Effect of Methotrexate Therapy on Bone Mineral Density and Body Composition in Rat Adjuvant Arthritis

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ABSTRACT. ***Objective.*** To test whether methotrexate (MTX) therapy of rat adjuvant arthritis (AA) prevents loss of bone mineral density (BMD) and loss of adipose and lean body mass compared to pair-fed controls with untreated rat AA (positive controls) and rats without AA (negative controls).

Methods. AA was induced by a *Mycobacterium butyricum* injection at the base of the tail of 5-week-old female Lewis rats. The MTX-treated group was injected with adjuvant and then treated twice weekly with MTX (1.0 mg/kg/wk intraperitoneally). To control for the effects of AA on appetite and weight, food given to control animals and MTX-treated rats with AA was limited to that consumed by rats with untreated AA. At 42 days post-adjuvant injection, the animals were sacrificed and tibial BMD was measured. Body composition was analyzed for percentage fat, protein, ash, and water.

Results. There was no difference in ankle edema score or ankle width between the negative controls and MTX-treated group at necropsy. BMD was significantly higher in the negative controls versus positive controls and MTX-treated and in MTX-treated versus positive controls. There was significantly less body fat and protein and greater body water in the positive controls and MTX group compared to the negative controls.

Conclusion. MTX prevents loss of BMD in the tibia in the rat AA model compared to positive controls. While MTX is effective in lowering inflammation in rat AA, there are still significant losses in BMD and body composition, which may have implications for rheumatoid arthritis. (J Rheumatol 2004;31:1693–7)

Key Indexing Terms:
ADJUVANT ARTHRITIS
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Rat adjuvant arthritis (AA) shares characteristics with rheumatoid arthritis (RA) such as genetic linkage, involvement of peripheral joints and cartilage, bone destruction, pannus formation, synovial CD4+ cells, and T-cell dependence[1,2]. Disease modifying antirheumatic drugs (DMARD) such as methotrexate (MTX) are effective in lowering joint inflammation in rat AA[3-9].

Bone mineral density (BMD) is decreased in rat AA in both trabecular and cortical bone compared to rats without arthritis[10]. The effect of MTX on BMD in rat AA is controversial. MTX in high dose (0.75 mg/kg/day) for 5 days caused a 27% reduction in net trabecular volume and decreased bone formation by 60%[11]. The numbers of osteoclasts were not different from untreated rats and osteoblast toxicity was evident by reduced volume and thickness of osteoid. A lower MTX dose (3 mg/kg/week) for 16 weeks has been shown to cause osteopenia in rat AA by suppression of osteoblast activity and stimulation of osteoclast recruitment with resultant increases in bone resorption[12]. However, in another study, the same MTX dose improved osteogenic activity of bone marrow cells and reduced bone resorption and periarticular osteopenia was partially normalized[13]. Therefore, the effect of MTX on rat AA is unclear.

AA affects body composition. Rats with AA lose 20% of their body weight by 28 days post-adjuvant injection and lean body mass decreases significantly compared to negative controls[14]. Pair-fed rats lose only one-quarter of the weight of rats with AA, indicating that anorexia alone is not the only reason for the cachexia[14]. Weight loss was correlated with tumor necrosis factor-α (TNF-α) production by splenic mononuclear cells[14]. MTX is used in the treatment of RA[15] and has been shown to prevent body protein break-

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down in 4 adults with RA[16]. It is unknown if MTX has the potential to alter body composition changes in rat AA.

We hypothesized that MTX, at a dose that lowers clinical joint inflammation in rat AA, would prevent loss in tibial BMD and reduce losses in ash and lean body mass compared to positive controls (i.e., untreated rat AA).

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved this study. Female Lewis rats, obtained from Harlan Laboratories (Indianapolis, IN, USA) at 5 weeks of age, were housed in individual cages. Rats were fed Harlan Teklad 4% Mouse/Rat Diet with a nutrient composition of 46% carbohydrate, 24% protein, 5% fiber, 4% fat, and a metabolizable energy of 2.94 kcal/g[17].

Randomization and treatment groups. A total of 89 rats at an age of 5 weeks were used and the experiment was performed in 6 blocks. On Day 0, the animals were randomized into 3 groups to assure initial equal mean body weights.

The experimental groups were (1) rats injected with mineral oil and subsequently injected intraperitoneally (IP) with phosphate buffered saline (PBS) (negative controls); (2) rats injected with mineral oil containing *Mycobacterium butyricum* and subsequently injected IP with PBS (positive controls); and (3) rats injected with mineral oil containing *M. butyricum* and subsequently injected IP with MTX in PBS at a dose of 1 mg/kg/wk (MTX group) starting at 3 days after adjuvant injection on Tuesdays and Fridays. The dose of MTX was selected on the basis of our earlier study, which concluded that 1 mg/kg/wk was the most effective dose with the least toxicity[3].

Induction of AA. AA was induced in the positive control group and MTX-treated group by an intradermal injection of 0.1 ml heat-killed *M. butyricum* (6 mg/ml) in light mineral oil into the base of the tail at Day 0. Rats were under light ether anesthesia during the adjuvant injection.

Modified pair-feeding protocol. Food consumption in the positive control group was determined by weighing the food given, weighing the food the next day on an electronic scale to the nearest 0.1 g, and calculating the difference. An amount of food that maintained equal mean body weights among the groups was fed to the negative controls and MTX-treated group. The amount of food given to the negative controls and MTX-treated group was adjusted daily (i.e., after daily weighing). A modified pair-feeding design was used so that changes in body composition could not be explained by changes in body weight.

Clinical evaluations. Body weights were monitored daily on an electronic scale to the nearest gram. Clinical disease activity was scored twice weekly for edema and erythema separately on a scale of 0–4[3]. Edema was also quantitated twice weekly by measurements of maximal lateral ankle diameters using a digital caliper[7,13]. Each block of the protocol was continued for 42 days post-adjuvant injection. At the end of the protocol, the rats were euthanized by CO_2 asphyxiation.

Dual energy x-ray absorptiometry (DEXA) and body composition analysis. Tibial BMD was determined using a Lunar/GE PIXImus densitometer (Lunar, Madison, WI, USA)[18]. Carcass composition was determined by a modified analysis procedure[19].

Methods of analysis and statistical analysis. Descriptive statistics including means, standard deviation (SD), and sample size were calculated for each variable[20]. Two parametric approaches, Student's t test and Pearson correlation, were used for data analysis because they tended to be more powerful for detection of group differences than nonparametric approaches, even for small samples and/or non-normality[21]. The 2-sample t test was employed to test whether there were differences between group means for each response variable. In addition, we used Pearson correlation analysis to examine the relationship of clinical joint involvement with body composition and DEXA in the positive control group. A p value < 0.05 was considered significant.

RESULTS

A total of 89 animals were entered into the protocol; 30, 29, and 30 animals were in the negative control, positive control, and MTX groups, respectively. One group underwent body composition analysis prior to the completion of DEXA analysis for BMD; therefore a total of 74 animals underwent DEXA analysis.

Body weights. Mean body weights were not different at any of the time points prior to the end of the trial ($p > 0.05$), indicating that the modified pair-feeding protocol was successful. At the time of necropsy, the mean weight ± SD for the MTX group was 148 ± 17 g, for the negative control group 149 ± 16 g, and for the positive control group 150 ± 20 g. There was no difference in weights between any groups.

AA disease course. There was evidence of arthritis (clinical joint inflammation) in 90% of the positive controls and 17% of the MTX group during the 6-week treatment period. The onset of clinical arthritis occurred at approximately 17 days post-adjuvant injection. Clinical joint inflammation as gauged by positive swelling scores at 42 days post-adjuvant injection is shown in Table 1. There was significantly more disease activity, gauged by ankle width and mean edema score, in the positive controls compared to the negative control and MTX-treated groups. There was no difference in disease activity between the negative control and MTX-treated group at any time during the experiment.

BMD. BMD is shown in Table 2. There were significant differences ($p > 0.05$) with all pairwise comparisons of BMD. There were no significant differences in any of the pairwise comparisons of area in the 3 groups.

Body composition. Table 3 shows body composition at the time of necropsy. There were no significant differences in the final carcass weights between the 3 groups. There were significant differences in percentage fat, water, and protein between the negative control and the MTX-treated groups and negative and positive control groups ($p < 0.05$). There was no difference in ash between any of the pairwise comparisons between the groups.

Correlation between disease activity and BMD and body composition. Table 4 displays the correlation coefficients between final rear ankle width and rear paw edema score (as a gauge of disease activity) and BMD. There was a significant negative correlation between final ankle width and swelling score and BMD in the positive control group. When a similar analysis was completed in the MTX-treated group, there were no significant relationships.

Table 4 also shows the correlation coefficient between final rear ankle width and swelling score and body composition in the positive control group. There was a significant negative correlation between final ankle width and edema score and protein content in the positive control group. There was a significant positive correlation between final

Table 1. Mean width of rear paws in mm ± SD and mean edema score in the rear paws at 42 days after adjuvant injection. Means with different letter superscripts are different, $p < 0.05$.

Group (n)	Mean Width	Mean Edema Score ± SD
Negative control (30)	4.49 ± 0.14[a]	0.00[a]
Positive control (29)	6.26 ± 1.96[b]	1.83 ± 1.43[b]
MTX (30)	4.59 ± 0.55[a]	0.183 ± 0.50[a]

Table 2. Mean bone mineral density (BMD) in the tibia in the 3 treatment groups ($p < 0.05$ for all pairwise comparisons).

Group (n)	BMD, g/cm^2 ± SD
Negative control (25)	0.1355 ± 0.0550
Positive control (24)	0.1116 ± 0.0141
MTX (25)	0.1217 ± 0.0094

ankle width and edema score and water content in the positive control group. There was no significant correlation between ash content and final rear paw width and final edema score in the positive control group. There were no significant correlations in the MTX-treated group between ankle width and final edema score and body composition.

DISCUSSION

Clinical arthritis was present in 90% of the positive controls, indicating that the rat AA model was functional. The efficacy of MTX as a treatment for rat AA at a dose of 1 mg/kg/wk IP was confirmed, since joint inflammation was significantly lower in the MTX group compared to the positive controls at all days after day 17 post-adjuvant injection[3,5-9].

There was a significant difference between all 3 pairwise comparisons related to BMD. Low-dose MTX, 1 mg/kg/wk, did improve BMD in rat AA relative to positive controls, but did not normalize BMD relative to negative controls. Our data also confirm a previous finding that rat AA is associated with a loss of BMD[10]. MTX has been shown to have a variety of effects on bone metabolism in animal models[11-13]. Friedlaender, *et al* showed that administration of MTX in Lewis-Wistar rats (0.75 mg/kg/day) caused a 27% reduction in net trabecular bone volume[11]. Injections of MTX (3 mg/kg) to female Sprague-Dawley rats showed decreased bone formation and increased osteoclast recruitment[12]. Suzuki, *et al* administered MTX (3 mg/kg/wk) to controls and rats with AA[13]. In controls, MTX decreased the growth of fibroblast colony-forming units in the marrow and lowered serum osteocalcin levels, and in rats with AA, partially normalized bone resorptive activity and osteogenic activity of bone marrow cells. Our results agree with the results of Suzuki, *et al*, since MTX in a dose of 1 mg/kg/wk significantly improved BMD relative to positive controls, but did not restore BMD to levels seen in the negative control group.

Table 4. Correlation coefficients between final rear ankle width and rear paw edema score and the BMD and body composition in the positive control group (p values in parentheses).

Variables	Final Rear Paw Width	Final Edema Score
BMD	–0.43 (0.04)	–0.45 (0.03)
Water, %	0.63 (< 0.001)	0.57 (0.001)
Ash, %	0.24 (0.21)	0.29 (0.13)
Fat, %	–0.61 (< 0.001)	–0.55 (0.002)
Protein, %	–0.44 (0.02)	–0.47 (0.01)

There have been conflicting reports of the effect of MTX on human bone metabolism. Increased fractures have been reported in individuals taking MTX for RA or psoriatic arthritis[22-25]. Other studies have reported that low dose MTX therapy does not seem to affect trabecular or cortical BMD[26-31]. Buckley, *et al* concluded that MTX alone did not lower BMD over a 3-year period in patients with RA, but that the combination of MTX plus ≥ 5 mg/day of prednisone was associated with more bone loss than MTX without prednisone[26]. Our MTX-treated rat AA model differs from MTX-treated RA, because in this experiment, MTX is administered in young, growing animals, not adults. This may explain why MTX improved BMD relative to positive controls in rat AA and why this finding has not been seen in RA. A limitation of our study is that there was no MTX therapy in normal rats; therefore we can make no comment on the effect of MTX on normal bone metabolism.

There was no difference in mean weight at any time during the protocol between groups and the carcass weights were the same at the time of necropsy. Therefore it is unlikely that changes in body composition result from artifacts caused by differences in body weight. The MTX-treated and positive control animals had significantly higher

Table 3. Mean percentage body composition and final carcass weight ± SD. Means with different letter superscripts were significantly different, $p < 0.05$.

Group (n)	Final Carcass Weight, g	Fat, %	Water, %	Ash, %	Protein, %
Negative control	149 ± 16	7.77 ± 1.78[b]	66.12 ± 2.32[b]	4.30 ± 0.56	21.80 ± 1.50[b]
Positive control	150 ± 20	6.68 ± 1.62[a]	68.00 ± 1.90[a]	4.14 ± 0.26	21.17 ± 0.58[a]
MTX	148 ± 17	6.70 ± 1.69[a]	68.14 ± 2.39[a]	4.25 ± 0.44	21.20 ± 0.69[a]

body water and lower body fat and protein than the negative controls, which may reflect the edema of joint swelling and cachexia. These data suggest that a MTX dose that lowers joint inflammation is not sufficient to protect against body composition changes. There was no difference in percentage body ash between any of the 3 groups. This is likely explained by the fact that percentage body ash and tibial BMD are measurements of different variables.

There was an inverse relationship between BMD and disease activity and body composition in the positive control group. Animals with more active disease (larger edema scores and ankle widths) had lower BMD. Our data are similar to the data presented by Roubenoff, *et al*, who showed impressive weight loss and cachexia in rat AA compared to negative controls[14]. There is a strong negative correlation between body fat and protein and disease activity as measured by final ankle width and edema score.

RA is a chronic wasting disease. Resting energy expenditure was found to be higher in subjects with chronic inflammation even when the disease was clinically well controlled[32]. Lower sustained inflammation has been linked to better outcomes and sustained disease activity is inversely related to survival[33,34]. Lean body mass was also found to be inversely related to number of swollen joints[35]. Roubenoff, *et al* suggested that "hypermetabolism of chronic inflammation smolders," even during good control of disease[32], and that rheumatoid cachexia is not apparent on clinical evaluation[35]. In a study evaluating protein metabolism using ^{13}C-leucine, patients with RA had increased whole-body protein turnover that was positively correlated with growth hormone, glucagon, and TNF-α production. Patients receiving MTX were found to have protein kinetics similar to young healthy subjects[16]. The authors speculated that MTX "may be effective in normalizing protein kinetics in RA," but suggested that this needs to be confirmed in a larger study[16]. Our results in an AA model suggest that despite relatively good control of disease (i.e., only 17% of the MTX-treated group had clinical disease), there was incomplete protection from lean body loss. The addition of hormone and cytokine analyses to our protocol would have been useful[36].

We conclude that rat adjuvant arthritis is a wasting disease and that MTX in a dose of 1 mg/kg/wk does not maintain tibial BMD compared to negative controls, but improves BMD relative to positive controls. MTX therapy does not protect from loss in fat mass and protein compared to negative controls. Therefore, erosion of body composition can coexist with suppression of joint inflammation. These results have possible implications in RA. The clinical endpoint currently sought with disease modifying antirheumatic drug (DMARD) treatment of RA is a reduction of inflammation. More attention may need to be focused on changes in body composition and BMD in RA and the effect of DMARD therapy on these variables.

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