# Methotrexate inhibition of bone mineral density increase in growing rabbits: Prevention by folinic acid

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## Abstract

### Objective

*Methotrexate (MTX) action on bone metabolism is as yet not completely understood. The results of clinical studies are controversial, since it is difficult to distinguish the side effects of MTX from those of the primary disease. This study assessed the effect of MTX, with and without folinic acid supplementation, on bone mineral density in growing normal rabbits.*

### Methods

*Three groups of young NZW growing female rabbits were treated with: saline (n=6) or MTX (0.25 mg/kg/week, n=5) or MTX (same dose as above) plus folinic acid (0.25 mg/kg/week, n=6) for a period of 3 months. The dose, duration and frequency of MTX administration were similar to the treatment of RA patients. The animals were submitted to dual-energy absorptiometry densitometry (HologicQDR 2000) before and after treatment; total body and L4-L5 BMD were evaluated. Histomorphometric analysis (L4 vertebrae) was also performed.*

### Results

*Growing control rabbits showed increased total body BMD from a baseline of $0.180 \pm 0.006$ to $0.198 \pm 0.007$ $gm/cm^2$ (mean $\pm$ S.E.M, $p < 0.006$). In contrast, no increase in BMD ($0.182 \pm 0.006$ versus a baseline of $0.184 \pm 0.004$, ns) was observed in the group treated with MTX, while the addition of folinic acid resulted in an increase in BMD values similar to controls, from a baseline of $0.181 \pm 0.004$ to $0.198 \pm 0.003$, $p < 0.02$), thus preventing adverse MTX bone effects. Average percent variations in BMD were +7.7%, -1% and + 8.4% respectively. Spine (L4-L5) BMD showed analogous results, in line with the histomorphometric data.*

### Conclusion

*These results strongly support a deleterious action of MTX on bone metabolism, which is prevented by folinic acid supplementation. The potential clinical implications of our data are particularly significant for paediatric therapy.*

### Key words

Folinic acid, methotrexate, bone mineral density, absorptiometry, rabbit

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## Introduction

Methotrexate (MTX) has been widely used in the treatment of RA and more recently has been indicated for JCA (1). While the therapeutic efficacy of MTX is well recognised (2,3), its mechanism of action is still poorly understood. MTX interference with folate metabolism, as a competitive inhibitor of the enzyme dehydrofolate reductase blocking *de novo* purine synthesis, does not seem an adequate explanation for either the rapid onset of its anti-inflammatory effects or the lack of notable worsening effects of the concomitant use of folic or folinic acid on disease activity (4,5). Although still an unsettled subject, recent studies have supported the beneficial effect of folate supplementation in reducing oral, hepatic and gastrointestinal side effects (6-8).

MTX action on bone metabolism is a further controversial topic. MTX osteopathy described in paediatric oncology refers to a syndrome of reversible osteopenia, bone pain and fracture (9). Furthermore, after complete oncological treatment, MTX modulated the post-glycocorticoid increase in bone turnover. It impaired osteoblast recovery and enhanced osteoclast activity (10). While stress fractures induced by MTX continue to be reported in adult patients using MTX for psoriatic arthritis and RA (11-14), data from recent studies have suggested that MTX alone has no effect on BMD in RA patients (15-17).

Whether or not low-dose MTX in RA interferes with bone metabolism remains to be established, mainly because it is difficult to separate the direct action of MTX on bone from its anti-inflammatory activity, as well as from the deleterious effects of the primary disease itself. The generalised osteopenia well known to occur in these patients involves multiple factors, including systemic inflammation, decreased physical activity, malnutrition, postmenopausal status and corticosteroid administration.

The objective of this study was to identify the effect of long-term low dose MTX administration and of the addition of folinic acid to MTX treatment on bone metabolism in growing healthy rabbits.

## Materials and methods

### Animal model

The experimental protocol was approved by our institutional ethical committee in accordance with COBEA (Brazilian College of Animal Experimentation) and with the procedures laid down by UFAW (University Federation for Animals Welfare). It established experimental conditions similar to the treatment of human RA in terms of duration, dose, and frequency of MTX use. Three groups of young female NZW rabbits (3 months old in terms of growth stage) received by the intramuscular (i.m.) route either saline (n=6), MTX (0.25 mg/kg/week, n=5) or MTX followed by folinic acid (0.25 mg/kg/ week, n=6) after 6 hours. Over a period of 3 months each animal received two i.m. injections 6 hours apart once a week: saline and saline in the control group, MTX and saline in group II and MTX followed by folinic acid in group III rabbits. The animals had water and dry food *ad libitum* and the caretakers were kept blind regarding the medication administrated. Initial and weekly body weights and blood samples were drawn from all animals. Blood cell counts (number of erythrocytes, leukocytes and platelets) were manually performed in a Neubauer chamber.

### Densitometry

Anaesthetized (xylazine 5 mg/kg, i.m. and ketamine 50 mg/kg i.m.) rabbits were submitted to dual-energy absorptiometry densitometry (Hologic QDR 2000 PLUS) of the total body (infant whole body program – V5.73P) and of the L4-L5 region (small animal software-Hi-Res V4.76) (18-20) before and after treatment. The coefficient of variation (CV = 100 x SD / mean of 8 repeated BMD measures in one animal) was lower than 3% for the total body as well as for the L4-L5 measurements. After 3 months the percent variation in total body and spine BMD was established through the formula: percent of variation = 100 x (final BMD - initial BMD/initial BMD) which was

applied to the BMD values of each animal. The results are expressed as the mean value for each group. The investigators in charge of animal manipulation and BMD measurements were unaware of the group allocation, drug administration and outcomes.

*Histomorphometry*

After sacrifice (anaesthetized animals received 2 ml of 20% KCl i.v.), the fourth lumbar vertebra was removed for histomorphometric analyses and prepared as previously described (21). Static and structural parameters of bone formation and resorption were measured at a standardised site using Osteomeasure software (Osteometrics Inc., Atlanta, GA). Histomorphometric data are expressed in accordance with the guidelines of the American Society of Bone and Mineral Research (22).

*Statistical analysis*

Results are expressed as the mean ± S.E.M. or percent of variation. Data before and after treatment in the same animal were analysed by the paired T test and comparisons between groups were analysed by repeated measures analysis of variance. Post-analysis was conducted by Newman-Keuls multiple comparison tests. A logarithm transformation was performed on all histomorphometric variables. The level of significance was 0.05.

**Results**

All rabbits exhibited increased body weight after 3-month treatment (Fig. 1), with no difference among the three treatment groups, i.e. saline, MTX, and MTX plus folinic acid (FA). No adverse effects (vomiting, diarrhoea or abnormalities in the blood counts) were observed.

The control group, comprising 6 saline-treated young rabbits followed for 3 months, presented an expected 7.7% increase in total BMD (from a baseline of 0.180 ± 0.006 to 0.198 ± 0.007 g/cm², p < 0.006) (Fig. 2A). An analogous 19.7% increase was observed on spine (L4-L5) BMD: 0.2407 ± 0.01 g/cm² vs. baseline of 0.2061 ± 0.02, p < 0.006 (Fig. 2B).

In contrast, rabbits treated with low-
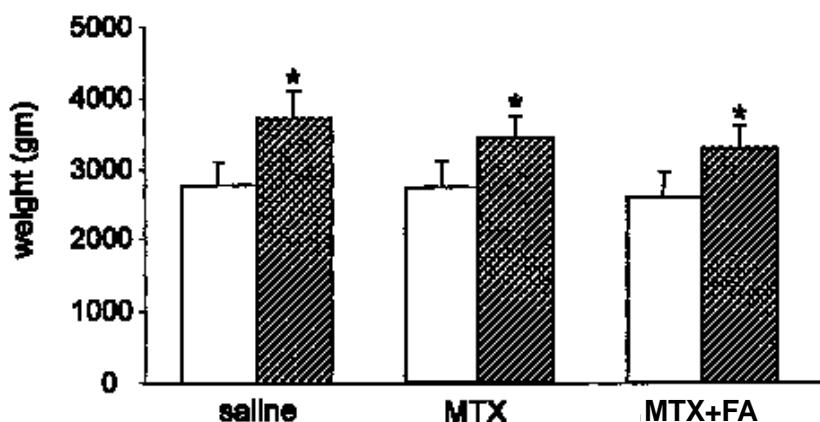


**Fig. 1.** Weight of growing rabbits ▭ before and ▨ after a three-month treatment with saline (n=6), MTX (0.25 mg/kg/week, n=5) or MTX plus folinic acid (FA) (0.25 mg/kg/ week, n=6). Each column represents the mean ± S.E.M. *p < 0.02
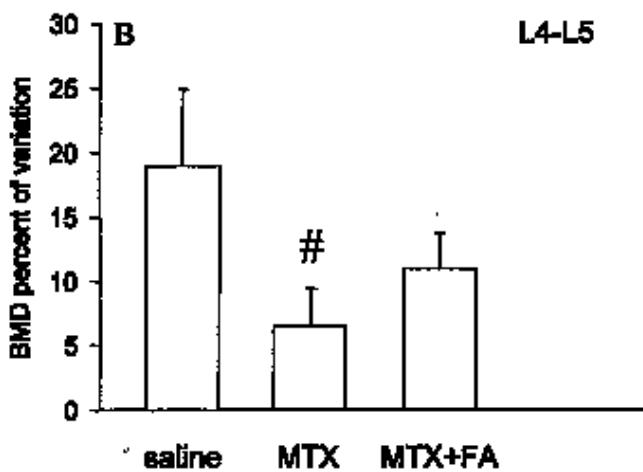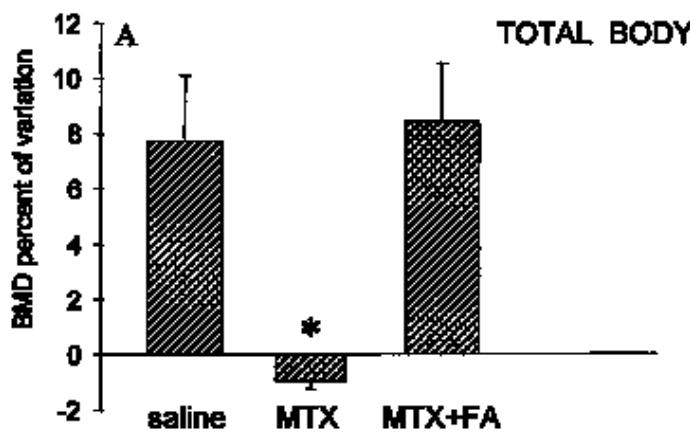


**Fig. 2.** BMD of growing rabbits, expressed as the percent variation from baseline values after a three-month treatment with saline (n=6),MTX (0.25 mg/kg/week, n=5) or MTX plus folinic acid (FA) (0.25 mg/kg/ week, n=6). Each column represents the mean ± S.E.M.
(**A**) ▨ total body BMD and (**B**) ▭ L4-L5 BMD, both expressed as the percent of variation from baseline. *p < 0.03 MTX vs.saline and MTX versus MTX + folinic acid (FA). #p < 0.05 MTX versus saline.

dose MTX showed no change in total body BMD (from a baseline of 0.184 ± 0.004 to 0.182 ± 0.006 gm/cm$^2$, ns), corresponding to an average -1.1% percent variation (Fig. 2A).

Simultaneous administration of MTX and folinic acid led to an average 8.4% increase in total body BMD, which was similar to that observed in control rabbits (from a baseline of 0.181 ± 0.004 to 0.198 ± 0.003gm/cm$^2$, p < 0.02 versus baseline, p < 0.03 versus MTX alone and p = ns versus control rabbits (Fig. 2A).

Comparable results, although less pronounced, were observed on spine (L4-L5) BMD: MTX-treated rabbits showed no significant increase in BMD (from a baseline of 0.2371 ± 0.02 to 0.2513 ± 0.01 gm/cm$^2$, p = 0.1), contrary to rabbits treated with MTX plus folinic acid (from a baseline of 0.2258 ± 0.01 to 0.2490 ± 0.01 gm/cm$^2$, p < 0.03) and the average percent BMD increases versus baseline respectively of 6.3% and 11%, are depicted in Figure 2B.

Histomorphometric analysis performed as described (22) was in accordance with the BMD results (Table I): MTX treatment led to a resorption surface (ES/BS%) of 2.34 ± 0.94 and to an enhanced osteoclast surface (Oc.S/BS% = 0.32 ± 0.17; p < 0.05), which were respectively a 2.6 and 4.4-fold increase versus control values. There was no corresponding increase in

osteoid (OS/BS% = 7.94 ± 2.0) and osteoblastic surfaces (Ob.S/BS% = 2.53 ± 0.78) versus respective control values of 6.48 ± 1.4 and 1.96 ± 0.77%. With concomitant folinic acid treatment, resorption measures returned to control levels (Table I). Overall, these data point to an increased bone resorption without corresponding bone formation in MTX-treated rabbits, which was prevented by co-administration of folinic acid.

## Discussion

Our results showed MTX-induced inhibition of BMD increase in growing normal rabbits and the ability of folinic acid to prevent such a deleterious effect. Interestingly, MTX associated fractures either in children or in RA and psoriatic patients (stress fractures) are not observed on the axial skeleton (9, 11-14) and the MTX effect on spine BMD was also less pronounced in our model.

Three-month old NZW rabbits were used for all studies because of the requested treatment duration comprising a significant period of the animal growing stage. The period of time required to reach skeletal maturity and peak bone density is well established in this species. Skeletal maturity, defined as the closure of the epiphyseal growth plates and the highest vertebral bone density, is achieved within 6-7 months of age, corresponding to the age of sac-

rifice for the rabbits in the present study (21).

In adult rat animal models, developed before the widespread use of low-dose MTX in rheumatic diseases, adverse effects on bone were reported with chemotherapeutic high dose MTX, pointing to a decrease in bone formation with conserved or increased bone resorption (23, 24).

Similar to our findings, a study analysing the action of MTX in rat adjuvant arthritis (25) showed a reduction in osteogenesis and the increased formation of osteoclast-like cells in control animals (MTX treatment without arthritis) (25). BMD was unchanged in these control animals, possibly because a 3-week study was not long enough to detect alterations in bone mass, particularly in adult animals.

In adult patients, iliac crest bone histomorphometry showed a low bone turnover state with reduction in osteoblastic surfaces, osteoid matrix parameters and tetracycline labelling (11).

In accordance with our results, *in vitro* studies (26) carried out on an osteoblast cell line (UMR rat osteoblast-like osteosarcoma) incubated with MTX for 48 hours showed a dose-dependent (40 nM – 100 nM) direct toxic effect of MTX that was reversible by folinic acid (1 – 10 uM). Although MTX concentrations detected in human trabecular and cortical bones were in the range of 0.511 to 0.0539 and 0.468 to 0.0793 nmol/gm respectively (27), the bone cell exposure to MTX is prolonged. Such a sustained exposure could contribute to the osteoblastic toxicity of MTX in humans. Additionally, in cultures of human bone-derived osteoblasts, MTX induced the strong inhibition of osteoblast proliferation (28, 29), although without any effect on the mature osteoblastic population (29). Also, the results of *in vitro* culture of bone mouse cells suggest that a dose-related decrease in mouse osteoblastic cell function occurs with low average concentrations of MTX, leading to slow osteoblastic matrix production. In this study MTX did not change the numbers of osteoclast and osteoblast cells (30). These differences could be due to experimental conditions or cell

**Table I.** Histomorphometry of the fourth lumbar vertebra after 3 months of treatment with either saline or MTX or MTX plus folinic acid).

|  | Control | MTX + folinic acid | MTX |
|---|---|---|---|
| Bone volume (BV/TV%) | 28.65 ± 4.35 | 29.14 ± 4.11 | 20.66 ± 1.27# |
| Resorption surface (ES/BS %) | 0.91 ± 0.21 | 1.11 ± 0.51 | 2.34 ± 0.94 |
| Osteoclast surface (Oc.S/BS%) | 0.09 ± 0.02 | 0.24 ± 0.09 | 0.42 ± 0.18* |
| Osteoid volume (OV/BV%) | 0.39 ± 0.12 | 0.59 ± 0.25 | 0.97 ± 0.50 |
| Osteoid seam width (O.th μm) | 2.65 ± 0.8 | 2.43 ± 0.4 | 2.67 ± 0.59 |
| Osteoid surface (OS/BS %) | 6.48 ± 1.4 | 7.39 ± 3.66 | 7.93 ± 2.0 |
| Osteoblastic surface (Ob.S/BS%) | 1.96 ± 0.77 | 3.38 ± 1.23 | 2.53 ± 0.78 |
| Trabecular thickness (Tb.th μm) | 68.07 ± 12.06 | 62.3 ± 5.59 | 54.91 ± 2.71 |
| Trabecular number (Tb.N no./mm) | 3.62 ± 0.73 | 4.84 ± 0.9 | 3.81 ± 0.39 |
| Trabecular separation (Tb.sp μm) | 216.37 ± 34 | 164.87 ± 33.8 | 216.27 ± 28.12 |

Values are expressed as the mean ± SEM; n = 4 for each group.
#p < 0.05 MTX vs MTX plus folinic acid; *p < 0.05 MTX vs control.

type sensitivity to MTX. Nevertheless, all of these studies point to an inhibitory effect of MTX on osteoblast cells.

The postulated mechanisms of action of MTX so far have not addressed bone metabolism (4, 5,31), although it is well known that MTX is present in high levels in the cortical and trabecular bone of RA patients receiving low dose MTX intramuscular injections (27). It has been reported that MTX can antagonize the actions of IL-1 (31, 32), a potent stimulator of osteoclastic bone resorption, and also has inhibitory activity on $PGE_2$ production (31, 32, 33), another inducer of osteoclastogenesis (34). Therefore, it is possible to speculate whether both of these MTX inhibitory activities should have an agonist effect on bone turnover in an inflammatory situation and, once the inflammatory process has been reduced, the deleterious activities of MTX on bone would prevail, unless simultaneous administration of folinic acid is added.

Another important issue that is still unsettled in clinical practice is the effect of the concomitant administration of folic or folinic acid on MTX efficacy. A recent meta-analysis (35) was unable to find consistent differences in disease activity parameters when comparing placebo and folic or folinic acid at low or high doses. Additionally van Ede *et al.* (37) showed not only no relationship of the activities of the purine enzymes (folate metabolism) studied with the therapeutic efficacy of MTX treatment, but also no difference in the efficacy, toxicity and enzyme activities associated with supplementation with folic or folinic acid and placebo.

Therefore our results, although preliminary, are the first to show that MTX inhibits the normal increase in BMD on growing bone in rabbits, as well as the ability of folinic acid to prevent this deleterious effect of MTX. This finding might be important in the treatment of children suffering from juvenile chronic arthritis with MTX.

## Acknowledgements

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