Macromolecular Prodrug of Dexamethasone Preserves Bone in Inflammatory Arthritis: A Micro-CT Study

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Aims
Rheumatoid arthritis (RA) is an autoimmune disease characterized by a chronic inflammation of synovium, leading to joint destruction¹. Local and systemic inflammation favors periarthritis² and generalized osteopenia/osteoporosis³, producing deformity, laxity, functional disability and increased risk for fractures⁴,⁵, which is a hallmark of RA¹. It is well acknowledged that bone loss is a major and, as yet, unsolved problem in RA⁴.

Recently, a water soluble, arthrotropic macromolecular prodrug of dexamethasone (Dex) based on N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer conjugate (P-Dex) showed evidence of superior anti-inflammatory properties and sustained amelioration of inflammatory arthritis⁵. We specifically aimed to look into the efficacy of P-Dex in ameliorating periarthritis bone damage using micro-CT.

Method
Collagen-induced arthritis (CIA)⁶ was induced in DBA/1J mice (7~8 weeks) by immunizing the animals with type II collagen in Freund’s adjuvant⁷ on day(s) 0 and 21. Following the day 21 booster injection, the mice were regularly monitored for the paw inflammation. Administration of therapeutic agents was initiated only after a paw of an animal achieved a clinical score of 2. Mice with established arthritis were selected and randomized into four groups (5-6/group): healthy, saline (control), free Dex (2mg/kg/day for 30 days) and P-Dex (60mg/kg, single dose).

Mice were sacrificed 1 month post treatment and both the hind limbs were isolated and fixed. Each hind limb (ankle) was scanned and reconstructed into a 3D-structure using micro-CT (Skyscan 1172, Skyscan, Aartselaar, Belgium) with a voxel size of 4.8 μm. The X-ray tube voltage was 60 kV and the current was 170 μA, with a 0.5 mm thick Al filter. Exposure time was 530 ms. The X-ray projections were obtained at 0.7° intervals with a scanning angular rotation of 180° and six frames were averaged for each rotation. 3D reconstructions were performed using NRECON (Skyscan) software. For arthritic joint bone quality analysis, we specifically focused on calcaneus (heel) bone as it is spatially isolated from other bones of the ankle, is of sufficient size, is relatively uniform in density and is consistently involved in arthritis development⁸. The final analysis was done on a volume of interest (VOI) of 75 slides, for which a constant spherical (0.55mm X 0.55mm) region of interest (ROI) was defined and placed centrally in the trabecular region of calcaneus. Specific threshold settings were used and kept constant for all samples. The following parameters were measured and calculated: bone mineral density (BMD) of trabecular bone, bone volume (BV), bone volume to tissue volume or bone volume density (BV/TV), bone surface to tissue volume or bone surface density (BS/TV), trabecular number (Th. N), and trabecular separation (Tb. Sp). For nonmetric indices, trabecular pattern factor (Tb. Pf), structural mean index (SMI) and connectivity density (Conn.D) were measured. 3D images were reconstructed using CT-Vox and CT-Vol software (Skyscan), to produce a visual representation of the results.

Data is expressed as mean ± SD. Statistical analysis was done by one-way analysis of variance (ANOVA) with Bonferroni/Tamhane’s T2 test as post-hoc test. Difference between
groups, healthy, Dex and P-Dex vs saline group is indicated as significant (p < 0.05) or non-significant (NS). For statistically significant groups, (*) denotes p<0.05 whereas (**) represents p<0.001 between groups.

Results

To evaluate the treatment effects on inflammation and disease progression, clinical scores and paw thickness were evaluated and found to be significantly decreased in Dex and P-Dex groups vs saline group. P-Dex was found to be significantly better in controlling inflammation and disease progression when compared to the Dex group (data not shown).

We next assessed the effects of treatment on BMD using micro-CT. It was found that BMD was significantly higher in mice treated with Dex (0.30 ± 0.04 g/cm³, p=0.012) and P-Dex (0.33 ± 0.07 g/cm³, p=0.001) vs saline (0.20 ± 0.09 g/cm³) (Fig.1a and 1c). BV/TV (%) also differed significantly between saline (18.27 ± 7.18) vs Dex (26.57 ± 3.25, p=0.004) and P-Dex (28.63 ± 4.99, p<0.001) (Fig. 1b, 1c and 2b).

Bone surface density (BS/TV) was used to measure surface roughness/erosion, which is a key pathogenic effect in RA leading to joint fusion and deformity. Untreated saline (14.17 ± 4.78 mm⁻¹) group showed significant erosion/roughness vs healthy (21.62 ± 1.78 mm⁻¹, p=0.004) group. Treatment groups, Dex (20.06 ± 2.13 mm⁻¹, p=0.022, +42%) and P-Dex (21.15 ± 1.97 mm⁻¹, p=0.007, +49%) groups showed significant preservation of bone and joint integrity vs saline group (Fig. 2 and 3). The preserved density and volumetric changes, in the treatment groups, can be attributed to the trabecular structural differences, i.e. increase in Tb.N and decrease in Tb.Sp, as shown in Fig.3b, 3c and 3e. For mean Tb.N, significant difference were found in Dex (4.90 ± 0.49 mm⁻¹, p=0.021, + 46%), P-Dex (5.22 ± 0.67 mm⁻¹, p=0.006, + 56%) vs saline (3.35 ± 1.26 mm⁻¹). Tb.Sp differed among the groups, Dex (0.12 ± 0.014 mm, p=0.069 (NS), - 20%) and P-Dex (0.11 ± 0.014 mm, p=0.036, - 27%) vs saline (0.15 ± 0.036 mm ) (Fig.2b and 3).

Figure 1. Density and volumetric Indices using micro-CT. (a) and (b) Graphical representation of BMD and BV/TV differences between the groups. (c) Similar density and volumetric differences are visualized in images from Dataviewer (coronal, transaxial and sagittal planes) using color coded bar, between the groups.
Figure 2. 3D-reconstructed images of the calcaneus using CT-Vol. (a) Note the eroded joint and bone surface in the untreated group which is reverted back to normal in treatment groups. (b) 3D representation of the trabecular bone inside the calcaneus.

Figure 3. Structural differences between groups. (a), (b) and (c) show graphical representation of differences between groups for BS/TV, Tb.N, and Tb.Sp respectively. (d) Images from CT-Vox between the groups. Note the increased porosity, eroded surface and exaggerated periosteal reaction of the untreated group. (e) 3D coronal cuts using CT-Vol, to have a visual depth perception of bony architecture inside the calcaneus.

In order to clarify the validity of the parameters calculated by micro-CT, we evaluated the correlation coefficients among micro-CT parameters, and found them to be significantly correlated (Table 1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BMD</th>
<th>BS/TV</th>
<th>Tb.N</th>
<th>Tb.Sp</th>
</tr>
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<tbody>
<tr>
<td>BV/TV</td>
<td>$r=0.900$, $p&lt;0.001$</td>
<td>$r=0.927$, $p&lt;0.001$</td>
<td>$r=0.954$, $p&lt;0.001$</td>
<td>$r=-0.887$, $p&lt;0.001$</td>
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<td>BMD</td>
<td>$r=0.916$, $p&lt;0.001$</td>
<td>$r=0.939$, $p&lt;0.001$</td>
<td>$r=-0.867$, $p&lt;0.001$</td>
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<tr>
<td>BS/TV</td>
<td>$r=0.950$, $p&lt;0.001$</td>
<td>$r=-0.969$, $p&lt;0.001$</td>
<td></td>
<td></td>
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<tr>
<td>Tb.N</td>
<td>$r=-0.951$, $p&lt;0.001$</td>
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Table 1. Correlation between micro-CT parameters. Pearson Product Moment Correlation ($r$) between parameters, all groups included ($n=41$).

BMD has limitations as a surrogate parameter of bone strength and risk of fragility fracture whereas Tb.N (increase) and Tb.Sp (decrease) alone cannot define all the
volumetric changes. To look for other surrogate parameters of bone strength, fracture risk and volumetric changes, we calculated nonmetric parameters using micro-CT (Table 2).

<table>
<thead>
<tr>
<th></th>
<th>Healthy (n=10)</th>
<th>Saline (n=10)</th>
<th>Dex (n=11)</th>
<th>P-Dex (n=10)</th>
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<tr>
<td>Tb.Pf (mm⁻¹)</td>
<td>3.24±3.65*</td>
<td>16.90±10.24</td>
<td>7.62±3.44</td>
<td>5.85±3.87*</td>
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<tr>
<td>SMI</td>
<td>0.90±0.315*</td>
<td>1.63±0.572</td>
<td>1.12±0.210</td>
<td>0.98±0.210*</td>
</tr>
<tr>
<td>Conn.D (mm⁻³)</td>
<td>581.59±87.46*</td>
<td>369.51±128.75</td>
<td>586.66±208.35**</td>
<td>508.81±117.91**</td>
</tr>
</tbody>
</table>

Table 2. Nonmetric Indices. Data expressed as mean ± SEM. Asterix (p<0.05) and double asterix (p<0.001) represent difference between groups, healthy, Dex and P-Dex vs saline group.

Trabecular microarchitecture was measured using Tb.Pf and SMI. Tb.Pf represents contour of the trabecular surface, the ratio of convex to concave surfaces. Thus, larger Tb.Pf values indicate that the convex³ (eroded) contour dominated the structure. Our results indicate a decrease of 55% (NS) (p=0.113) in Tb.Pf with Dex group in comparison to 65% (p=0.048) decrease by P-Dex vs saline. SMI represented the plate-rod characteristics of trabecular structure. Larger SMI values indicate that trabecular structure contained a more rod-like structure³ (osteopenic/osteoporotic). Dex showed 31% (NS) (p=0.122) decrease in SMI in comparison to 40% (p=0.035) decrease by P-Dex vs saline. The results seem to suggest P-Dex offers better protection of trabecular microarchitecture when compared to Dex group.

Conn.D is defined as the number of trabecular elements that may be removed without separating the trabecular network. Loss of connectivity due to perforation/removal of trabeculae decreases modulus twice as much as by trabecular thinning (i.e. increase in Tb.Sp). Therefore it is very important in defining bone fragility³. Our results showed both P-Dex and Dex treatment provide statistically higher Conn.D than saline group, suggesting stronger bone mechanical properties. To validate the result, we calculated the correlation co-efficient (r) between Conn. D and Tb. Sp and found them to be negatively correlated with significance (r = -0.760, p<0.001).

Though no significant differences were found between Dex and P-Dex groups, using micro–CT data, the overall general trend suggests a better bone protecting benefit of P-Dex. It should also be taken into consideration that even though Dex was given daily and P-Dex only once throughout the treatment, P-Dex seems to result in better bone preservation (though not statistically significant) in comparison to Dex. To further explore the difference between Dex and P-Dex, we are using micro-CT to analyze bone quality at peripheral site (e.g. femoral head), which may reveal the potential reduced skeletal side effect of P-Dex comparing to Dex.

Conclusion

The prevention of joint damage is a key goal of treatment for RA¹. Quantitative as well as qualitative micro-CT results from this study showed that the dexamethasone prodrug (P-Dex, single dose) could not only provide sustained amelioration of the joint inflammation in an inflammatory arthritis model but also preserves joint bone quality. Different from regular dexamethasone, the prodrug could target the inflammatory joints and therefore has the potential to reduce the off-target toxicities of glucocorticoids such as systemic osteopenia. In this study, micro-CT was proved to be a very effective tool in evaluation of the therapeutic benefits of the novel prodrug developed.

References:
1. Le Goff B, Soltner E, Charrier C, Maugars Y, Rédini F, Heymann D, Berthelot JM, "A combination of methotrexate and zoledronic acid prevents bone erosions and...


