The Effect of Low Dose Methotrexate on Fracture Healing

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**Introduction**

Low dose methotrexate treatment (10-25 mg./week) is today considered the first choice disease-modifying agent (DMARD) for adult and juvenile rheumatoid arthritis. Methotrexate (MTX) has proven to be a safe and effective drug with relatively few side effects. Being a folate antagonist, MTX was originally designed for use in the treatment of malignancy. From the oncological practice, where MTX is used in high doses (500-1500 mg. repeatedly), there have been reports on an increased risk of spontaneous fractures and osteopathy. From this reason, ongoing anti-rheumatic MTX treatment is often discarded pre- and postoperatively when orthopedic surgery is performed in the patients with rheumatoid arthritis (RA). Many times this will cause a flare up in the disease activity, leading to increased pain, joint stiffness and thus problems during the rehabilitation period. However, when used as a DMARD in RA, the impact of MTX on bone metabolism, fracture healing and prosthesis in growth is not well understood.

The aim of the present study therefore was to assess the early process of fracture healing during low dose MTX treatment, in an experimental fracture model. The evaluation included histomorphometrical analysis of new bone formation and an assessment of the local inflammatory reaction, using immunostaining of tissue macrophages.

**Methods**

**Animals**

18 male Sprague-Dawley rats were allocated to 3 groups. 1: Low Dose MTX Group (LD; n=6). Pretreatment by intraperitoneal injection of 3 mg/kg of MTX, once every week for 4 weeks before surgery. 2: Normal Control Group (C; n=6) No MTX treatment. 3: High Dose MTX Group (HD; n=6) as Positive Control. Pretreatment by intraperitoneal injection of 250 mg/kg of MTX as a single dose at day of operation. All MTX doses were chosen to yield serum concentrations corresponding to human doses.

The project was approved by the local animal research ethics committee.

**Experimental fracture model**

Before operation, rats were anaesthetized by an intraperitoneal injection of 1ml of mixed solution with Ketamine and Diazepam in proportions 4:1.

A curved incision was made through the skin from the base of the tail to the knee. A fixator was fastened to the middle femur and an osteotomy was performed. Using a distraction/compression screw of the fixator, the bone fragments were moved to a 2 mm fracture defect. The rats were then allowed to move freely for one week, then they were sacrificed, perfusion fixated and the fractured leg was dissected free. Specimens were decalcified in EDTA solution for 3-4 weeks, transversely cut, dehydrated, embedded in paraffin and cut in 5 micrometer thick sections

**Histomorphometry**

The sections were stained with alcian blue and haematoxylin/eosin. A computerized image analysis system, (Easy Image 2000, Bergström Instrument AB, Solna, Sweden) was used. A standardized rectangular frame (5x3mm) was placed centrally over the fracture gap. The area of new bone was measured in the periosteal zones within the frame. The total area of new bone was calculated as the sum of the periosteal areas. The measurement accuracy of this technique was defined by separate evaluation.

**Immunohistochemistry**

The sections were incubated with monoclonal anti-rat ED2 (Tissue macrophages) antibody (Serotec, Oxford, UK). They were further incubated with a rabbit anti-mouse IgG antibody (DAKO, Copenhagen, Denmark), linked to peroxidase. A computerized image analysis system (Easy Image 2000, Bergström Instrument AB, Solna, Sweden) was used to count the number of positively staining cells, within a standardized rectangular frame (0.8x0.8mm), placed over the muscle tissue close to the fracture gap.

All specimens were assessed by an independent observer.

**Results**

One week follow up:

<table>
<thead>
<tr>
<th>Group</th>
<th>New bone formation</th>
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<tbody>
<tr>
<td>Control (n=6)</td>
<td>2.9 mm$^2$ SD: 1.1</td>
</tr>
<tr>
<td>Low Dose (n=6)</td>
<td>3.1 mm$^2$ SD: 0.7</td>
</tr>
<tr>
<td>High Dose (n=6)</td>
<td>1.2 mm$^2$ SD: 0.6</td>
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Both the low dose group and the control group demonstrated similar amounts of periosteal new bone formation after one week, while the high dose group compared with the control group, had a statistically significant lower production of new bone; p < 0.01.

The macrophage count per mm$^2$ was at its lowest in the control group and at its highest in the high dose group. Comparing between the groups, all groups demonstrated significantly different amounts of macrophages per mm$^2$; p < 0.001

Statistical method: Wilcoxon two sample test.

**Discussion**

The results of the present study indicate that the early process of periosteal new bone formation during fracture healing, is unaffected by an ongoing treatment with low dose MTX. The slower progress of new bone formation in the HD group, demonstrates that MTX may have a negative influence on bone regeneration, when used in higher doses. The etiology behind the big differences in the abundance of macrophages, is not easily explained. The high number of macrophages in the LD and especially in the HD group, might be caused by a prolonged abundance of macrophages locally, increased recruitment or tissue injuries due to combined effects of MTX and surgery. To clarify these issues, further research will be needed.

In conclusion, the impact of low dose and especially high dose MTX on the inflammatory reaction in the soft tissues surrounding an ongoing fracture repair, is still unclear. However, the cells directly involved in the formation of new bone, seems to be unaffected by low dose MTX treatment.