24, 48, and 96 h of experimentation (Student's t-test, $p < 0.05$) (Fig. 10). Furthermore, the level of these cells of the white blood series in animals of both groups was similar to the referential concentration of those cells obtained in healthy BALB/C animals.[23] The obtained results indicated that MTX controlled-released from the PCL implants did not affect the expression of the systemic hematological parameters; consequently, the myelosuppression was absent.

DISCUSSION

The MTX is clinically used to treat arthritis rheumatoids, psoriasis, Chron's disease, and other immune-mediated inflammatory pathologies. Besides MTX being the most common drug used in the management of these chronic diseases either in monotherapy or in association with other therapeutic agents, it can induce severe adverse effects, which force the discontinuity of the therapy, the continuous monitorization, and/or the modification of the MTX doses. The administration of MTX can provoke toxicity to healthy cells because of its low specifity, bone marrow suppression, interstitial pneumonitis, acute and chronic hepatotoxicity, nephrotoxicity, among others. To overcome these limitations of the MTX, administrated using oral and parenteral conventional dosage forms, a variety of sustained-controlled MTX delivery systems has been developed and evaluated in preclinical trials considering their clinical response and tolerability.[24,25]

Accordingly, in this study, PCL implants containing MTX were elaborated and an attempt was made to improve the efficacy and reduce the toxicity of this drug by inserting these implantable devices directly at the site of inflammation and angiogenesis. These implants based on a biodegradable synthetic polymer incorporated into MTX demonstrated their capability of controlling and sustaining the drug release in the target site for a prolonged period, as demonstrated by the *in vivo* study of MTX release, inhibiting locally the development of the fibrovascular tissue in the sponge discs without inducing renal and/or hepatic dysfunctions and myelosuppression.

The enhanced vascular permeability produced in response to local inflammation stimuli, induced by the insertion of the nonbiocompatible sponge discs into the subcutaneous tissue of mice, increased the deposition of a provisional matrix in the sponge because of the extravasation of serum proteins, which provided the migration of leukocytes and a template for capillary sprouting. Capillary sprouting is one of the main mechanisms for neovascularization.[1] Additionally, the formation of new vessels involves soluble and cell–surface bound mediators, including vascular endothelial growth factor (VEGF) and hypoxia-inducible factors, as well as cytokines such as the TNF-α and chemokines.[22] In this study, the Hb content of the sponge granuloma tissue was an important marker for angiogenesis.[26]

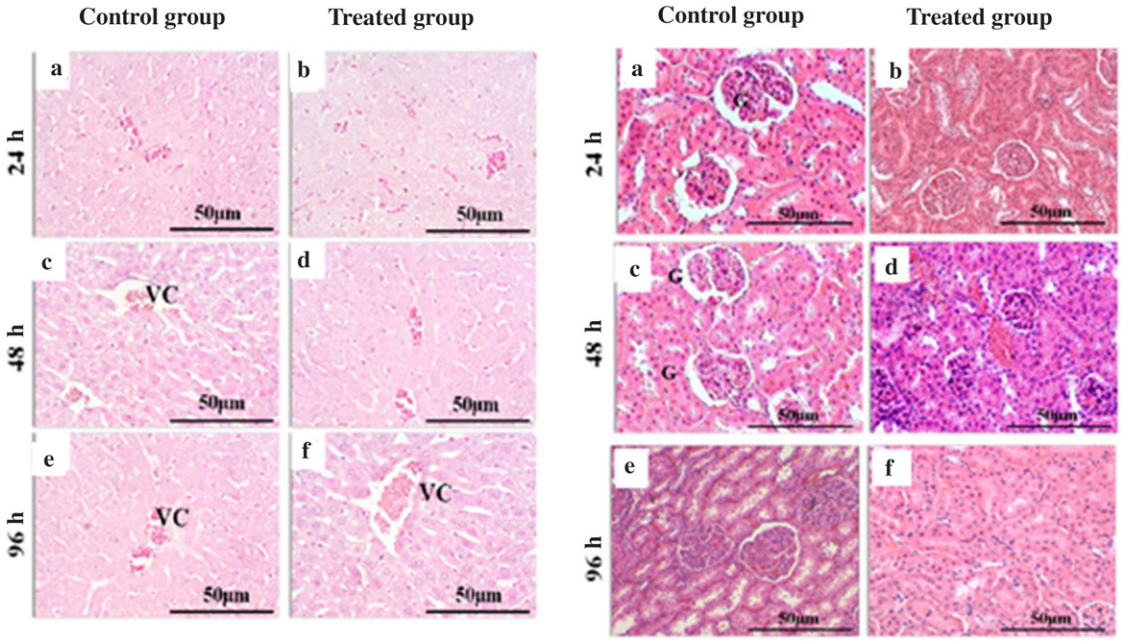

Figure 8. Representative histological sections (4 mm, stained with HE) of the liver and kidney collected after 24, 48, and 96 h after implantation of the polymeric drug delivery systems. Photomicrographs in (a), (c), and (e) represented the hepatic and renal tissues of animals of the control group (receiving PCL implants without drug). Photomicrographs in (b), (d), and (f) demonstrated the hepatic and renal tissues of animals of the treated group, in direct contact with the MTX PCL implants (n = 4–5 animals per each time point). V, blood vessel; G, glomerulus. 40× magnification. Bar = 50 μm.

The MTX controlled-released from the PCL implants presented an explicit antiangiogenic effect as the Hb levels in the sponge discs of treated animals was significantly decreased as compared with the Hb levels of the untreated group. It was hypothesized that the antiangiogenic mechanism of the MTX involved the downregulation of the VEGF expression. This hypothesis is in accordance with the data previously published by Shaker et al.,[27] who demonstrated that MTX regulated the VEGF expression in lesional psoriatic skin of patients. Furthermore, the antiangiogenic mechanism of the MTX was also related to the inhibition of the TNF-α expression, a potent angiogenic factor, as the TNF-α levels in the animals receiving MTX-loaded PCL implants were significantly reduced in a period of 96 h of the experiment. The obtained result corroborated with data previously described by Dolhain et al.,[28] who indicated that the MTX therapy reduced the production of TNF-α, the expression of adhesion molecules and the signs of cell proliferation in synovial tissues of patients with arthritis rheumatoid.

The migration and accumulation of leukocytes at the compartment of the nonbiocompatible sponge discs led to the establishment of an acute inflammatory response. The stimulated neutrophils released inflammatory mediators, such as MPO. In this study, the presence of the neutrophils in the injury could be detected by quantifying this inflammatory marker. MTX sustained released from PCL implants led to a reduction of the MPO levels, and consequently, an inhibition of the neutrophil migration in the sponge discs, resulting in a regression of the acute inflammatory process. Dalmarco et al.[29] also demonstrated the regression of the MPO activity, and consequently, the inhibition of activated neutrophils in the pleurisy induced by carrageenan because of the administration of a MTX solution by intraperitoneal route. Concomitantly with the neutrophil influx, circulating monocytes entered at the compartment of the sponge discs and differentiated into macrophages. Activated macrophages contribute to all phases of the inflammatory response. In the proliferative phase, they promote inflammation, stimulate cell proliferation, and release proinflammatory cytokines, such as IL-1, IL-6, and TNF-α. In the remodeling phase, they induce the repair of the tissue, by expressing anti-inflammatory cytokines, such as transforming growth factor. Eventually, as remodeling is complete, macrophages adopt a deactivated phenotype and inflammation resolves.[30] As the nonbiocompatible sponge discs were maintained in the subcutaneous tissue, the inflammatory stimuli persisted, leading the continuous activation of the macrophage, which induced a chronic inflammatory response in the animals of the untreated group. By contrast, in the sponge discs of the animals receiving MTX PCL implants, the persistent inflammation was not visualized because of the inhibitory effect of the MTX not only upon the activated macrophages, but also upon the recruitment of these cells, which was confirmed by the reduced NAG activity. Furthermore, as previously mentioned, the TNF-α is considered a potent angiogenic factor; however, this substance is also an important proinflammatory cytokine secreted by activated macrophages. As these cells were inhibited by the MTX released from the PLC implants, there was a significant regression of the expression of this proinflammatory cytokine. Finally, the IL-6 is considered a potent proinflammatory cytokine, and it can be synthesized by a variety of cells, including macrophages. The IL-6 was reported to control vascular permeability through inducing VEGF production, and increased permeability enhances the recruitment of inflammatory cells into the tissues and aggravates the damage.[31,32] As the macrophages were inhibited by the MTX leached from the PCL implants, an expressive reduction of the IL-6 levels was verified, resulting in the suppression of the progression of the

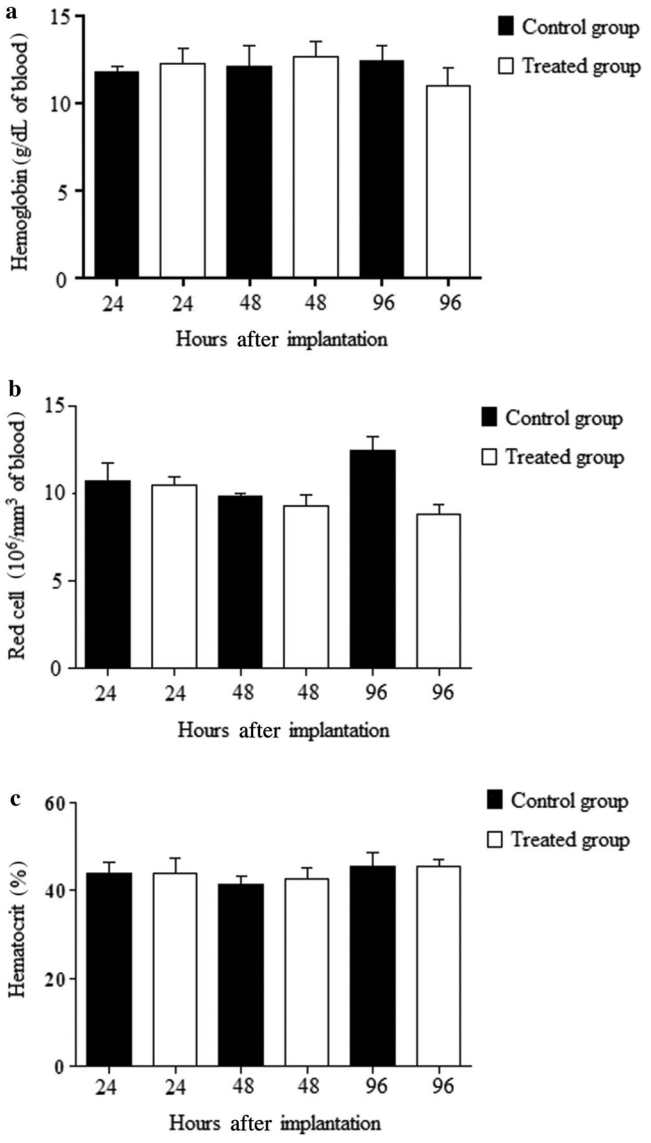

Figure 9. Quantitative evaluation of the effects of MTX released from PCL implants on the number of red blood cells. Number of Hb/dL of blood (a). Number of red blood cells 10^6/mm^3 of blood (b). Percentage of hematocrit (c). Results represent mean ± standard deviation (n = 3–4 animals per each time point). Results were analyzed by using the Student's t-test ($p < 0.05$).

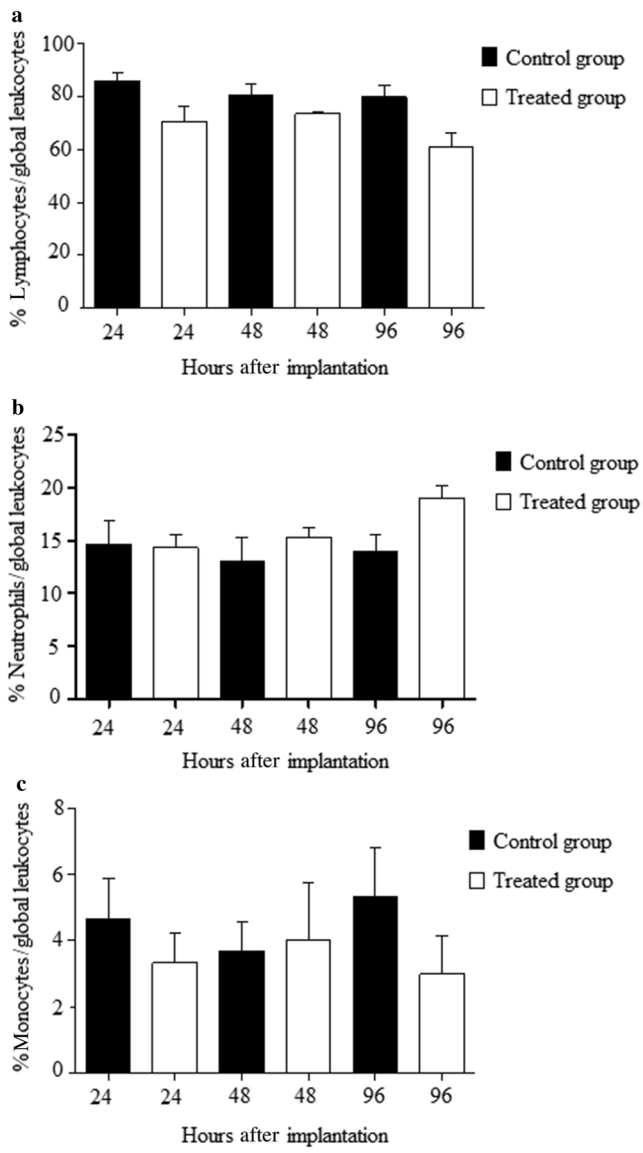

Figure 10. Quantitative evaluation of the effects of MTX released from PCL implants on the number of white blood cells. Percentage of lymphocytes relative to global leukocytes (a). Percentage of neutrophils relative to global leukocytes (b). Percentage of monocytes relative to global leukocytes (c). Results represent mean ± standard deviation (n = 3–4 animals per each time point). Results were analyzed by using the Student's t-test ($p < 0.05$).

chronic inflammation in the animals of the treated group. The obtained results corroborated with the descriptions of Borchers et al.[33] and Shaker et al.,[27] who mentioned that the MTX is converted to MTX polyglutamates, which induce the intracellular adenosine concentration, and the subsequent release of the adenosine into the extracellular fluid. The adenosine exhibits a variety of anti-inflammatory activities, including the prevention of the neutrophils and macrophage influx at the site of inflammation and the reduction of the TNF-α and IL-6 synthesis by macrophages.

The proliferative phase of the inflammation is followed by wound healing, characterized by the remodeling phase. At this phase, the inflammatory cells, together with resident cells, release mediators that stimulate fibroblast proliferation and collagen deposition.[34] The deposition of collagen in the sponge discs of the animals that received the polymeric implants loaded with MTX was lower than that observed for the sponge discs of the animals of the untreated group. As the MTX, constantly released from the implants, inhibited the cascade of events related to the inflammatory process, the suppression of the repair phase was expected, and consequently a decrease of the number of fibroblasts and the regression of the accumulation of the fibrotic deposits.

Finally, the MTX released from the PCL implants provided the regression of the of the levels of proangiogenic mediator, represented by the Hb and TNF-α, the levels of proinflammatory enzymes, characterized by the MPO and NAG, and the concentration of TNF-α and IL-6 at the site of the inflammation. Consequently, an antifibrotic effect was also confirmed.

These evidences could be verified not only by quantifying the biochemical parameters, but also by assessing the qualitative histological data. The histological analysis revealed that the MTX locally released from the polymeric implants inhibited the cellular components of the angiogenesis and inflammation, resulting in the decreased fibrovascular tissue in the sponge discs.

The effectiveness of the MTX therapeutically leached from the polymeric implants was evidenced by the suppression/minimization of the angiogenic and inflammatory processes created by the subcutaneous implantation of the nonbiocompatible sponge discs. However, the efficacy of these MTX systems could be limited by the occurrence of hepatotoxicity in the animals. The hepatocellular dysfunction induced by the MTX represents one of the major clinical complications of the MTX treatment. To monitor the liver function by measuring the hepatic enzyme levels represents a clinical assessment of the possible MTX toxicity.[35] In this study, the AST and ALT were quantified in the serum of animals that received MTX PCL implants and PCL implants without drug, and the levels of these enzymes were not significantly different in both groups, suggesting that the MTX controlled and locally released from the implantable devices did not interfere the hepatic function. Additionally, the histopathological analysis of the hepatic tissue revealed the integrity of the anatomical structures of this organ, as it was not evidenced degenerated hepatocytes, an increased number of activated Kupffer cells, cirrhosis, fibrosis, and other signs of toxicity. Therefore, the polymeric implantable devices containing MTX provided the local release of the therapeutic levels of the drug, preserving the structural integrity of the liver. By contrast, it was previously demonstrated that the MTX administrated by intraperitoneal injection in rats caused an increase in the levels of AST and ALT. High levels of AST and ALT may be related to the release of these enzymes from the cytoplasm into the blood circulation after the hepatocellular damage.[36]

When starting the treatment of chronic inflammatory pathologies such as arthritis rheumatoid, with MTX or increasing the dose, ALT with or without AST, creatinine, and complete blood count should be performed every 1–15 months until a stable dose is reached and every 1–3 months thereafter.[35] Therefore, besides the possibility of inducing hepatotoxicity, the MTX may be responsible to induce nephrotoxicity and myelotoxicity, then the biochemical markers and the cellular components should be monitored. It has been previously reported that the MTX promotes cell death through apoptosis. Multiple factors are known to induce renal tubular cell apoptosis. The MTX inhibits cytosolic nicotinamide adenosine diphosphate (NADP)-dependent dehydrogenase and NADPmalic enzyme, resulting in decreased availability of NADPH in cells. NADPH is normally used by glutathione reductase (GSH-Rd) to maintain cell glutathione (GSH), known as an important protective agent against reactive oxygen species (ROS). Thus, when the antioxidant defense system is reduced, cells from the kidney begin to sensitize to ROS-related cellular injury.[37–39] Therefore, the kidney is susceptible to the oxidative stress, resulting in apoptosis induced by the MTX. Additionally, as the MTX is primarily cleared by renal excretion, the MTX-induced renal dysfunction leads to delayed elimination of this drug and its metabolites, resulting in the precipitation of them in the renal tubular cells, which could cause direct renal damage.[40] The glomerular and tubular injury promoted by the MTX could be evaluated by measuring the creatinine and urea levels in the plasma. In this study, these markers of the renal function were assayed in the plasma of the animals in contact with the MTX PCL implants and with the PCL implants without drug. Accordingly, the MTX released from the PCL implants did not induce an augmentation of the creatinine and urea concentrations, demonstrating the normality in the renal function. The histopathological analysis of the kidney also evidenced the absence of nephrotoxicity because of the delivery of the MTX. The MTX was implanted in the subcutaneous tissue at a dose of 2 mg/animal. Although the amount of drug administered was enormous and capable of triggering toxicity in an animal weighing approximately 30 g, damages in the kidney or liver were not exhibited, demonstrating the ability of PCL implants of controlling the MTX release in therapeutic doses without inducing dysfunction of these organs.

The administration of MTX could be associated with leukopenia, thrombocytopenia, neutropenia, pancytopenia, and megaloblastic anemia because of the bone marrow depression.[41] The immunomodulating effect of the MTX may be related to its potential to inhibit the synthesis of purine/pyrimide or proinflammatory cytokine production, promoting adenosine release or activated T-cell apoptosis, suppressing lymphocyte proliferation, neutrophil chemotaxis, or neutrophil adherence, and reducing serum Ig levels.[42] The immunosuppressive effect of the MTX should be monitored by measuring the complete blood count. In this study, the red and white blood series of animals receiving MTX PCL implants and PCL implants without drug were assessed to evaluate the possibility of myelosuppression of the drug. However, the MTX controlled-leached from the polymeric implantable devices did not affect the proliferation of these systemic hematological cells, and consequently did not induce bone marrow depression. It was suggested that the MTX locally accumulated was capable of inhibiting the proliferation of the inflammatory cells and the expression of cytokines specifically at the site of inflammation and angiogenesis, without promoting suppression of systemic cells because of the absence of hematological alterations. As a result, the myelotoxicity was not presented.

CONCLUSIONS

Poly(ε-caprolactone) implants control the delivery of the MTX in therapeutic concentrations for a prolonged period directly at the site of inflammation and angiogenesis responses. The polymeric implants demonstrated their efficacy in a murine sponge model of inflammatory angiogenesis, as the MTX suppressed/minimized the influx and proliferation of neutrophils and monocytes/macrophages and the expression of proinflammatory (TNF-α and IL-6) and proangiogenic cytokines (TNF-α), preventing the progression of the inflammatory and angiogenic processes. Furthermore, the polymeric implants provided local accumulation of the MTX at the site of the pathology, leading to the modification of the pharmacokinetic parameters of this drug in the organism. The entire dose of the MTX was not distributed by the blood circulation to the healthy tissues because it was a controlled delivery from the implants, the drug did not induce systemic adverse side effects represented by hepatotoxicity, nephrotoxicity, and myelosuppression. Finally, the MTX-loaded PCL implants symbolized targeted drug delivery systems potentially useful in controlling the chronic

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autoimmune inflammatory pathologies, in which inflammation and angiogenesis are concomitant and synergistic processes, without promoting organ damages, and consequently, systemic side effects.

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