BASIC RESEARCH

No deleterious effect of low dose methotrexate on titanium implant osseointegration in a rabbit model

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OBJECTIVE: To evaluate the effect of low dose methotrexate alone or in combination with glucocorticoid treatment on titanium implant osseointegration.

METHODS: Groups of 6–8 adult New Zealand White rabbits were treated for 18 weeks with saline (control), methotrexate, glucocorticoid, or methotrexate plus glucocorticoid. The animals received a titanium implant in the tibia at week 6. Lumbar spine and tibia bone mineral densities were analyzed before and after treatment. Histomorphometric analysis of bone cortical thickness, total bone area around the implant, and % of bone to implant contact was performed.

RESULTS: After 18 weeks, the change in the bone mineral density in the lumbar spines and tibias in the methotrexate group was comparable to the control group (0.035 vs. 0.055 g/cm^2 and 0.021 vs. 0.041 g/cm^2, respectively). In contrast, both the glucocorticoid group and glucocorticoid plus methotrexate group had significant reductions at both sites. Histomorphometric analysis of the tibia in the control and methotrexate groups revealed no significant changes in cortical thickness (133 vs. 126 μm), total bone area around the implant (33 vs. 30%), or bone to implant contact (40 vs. 38%). In contrast, glucocorticoid group had significant reductions compared to controls in tibia cortical thickness (99 vs. 133 μm), total bone area around the implant (24 vs. 33%), and bone to implant contact (27 vs. 40%). Similar reductions were observed in the glucocorticoid plus methotrexate group.

CONCLUSIONS: Our results demonstrate that low dose methotrexate treatment does not affect titanium implant osseointegration, suggesting that this therapy is safe for surgical procedures requiring a titanium implant.

KEYWORDS: Methotrexate; Osseointegration; Dental Implant; Glucocorticoid.

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INTRODUCTION

Bone implants are a valuable tool for the reconstruction of tissue affected by trauma or inflammatory diseases and for orthodontic anchorage. The success of endosseous implants is dependent on osseointegration, a cicatricial process of implant/bone interaction defined histologically as direct bone apposition on the implant surface with nearly no interposition of soft tissue that leads to bone-to-implant fixation.[1] Titanium is an excellent material for bone implants due to its biocompatibility, augmented resistance to corrosion, lack of toxicity on macrophages and fibroblasts, and reduced inflammatory response in peri-implant tissues.[2] However, osseointegration also requires the integrity of the bone remodeling process, which is influenced by various factors. Issues affecting osseointegration include the properties and geometry of the implant, implant surface treatment and coating, host bone bed,[3] mechanical stability and loading conditions,[4] and the use of adjuvant treatments.[5,6] In particular, non-steroidal anti-inflammatory therapies like selective COX-2 inhibitors,[7] glucocorticoids (GC), cyclosporin A,[8] and other immunosuppressants, which are drugs commonly used in rheumatologic patients, have deleterious effects on osseointegration.

Low dose methotrexate (MTX) is an antirheumatic drug widely prescribed to patients with rheumatoid arthritis (RA). However, its effect on bone metabolism remains controversial. Low doses of this drug in patients with active RA had a protective effect on bone metabolism from controlling the disease activity.[9] In fact, MTX therapy has been reported to inhibit generalized bone loss in patients with RA.[10] Conversely, a deleterious effect on bone metabolism has been described in RA patients[11] and in animals.[12] There is only a single case report on the effect of MTX on osseointegration, which describes a successful rehabilitation of a titanium implant in an elderly patient with severe osteoporosis and chronic polyarthritis whose treatment included MTX.[13] In contrast, GC, which is an anti-inflammatory drug frequently

prescribed in association with MTX for rheumatologic patients, was found to negatively affect osseointegration of titanium implants.[14-16] The distinction of the deleterious effect of these drugs on bone metabolism is hampered by their concomitant use with multiple therapies in autoimmune diseases. Additionally, systemic conditions *per se*, such as RA and other inflammatory conditions, may also affect this process.[17,18]

In this context, experimental models provide a unique condition to discriminate between the effects induced by the disease itself and those caused by the therapy. Therefore, we have evaluated whether low dose MTX influences bone density and histomorphometric parameters of peri-implant bone healing around titanium implants placed in the tibia in a rabbit experimental model.

MATERIALS AND METHODS

Animals and treatments

All experimental procedures performed on animals were in accordance with UFAW (The Universities Federation for Animals Welfare) and the Animal Ethics Committee of COBEA (Brazilian College of Experimental Animals). This protocol was approved by the Institutional ethics committee # 453/05. Male New Zealand adult rabbits 16 weeks old and weighing 2.67 ± 0.067 kg were divided into four groups of 6–8 rabbits each. The animals were kept in individual cages with food and water *ad libitum* and treated as follows: subcutaneous saline (0.3 mL, control group); intramuscular methotrexate (3 mg/kg/week, MTX group)[12], subcutaneous glucocorticoid (0.35 mg/kg methylprednisolone 3 times/week, GC group),[19] MTX (3 mg/kg/week) plus GC (0.35 mg/kg methylprednisolone 3 times/week, MTX+GC group).

Endosseous implant model

The implant surgery was performed 6 weeks after the initial drug administration. The animals were anesthetized with a mixture of xylazine (5 mg/kg) associated with ketamine (50 mg/kg) by the intramuscular route. After trichotomy, the skin was cleansed, an incision of approximately 2 cm was made, and the tibia was exposed by blunt dissection. A unicortical implant bed was prepared and a screw-type, commercially pure titanium implant with a rough surface, 8.5 mm in length and 3.75 mm in diameter (Conexão Sistema de Protese Ltda, 1–2 μm porosity), was placed such that the screw thread was completely perpendicular into the bone cortex.[16] Soft tissues were replaced and sutured. A single dose of Enrofloxacin was administered just before surgery and dipyrone was given for three days afterwards.

Densitometric evaluation

Bone density was measured by dual-energy X-ray absorptiometry (DXA) with a densitometer (QDR 2000 Hologic, Waltham, MA) in high-resolution mode using the ''small animals'' software supplied by the equipment manufacturer. The technique was standardized by positioning the anesthetized rabbits such that the lumbar spine (vertebrae L4-L5) and the proximal portion of the tibia that was not operated on (right) was analyzed. The region of interest was defined as the same where the implant was inserted but in the contralateral tibia. The initial assessment was performed on the first day of the experiment and the final at end of the treatment (week 18). Results are expressed as mean $\pm$ SE of bone mineral density (BMD) variation (ΔBMD = final BMD - initial BMD).

Analysis of bone parameters

After the animals were euthanized (week 18), the left tibia (implanted) and right tibia (non-operated) were removed and fixed in 4% neutral buffered formalin for 14 days. The specimens were prepared for non-decalcified histology.[20] Briefly, pieces were washed in running water for 24 hours, dehydrated in an ascending series of ethanol (40% to 100%), and subsequently embedded in methyl metacrylate blocks. Sections (80 microns) were obtained and stained with toluidine blue as described previously.[16]

Bone cortical thickness, tibia size (diameter), ratio of bone to implant contact, total bone area of the tibia section, and peri-implant bone density were analyzed by light microscopy. Images were captured and digitalized with Image Pro Plus 6 (Media Cybernetics, Bethesda, MD). The cortical thickness (μm) was determined as the mean of multiple measurements of the non-operated right tibia taken at 30 μm intervals along the tibia perimeter.

Osseointegration was observed via light microscopy as direct bone (toluidine blue-stained) deposition on the implant surface without any other detectable tissue interposed. The total bone area of the tibia section was evaluated by software that determines the area of bone tissue (BT) stained with toluidin blue present in the total area [tibia + implant (TA, μm^2)] and expresses it as the percentage of total bone area = BT/TA $\times 100$.[24] The ratio of bone to implant contact (BIC) was calculated from the measurement of the total perimeter of the implant (TPI) and all the osseointegrated spaces around the implant (OSI), both of which were obtained manually, and expressed as percent bone to implant contact (% of BIC = OSI/TPI $\times 100$). Two specialists blinded to the treatments acquired all the bone parameter data.

STATISTICAL ANALYSIS

Bone parameter results were analyzed by repeated measures ANOVA and compared with the Newman-Keuls test (when normal distribution was detected). The ratio of bone to implant contact was expressed as the median and the comparisons between groups were carried out with the Mann-Whitney U test. Based on the ratio of bone to implant contact difference between groups, the observed power of analysis was 77% (β risk = 23%). Results are expressed as mean $\pm$ SE and the chosen level of significance was 0.05.

RESULTS

General outcomes

Gain of body weight was comparable among the groups (control, 0.82 ± 0.15 kg; MTX, 1.18 ± 0.24 kg; GC, 0.58 ± 0.10 kg and GC+MTX, 0.65 ± 0.22 kg, $p = 0.105$). No adverse gastrointestinal effects (vomiting or diarrhea) were observed. Mortality, infection, and wound dehiscence was not recorded with this protocol.

Bone mass density variation

Initial bone mass densities were similar among the experimental groups for the lumbar spine (control, 0.269 ± 0.008 g/cm^2; MTX, 0.274 ± 0.011 g/cm^2; GC, 0.268 ± 0.010 g/cm^2; GC+MTX, 0.284 ± 0.011 g/cm^2, $p = 0.674$) and tibia (control,

0.371 ± 0.012 g/cm^2; MTX, 0.385 ± 0.008 g/cm^2; GC, 0.387 ± 0.011 g/cm^2; GC+MTX, 0.387 ± 0.011 g/cm^2, $p = 0.641$).

After 18 weeks, control animals had a positive lumbar BMD variation (ΔBMD, 0.055 ± 0.009 g/cm^2) comparable to the MTX group (0.035 ± 0.015 g/cm^2, $p = 0.280$, Fig. 1A). However, there was a significant reduction of ΔBMD in the GC group (-0.004 ± 0.012 g/cm^2, $p = 0.003$) and GC+MTX group (-0.003 ± 0.012 g/cm^2, $p = 0.003$, Fig. 1A). Tibia ΔBMD were also positive and similar in control and MTX groups (0.041 ± 0.011 g/cm^2 vs. 0.021 ± 0.009 g/cm^2, $p = 0.190$), but significantly decreased in GC (-0.018 ± 0.008 g/cm^2, $p < 0.001$) and GC+MTX groups (-0.003 ± 0.012 g/cm^2, $p = 0.022$, Fig. 1B).

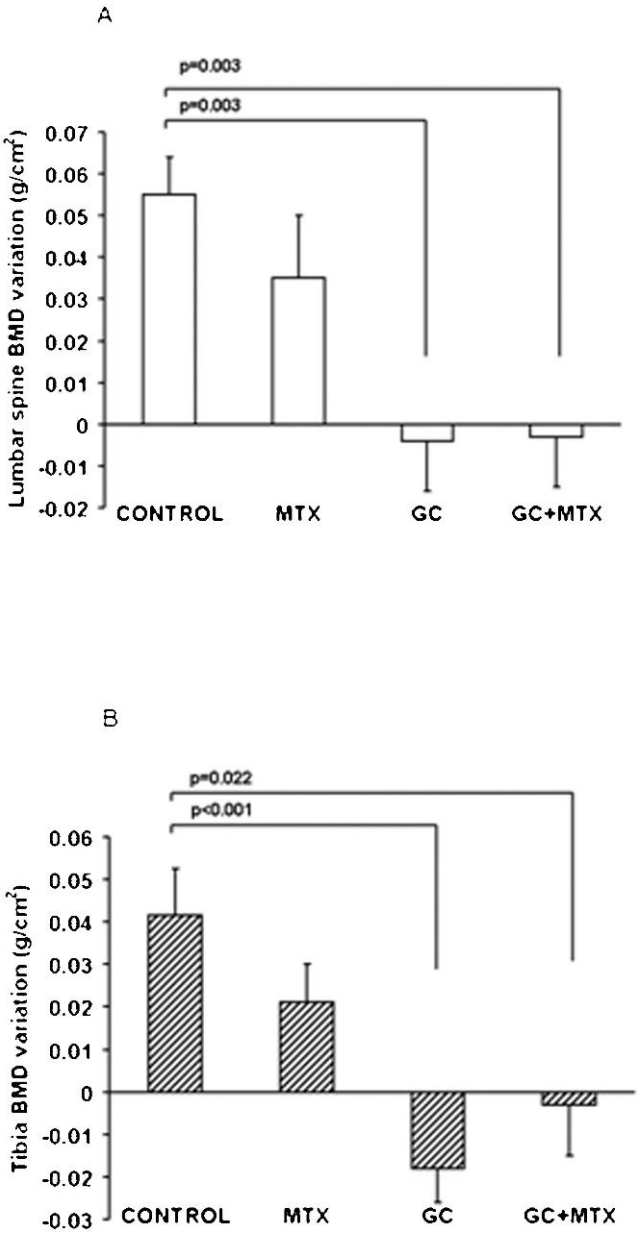

Figure 1 - Lumbar spine and tibia BMD variation (ΔBMD) in control animals (saline injected), methotrexate (MTX, 3 mg/kg/week), glucocorticoid (GC, 0.35 mg/kg/week), and GC+MTX. **Panel A**: lumbar and **Panel B**: tibia ΔBMD after 18 weeks of treatment. Results are expressed as the mean of 6–8 animals ± S.E. *$p < 0.05$ by comparison to control animals by ANOVA followed by the Newman-Keuls test.

Histomorphometric analysis

Histomorphometric analysis at 18 weeks revealed that the cortical thickness was comparable in the control and MTX groups (133.08 ± 2.36 vs. 126.24 ± 2.42 μm, $p = 0.071$). In contrast, at the final evaluation GC (98.81 ± 2.28 μm, $p < 0.001$) and GC+MTX groups (96.41 ± 3.12 μm, $p < 0.001$) had significant reductions. In addition, the percentage of bone tissue around the implant in control and MTX groups was similar (33.16 ± 1.29 vs. 30.13 ± 1.04%, $p = 0.097$) whereas GC (24.40 ± 1.51%, $p < 0.001$) and GC+MTX (25.65 ± 1.63%, $p = 0.005$) groups had significantly lower percentages of bone tissue around the implant compared.

The effects of these treatments on the percent of bone to implant contact (BIC) are summarized in Fig. 2A. Again, control and MTX groups had comparable values of osseointegration [median 39.56% (31.63–55.57%) and 37.99% (22.34–42.97%), respectively, $p = 0.101$], while GC [27.12% (14.53–37.45%), $p = 0.003$] and GC+MTX groups [31.94% (18.43–46.55%), $p = 0.03$] reduced values. The osseointegration of titanium implants in rabbit tibia was observed as direct bone (toluidine blue stained) deposition on the implant surface without any other detectable tissue interposed (Fig. 2B & C).

DISCUSSION

The present study is the first to provide compelling evidence that low dose methotrexate does not have a deleterious effect on titanium implant osseointegration. Patients with inflammatory diseases are often treated with MTX and novel biological therapies are frequently prescribed in combination with this drug.[21] The increase in quality of life for rheumatologic patients achieved in the last decade[22] may increase the demand for arthroplasty and prosthodontic treatments.

The advantage of the present study's design using normal rabbits is that we were able to discriminate between the effects of MTX therapy and those of inflammatory disease on bone metabolism. Analysis of intracellular signaling mechanisms in osteoclasts has demonstrated that various immunomodulatory molecules play a role in the control of bone metabolism in RA.[23] In addition, there is growing acceptance of simultaneous therapy in RA with multiple drugs associated with GC[24] and the present model provides a clear analysis of the effects of each drug on the osseointegration process.

Another strength of our study is that we administered doses equivalent to what is routinely prescribed in the clinical setting[12] and we treated animals with tibias of almost complete (94%) length.[25] This allowed us to minimize complications, since long-term therapy with high cumulative MTX-doses in children is associated with osteopathy, which is characterized by osteopenia, zones of calcification, growth arrest lines, and fractures.[26] This complication seems to be due to intracellular accumulation of MTX and formation of methotrexate-polyglutamates in the rapidly growing skeletal structures of infants.[27] MTX-osteopathy in adult rheumatic disease patients under low dose therapy is uncommon and restricted to a few case reports.[11,28] The causal relationship is still under debate since these five patients had other important risk factors for

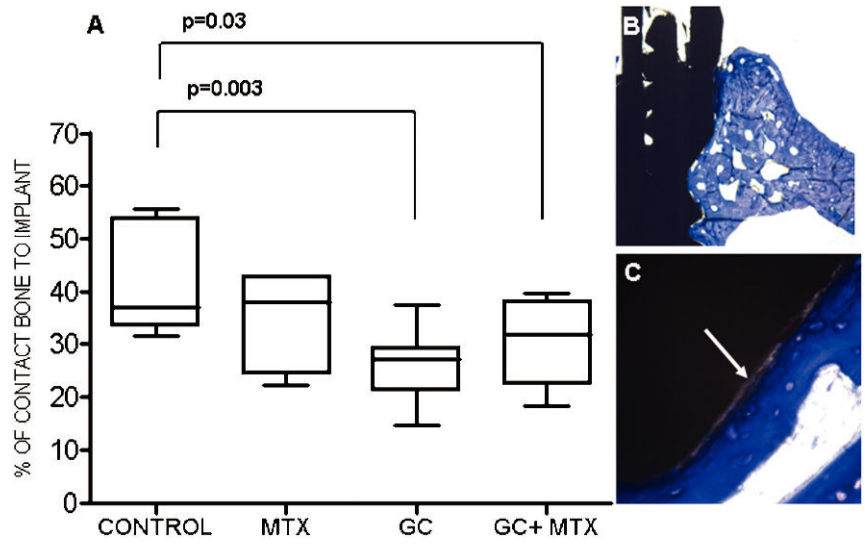

Figure 2 - Panel A: Percentage of bone to implant contact in control animals (saline injected), methotrexate (MTX, 3 mg/kg/week), glucocorticoid (GC, 0.35 mg/kg/week), and GC+MTX. Data are expressed as the median of 6–8 animals and the vertical axis show the maximum and minimum values. **Panel B:** Light microscopy of sections of titanium implant inserted in tibia of control rabbit (original magnification ×4), **Panel C:** Complete bone to implant contact (white arrow) (original magnification ×40).

osteoporotic fractures, emphasizing the relevance of the present study.[29] In addition, the observation period in the present study was an appropriate exposure time to the drug considering the comparative life spans of rabbits and humans,[30,31] which is relevant since MTX-osteopathy in adults is associated with prolonged periods of therapy.[28,32]

Our data confirms and extends previous observations that low dose MTX does not have a negative effect on BMD in patients with RA and psoriasis.[32,33] The complete exclusion of disease interference in our model allows for an accurate conclusion regarding the lack of a deleterious effect of this drug on bone mass at either cortical or trabecular sites. In addition, reproduction of the clinical conditions often observed in rheumatologic patients revealed that MTX had no effect on BMD when associated with GC. The reductions recorded in the GC and GC+MTX groups are comparable and are attributable to the GC treatment since this drug can cause rapid bone loss, decreased bone formation, and increased bone resorption.[34]

Our results reveal that MTX treatment preserves cortical thickness, percent of bone around the implant, and percent of bone to implant contact (BIC), all of which are histological criteria for osseointegration, in spite of the limited number of animals. In contrast to zoledronic acid,[18] MTX was unable to reverse the deleterious effect of GC. In fact, the previously reported protective effect of MTX is only seen under inflammatory conditions when several cytokines and other chemical mediators such as PGE_2, which affect bone cells, were involved.[35]

Together our data suggest that low dose MTX therapy should be maintained in individuals requiring implant surgery since this drug does not affect the osseointegration process.

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