Effects of different corticosteroids on the development of osteonecrosis in rabbits

K. Miyanishi¹, T. Yamamoto¹, T. Irisa¹, G. Motomura¹, S. Jingushi¹, K. Sueishi² and Y. Iwamoto¹

Objectives. Osteonecrosis (ON) of the femoral head is a devastating complication occurring in patients receiving corticosteroid treatment. This study examined the effect of three corticosteroids on the development of ON in rabbits.

Methods. Thirty-nine rabbits were injected once intramuscularly with either 25 mg/kg prednisolone sodium succinate (PSL; 13 rabbits), 20 mg/kg methylprednisolone acetate (MPSL; 13 rabbits) or 20 mg/kg triamcinolone acetonide (TR; 13 rabbits). Four weeks after corticosteroid injection, the bilateral femora and humeri were examined histopathologically for the presence of ON. Haematological examinations were performed before and after corticosteroid injection.

Results. MPSL treatment (17/26 proximal femora, 65%) significantly increased ON incidence in the proximal femora compared with the levels seen after TR (4/26, 15%) or PSL (3/26, 12%) treatment (P < 0.01). Although not significantly increased in comparison with rabbits receiving PSL treatment (1/26 proximal humeri, 4%), ON incidence within the proximal humeri was significantly increased in MPSL-treated rabbits (6/26, 23%) in comparison with those seen in rabbits receiving TR (0/26, 0%) treatment (P < 0.05). Serum levels of cholesterol, triglyceride and free fatty acid were significantly higher 1, 2 and 4 weeks after corticosteroid treatment in rabbits treated with MPSL relative to rabbits receiving TR and rabbits with PSL treatment (P < 0.05).

Conclusions. MPSL treatment significantly increased ON incidence in rabbits over levels seen after TR or PSL treatment.

Key words: Osteonecrosis, Corticosteroid, Rabbit, Hip.

Materials and methods

A rabbit model of corticosteroid-induced ON was used in this study [8]. Administration of a single high dose (20 mg/kg) of MPSL, simulating a dose of human steroid pulse therapy, was used to cause ON lesions reproducibly in this model [8]. All experiments, after review by the Common Ethics Committee for Animal Experiments at Kyushu University, were conducted in accordance with the Guidelines for Animal Experiments of Kyushu University, Law no. 105, and the notification (no. 6) by the government and the Committee on Ethics in Japan.

Animals

Adult (defined as having the growth plate already closed) male Japanese white rabbits (Kyudo, Tosu, Japan), weighing 3.0–4.0 kg, were housed at the Animal Center of Kyushu University and maintained on a standard laboratory diet and water. Rabbits ranged in age from 28 to 32 weeks.

Treatment

Thirty-nine rabbits were injected once intramuscularly with 25 mg/kg body weight PSL sodium succinate (Shionogi, Osaka, Japan; 13 rabbits), 20 mg/kg body weight MPSL acetate (Upjohn, Tokyo, Japan; 13 rabbits) or 20 mg/kg body weight TR acetonide (Bristol-Myers Squibb, Tokyo, Japan; 13 rabbits) into the right gluteus medius. The corticosteroid doses used here were...
determined to give equal glucocorticoid activities for each administration based on previously reported relative potencies (PSL:MPSL:TR = 4:5:5) [5]. Intramuscular injection of physiological saline was previously shown to produce no ON lesions in rabbits [8] and was not performed in this study.

**Tissue preparation**

Four weeks after corticosteroid injection, animals were anaesthetized by intravenous injection of pentobarbital sodium (25 mg/kg body weight; Abbott, Chicago, IL, USA), then killed by exsanguination via aortectomy. For examination by light microscopy, both femora and humeri (a total of four bone samples per rabbit) were obtained at the time of death and fixed for 1 week in 10% formalin, 0.1 M phosphate buffer (pH 7.4). Bone samples were decalcified with 25% formic acid for 3 days, then neutralized with 0.35 M sodium sulphate for 3 days. Samples were sectioned along the coronal plane for the proximal one-third and cut along the axial plane in the distal part (condyle). Lastly, specimens were embedded in paraffin, cut into 4-μm sections, and stained with haematoxylin and eosin.

**Evaluation of ON**

The entirety of the proximal one-third and distal condyles of both femora and humeri (a total of eight regions) were examined histopathologically for the presence of ON. A diagnosis of ON was made in blinded fashion by three of the authors (K.M., T.Y., T.I.), based on the diffuse presence of empty lacunae or pyknotic nuclei of osteocytes within the bone trabeculae, accompanied by surrounding bone marrow cell necrosis on the basis of published criteria for rabbit ON [8, 10]. A proximal or distal part of a bone is considered necrotic if it contains an ON lesion.

**Haematological examination**

Blood samples were obtained from fasting rabbits prior to experimentation (time 0) and 48 h and 1, 2, 3 and 4 weeks after corticosteroid injection. We examined the blood levels of cholesterol, triglycerides, free fatty acid and platelets.

**Statistical analysis**

Numbers of proximal or distal parts of femora and humeri with ON lesions were compared using the $\chi^2$ test with Bonferroni methods for multiple comparisons. Haematological data obtained at each time point were compared using Bonferroni tests. Statistical analyses were performed using StatView J-5.0 software (SAS Institute, Cary, NC, USA). $P$ values <0.05 were considered to be significant.

**Results**

**Macroscopic and histopathological features**

Macroscopically, regions exhibiting ON appeared as yellowish-coloured areas within the bone. Histologically, ON lesions exhibited an accumulation of bone marrow cell debris and the appearance of bone trabeculae with empty lacunae (Fig. 1). These findings were consistent for all osteonecrotic tissues. Little

![Fig. 1. Histological features of osteonecrosis in rabbits. Bone trabeculae demonstrate empty lacunae. The surrounding bone marrow tissue contains necrotic bone marrow cell debris. Little reparative tissues are observed in rabbits receiving MPSL treatment (A), while the osteonecrotic lesions are accompanied by adjacent reparative processes, including aggregation of macrophages and fibrous tissue invasion in rabbits treated with PSL (B). Haematoxylin and eosin, original magnification ×200.](https://academic.oup.com/rheumatology/article-abstract/44/3/332/2899362)
receiving TR treatment (triglyceride and free fatty acid between rabbits receiving PSL and TR. There was no significant difference in serum levels of cholesterol, triglyceride and free fatty acid between rabbits treated with MPSL and rabbits receiving TR (Fig. 2B). Significantly higher levels of free fatty acid were demonstrated in rabbits treated with MPSL in comparison with rabbits treated with PSL or TR (P<0.01) (Fig. 2D).

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Number of femora</th>
<th>Proximal (%)</th>
<th>Distal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSL</td>
<td>26</td>
<td>3 (12%)</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>MPSL</td>
<td>26</td>
<td>17 (65%)</td>
<td>8 (31%)</td>
</tr>
<tr>
<td>TR</td>
<td>26</td>
<td>4 (15%)</td>
<td>2 (8%)</td>
</tr>
</tbody>
</table>

*P<0.01 vs PSL and TR.

Table 2. Prevalence and location of osteonecrosis of the humerus in rabbits

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Number of humeri</th>
<th>Proximal (%)</th>
<th>Distal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSL</td>
<td>26</td>
<td>1 (4%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>MPSL</td>
<td>26</td>
<td>6 (23%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>TR</td>
<td>26</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

*P<0.05 vs TR.

Prevalence and location of ON

Prevalence and location of ON are shown in Tables 1 and 2. MPSL treatment significantly increased ON incidence in the proximal femur over the levels observed for TR or PSL treatment (Table 1) (P<0.01). No significant differences, however, were observed in ON incidence of the proximal femur in rabbits treated with PSL and TR. Incidence of ON in the distal femur was not significantly different among the treatment groups (Table 1).

While the number of proximal humeri with ON lesions in PSL-treated rabbits did not change significantly when compared with rabbits receiving MPSL or TR treatment, the number of necrotic proximal humeri significantly increased in rabbits receiving MPSL in comparison with rabbits receiving TR treatment (Table 2) (P<0.05). No ON lesions were observed in the distal humeri in any of the treatment groups (Table 2).

Haematological examination

Serum cholesterol levels were significantly higher in rabbits treated with MPSL than in rabbits with PSL treatment (P<0.01 at 1 and 2 weeks; P<0.05 at 3 weeks) and rabbits receiving TR (P<0.01 at 1, 2 and 3 weeks; P<0.05 at 4 weeks) (Fig. 2A). Serum levels of triglyceride were significantly higher in rabbits treated with MPSL than in rabbits with PSL treatment (P<0.01 at 1, 2 and 4 weeks) and rabbits receiving TR (P<0.01 at 1 and 2 weeks; P<0.05 at 4 weeks) (Fig. 2B). Significantly higher levels of free fatty acid were observed in MPSL-treated rabbits than in rabbits treated with PSL (P<0.01 at 1, 2 and 4 weeks; P<0.05 at 3 weeks) and rabbits receiving TR treatment (P<0.01 at 1, 2, 3 and 4 weeks) (Fig. 2C). There was no significant difference in serum levels of cholesterol, triglyceride and free fatty acid between rabbits receiving PSL and TR treatment at any time point tested.

Platelet numbers were decreased from pretreatment levels 1 week after corticosteroid injection in all treatment groups. The platelet levels were significantly higher in rabbits receiving PSL treatment than in rabbits with MPSL treatment and rabbits receiving TR at 1 (P<0.05) and 2 (P<0.01) weeks following corticosteroid treatment. At 3 and 4 weeks, levels of platelets were significantly lower in rabbits treated with MPSL in comparison with rabbits with PSL treatment and rabbits receiving TR (P<0.01) (Fig. 2D).

Discussion

In this study, we adjusted three variables—glucocorticoid activity, biological half-life and administration method—to exclude potential experimental biases. Glucocorticoid activity generally includes anti-inflammatory, anti-allergic and immunosuppressive effects [5]. Corticosteroid doses were determined to result in each administration producing equal glucocorticoid activities, based on their reported relative potencies (PSL:MPSL:TR = 4:5:5) [5]. In addition, PSL, MPSL and TR are all intermediate-acting corticosteroids with similar biological half-lives (12–36 h) [11]. These drugs were injected intramuscularly according to the original rabbit ON model [8].

To date, there have been no reports comparing the incidence of human ON according to the different corticosteroid compounds used. Intra-articular TR hexacetonide for juvenile rheumatoid coxitis did not increase the risk of ON [12]. There is a case report in which depot corticosteroid injections for hay fever that included TR and MPSL caused ON [13]. However, to our knowledge, there seem to be no or few adult ON cases which have proved to be caused by systemic administration of TR alone. This is consistent with the low ON incidence in rabbits treated with TR in our study. Although the results showed a lower incidence of ON in rabbits treated with PSLC than in those treated with MPSL, there are studies reporting on ON cases treated with MPSL or PSLC [3, 4, 14, 15]. The occurrence of clinical ON may be influenced by other factors, such as differences in administration methods, corticosteroid doses and underlying diseases [3, 4, 16].

It is difficult to draw a conclusion for human ON from this rabbit experiment due to the interspecies differences. On the basis of the results, we speculate that ON development in humans depends on the type of corticosteroid used; use of an optimal type of corticosteroid would be beneficial for patients with a high risk of ON. This hypothesis may be supported by the presence of several similarities between human and rabbit ON. First, increased lipid deposition and a rise in intraosseous pressure were reported in ON of both species [17–19]. Secondly, histological features of empty lacunae accompanied by surrounding marrow cell necrosis were shared in human and rabbit ON [8, 20]. Thirdly, ON is multifocal in both species [1, 8]. Fourth, haematological risk factors representing prominent lipid transport to the peripheral tissues were reported in humans and rabbits ON [10, 21].

Small differences in the structure of cortisol and its synthetic analogues result in remarkable differences in drug potency and duration of action [22]. All corticosteroid compounds have a common carbon skeleton. Addition of a 1,2 double bond to the corticosteroid nucleus creates PSL. The 6a-methylation (MPSL) or 9a-fluoride addition with 16-hydroxylation (TR) provides less salt-retaining activity but more glucocorticoid potency [22]. In this study, MPSL treatment produced striking findings differing significantly from those seen following PSL and TR treatment, including a high ON incidence. The precise mechanism for this difference is unknown. The 6a-methylation is the feature unique to MPSL, which may mediate long-term cytolysis [23]. Our observations may also be attributable to this component.

Differential binding capacities of the corticosteroid for albumin- and/or corticosteroid-binding globulin (CBG) may be another possible explanation for the high ON occurrence. Albumin and CBG are the primary corticosteroid-binding proteins that transport cortisol; free (unbound) cortisol is the active molecular form [22, 24]. Decreased protein binding in patients with liver disease and hypoalbuminaemia results in the major...
side-effects of corticosteroids [25], while increased protein binding may limit the bioactivity of corticosteroids in patients with Crohn’s disease [26]. Furthermore, the binding capacity of CBG to MPSL is lower than that to PSL [27]. The decreased binding capacity of CBG to MPSL may increase the free fraction of MPSL, resulting in increased ON incidence.

Coagulation abnormalities and hyperlipidaemia are among the postulated pathogenic mechanisms for ON development [1, 2, 8, 10, 14, 15, 21, 28–30]. Ischaemic events may result from vascular interruption through thrombi, lipid emboli or high intraosseous pressure associated with bone marrow fat-cell enlargement; these processes would subsequently lead to ON development [1, 2, 8, 10, 14, 15, 21, 28–30]. In this study, the platelet levels decreased at 1 week following corticosteroid treatment, suggesting a hypercoagulable plasma state due to increased platelet consumption. The platelet numbers recovered to pretreatment levels at 2 and 3 weeks in rabbits receiving PSL and TR treatment, respectively. However, the platelet levels did not reach pretreatment levels until at least 4 weeks after treatment in MPSL-treated rabbits (Fig. 2D). Significantly increased lipid levels were also observed following MPSL treatment. These data therefore suggest the presence of a hypercoagulable and hyperlipidaemic state of plasma in rabbits treated with MPSL compared with levels seen after PSL or TR treatment. Further study will be needed to clarify the mechanisms for steroid-induced ON, including morphological changes of bone marrow fat cells, the formation of thrombus and fat emboli, and expression of lipid- or coagulation-related genes in the marrow tissues [1, 2, 8, 10, 14, 15, 21, 28–30].

In conclusion, this study demonstrated that MPSL treatment significantly increased the incidence of ON in rabbits, in association with elevated lipid levels, from that observed for PSL or TR treatment. These results suggest that the type of corticosteroid given may be an important component determining human ON development.

Acknowledgements

This work was supported in part by a Grant-in-Aid for JSPS Fellows, a Grant for Intractable Diseases from the Ministry of Health and Welfare of Japan and a Grant from Uehara Memorial Foundation.

Rheumatology

Key messages

- Development of osteonecrosis in rabbits depended on the type of corticosteroid given.
- Use of an optimal type of corticosteroid may be beneficial for patients with a high risk of osteonecrosis.

Fig. 2. Sequential changes in the levels of cholesterol (A), triglycerides (B), free fatty acid (C) and platelets (D) in rabbits treated with PSL, MPSL or TR. *P < 0.05 or 0.01, MPSL vs PSL and TR; **P < 0.05, MPSL vs TR; ***P < 0.05 or 0.01, PSL vs MPSL and TR.
The authors have declared no conflicts of interest.

References