CHAPTER-III
HISTOPATHOLOGICAL AND RADIOLOGICAL EVALUATION OF EFFECT OF SMN AND CLX+SMN
Introduction

Osteoarthritis (OA) is a common chronic inflammatory disease affecting joints, which leads to disability, and pain mainly in weight bearing joints (Corti et al., 2003). Research findings from in vivo, in vitro human experiments, as well as animal models demonstrated that the pathogenesis of OA is multi-factorial, with aspects of local inflammation, joint stability, genetic predisposition, cartilage degeneration and bone remodeling in the affected joint (Felson, 2004). Diagnostic imaging has played an essential role in the study of OA pathogenesis. Radiographs have long served as the standard imaging study material for assessing OA (Felson et al., 1995). The most available and cost effective tool for diagnosing of OA is based on clinical orthopedic examination and X-ray radiology (Hart et al., 1991). Cartilage loss and joint space narrowing (JSN) are one of the critical radiographic signs (Resnick et al., 1995). Systems for evaluating radiographic OA such as Kellgren and Lawrence focus on osteophytosis and JSN (Kellgren et al., 1957).

Microscopic examination of tissue (histopathology) is the primary method to study the common histological changes of OA including: loss of cartilage surface, increased cell number, loss of proteoglycans, vascular invasion of calcified cartilage, clefts, surface fibrillation, chondrocyte cloning, erosion, and osteoclasts (Little et al., 1997).

To reassure tissue morphology in arthritis, microscopic evaluation using standard histochemical stainings are available. Safranin-O staining is one of the most preferred methods of staining instead of Toluidine Blue and and Hematoxyline-Eosin in many histology study of OA (Mitchell et al., 1976; Kiviranta et al., 1985; Wakitani et al., 1994; Mollenhauer et al., 2002; Okamura et al., 2010). It has been shown that Safranin-O retain and precipitate the proteoglycans in cartilage (Szirmai, 1963).

The microscopic inspection of OA cartilage was observed in experimentally induced OA in rat as an animal model. In order to study the pathogenesis of this disease and to evaluate the effectiveness of SMN and SMN+CLX on experimental groups, we evaluated X-ray images from tibia femoral (TB) angle of right knee joints and histopathological examination were done on the sagittal sections of cartilage following Safranin-O staining. The JSN and other morphological changes in bone were based on semi-quantitative scoring system adopted form Kellgren-Lawrence (KL) classification grades.
Materials and methods

Experimental design

Following a week of acclimation, the animals were assigned into five groups. The groups consist of control (treated with normal saline), animals with OA, treated with normal saline (OA⁺); OA⁺ treated with CLX (100 mg/kg b.w., orally), OA⁺ treated with SMN (50 mg/kg b.w., orally) and OA⁺ treated simultaneously with CLX and SMN (100 and 25 mg/kg, respectively). The dose levels were selected based on our previously study (Malekinejad et al., 2012). Animals received saline normal and/or test compounds via gastric gavages for 14 days.

OA induction

Osteoarthritis was induced according to Kalbhen method (Kalbhen, 1987). The detailed procedure of OA induction is given in the material and methods section chapter-II.

Safranin-O staining kit

Safranin-O staining kit was purchased from Asian chemistry company, Tehran, Iran.

The commercially available kit contained the following reagents: Hematoxyline Weigert solution, Fast Green solution, Safranin solution, Acid-alcohol solution, and Acetic acid solution.

Staining procedure: The staining procedure was followed as specified by the company. In brief, the slides were stained in Weigert’s iron hematoxylin solution for 4 min followed by destain in fresh acid alcohol solution for 4 min. The slides were thoroughly rinsed with tap water for 20 sec and stained in fast green solution for 3 min. After washing the slides in acetic acid solution for 30 sec, the slides were immersed in aqueous Safranin-O for 5 min. The dehydration procedure was carried out using 95 % methanol (2 x 5 min), 100 % methanol (3 x 5 min), and xylene (3 x 5 min). Following dehydration the slides containing stained sections were mounted using cover slips with the help of synthetic resin. The slides were allowed to dry overnight. The histology was observed using light microscope. The slides were analyzed by experienced pathologist.
Histopathological evaluation was performed on the sagittal sections of cartilage. Tissue samples that previously (24 h before) had been stored in 10% phosphate-buffered formalin, decalcified by solution containing 10% formalin and 10% formic acid. The tissues were embedded in paraffin and 5–6 µm sections were cut using a rotary microtome and stained with specific cartilage staining technique of Safranin-O.

The severity of OA lesions were graded on a scale of 0 to 3+, using parameters such as the presence of fibrillation on cartilage surface, density of osteoclasts, inflammatory cells infiltration, thickness of the cartilage layers on two epiphysial heads, connective tissue accumulation, and the destruction rate on cartilage surface of epiphyses. For each animal in the test and control groups at least three slides from each joint region were prepared and the averages of scored marks were analyzed.

**X-ray radiological analysis**

Before euthanizing the animals with pentobarbital sodium, X-rays were taken at the knee joint of animals for evaluating the bone and cartilage damage. Radiographs were taken using X-ray apparatus (Siemens-60MA, Germany) and industrial X-ray film (Kodak Diagnostic film). The X-ray apparatus was operated at 220V with a 40V peak, 0.2 sec exposure time, and a 60 cm tube-to-film distance for femur-tibia projection. The radiographic images were examined by experienced radiologist. We have evaluated the X-ray images from the tibia femoral (TB) angle of the right knee joints of control, animals with MIA induced arthritis and arthritis animals treated with SMN, SMN+CLX and CLX. The joint space narrowing (JSN) and other morphological changes in bone were classified based on the Kellgren-Lawrence (KL) classification grades.

According to the KL classification grades, the different stages of OA severity were classified as (0 = normal; 1 = possible narrowing of the joint space, possible osteophytes; 2 = small osteophytes, narrowing of the joint; 3 = multiple, moderately sized osteophytes, definite joint space narrowing, some sclerotic areas, possible deformation of bone ends; 4 = severe case; multiple osteophytes, severe joint space narrowing, bone deformity (Lawrence, 1964).
Statistical Analysis

For comparing the graded degree of pathological findings between groups, the Kruskal–Wallis test was used. A $p$ value < 0.05 was considered significant.
Results

The histopathological examinations with special staining technique of Safranin-O revealed that all examined sections in control groups were normal and no signs of cartilage destruction and infiltration of inflammatory cells were observed (Fig. 1A). In the sham group however, fibrillation and destruction of articular cartilage with presence of osteoclasts in margins of destructed areas, infiltration of lymphocytic inflammatory cells and increase of connective tissue deposits were observed ($p < 0.05$) (Fig. 1B). In the CLX treated animals no inflammatory cells infiltration could be seen and the severity of the cartilage injuries were significantly declined (Fig. 1C). The OA positive animals which were treated with SMN showed remarkable reduction in the number of inflammatory cells infiltration and a normal thickness of the cartilage surfaces were observed ($p < 0.05$) (Fig. 1D). Interestingly, the animals which received both CLX and SMN showed significantly ($p < 0.05$) almost no sign of inflammation and histological feature were similar to those of the control group (Fig. 1E). A normal cartilage thickness, normal synovial space with no destructive debris, a smooth cartilage surface with no articular fibrillations and no extra connective tissue depositions were manifested. The severity of histopathological lesions as scored numerical data is represented in table 1.

All examined images of the control groups by the help of specialized radiologist were revealed to be normal and no signs of joint space narrowing, osteophyte formations were observed (Fig. 1A). Joint spaces narrowing with KL3 grade and sclerosis with cortical/articular surface irregularity of 45 %, multiple osteophytes of 50 % were observed in the MIA injected OA group (Fig. 1B, 1C). In the CLX-treated animals no sclerosis was observed and the severity of joint space narrowing declined from KL3 to KL2 (Fig. 1D). The OA positive animals which were treated with SMN showed remarkable reduction in joint space narrowing and reduction from KL3 to KL1 of 50 % and a normal thickness of the cartilage surfaces was observed (Fig. 1E). In the SMN+ CLX treated animals significant reduction from KL3 to KL1 and KL0 grade of 66 % and normal joint space narrowing was observed (Fig. 1F). The severity of radiological features as percentage data are represented in table 2.
Discussion

It is known that higher doses of MIA (up to 3 mg) injection can cause cartilage erosion, and subchondral bone sclerosis (Guingamp et al., 2005). In OA cartilage, a higher percentage of the proteoglycans exist in a nonaggregated form, unbound to hyaluronate. With increased degeneration of the cartilage, fibrillation is first observed at the articular surface then extends down into the cartilage and the expression of pro-inflammatory mediators, mononuclear cell infiltration, fibrillation so-called ulcerated cartilage were also over expressed (Butlough et al., 2004; Benito et al., 2005). Further histologically, an increased number of lining cells and a mixed population of inflammatory cells has been shown in OA (Farahat et al., 1993).

In the present study, we documented histological changes in the cartilage at post-MIA injection, including loss of proteoglycans, cartilage fibrillation and osteoclasts, similar changes have been reported using other animal models of OA (Pritzker, 1994; Hayami et al., 2006). Previous studies have shown that the articular surface is smooth and the matrix is densely stained red with Safranin-O in the saline- injected normal rats groups (Yeh et al., 2008). In our study the normal groups and groups treated with combination of CLX and SMN showed a similar morphology. Evidence of changes consisted of increased osteoclastic and osteoblastic activity in the cartilage subjacent to the areas of cartilage loss and degeneration after injection of MIA was obvious (Dunham et al., 1993). Whereas in SMN treated animals the thickness of cartilage was normal. CLX is chosen as a reference treatment for this study (McKenna et al., 2001). In the CLX treated animals no significant increase in inflammatory mediators were observed (Fig. 2).

X-ray analysis could serve as an early marker of changes in the mechanical properties of cartilage and underlying bone. Radiographic evaluation is an important method for the assessment of structural change in OA (Dieppe, 1995). Classification of radiographic images of the knee OA was determined with regard to the grade of JSN, osteophytes formation in the joint margins, and subchondral sclerosis present in the radiographs. On the basis of these features, the Kellgren & Lawrence (KL) (Petersson et al., 1997) and Ahlback scores (Ahlbäck, 1968) were allotted for classification. Both of these methods are accurate and have been used frequently for scoring radiographs. The Kellgren-Lawrence (KL) scale (Deshmukh et al., 2011) was also accepted by the
World Health Organization as the reference standard for progression of OA. Previous reports have stated that changes in the articular cartilage in the medial tibiofemoral compartment were less marked, even where there was a linear correlation between mean cartilage volume and radiographic JSN (Cicuttini et al., 2003).

The results of our radiological analysis showed the severity of disease with X-ray pattern of JSN in arthritis group, whereas SMN treated groups show 50% improvement in JSN. The CLX treatment group demonstrated 35% improvement. Supporting our previous histological observations, the combined treatment groups which were administrated with both CLX and SMN showed 70% improvement in JSN (Fig.1). The report justifies the importance of X-ray analysis in monitoring the progression of arthritis and the significant effect of SMN and CLX treatment in prevention of OA damage in the joint. These results were further supported by our histopathology studies (Fig. 2).

To conclude, our results demonstrate that SMN and combination of SMN and CLX treatment in arthritis reduced JSN and prevented the morphological changes in bone. The above effects were very clearly observed using X-ray images and further reduced signs of inflammation in cartilage was observed by histology. Therefore, combination of SMN and CLX treatment can be considered as effective strategy for clinical treatment of OA.
Fig.1. Radiographic representation of the joints; (A): The normal knee joint of the control group, (B and C): The MIA injected OA group, OA is characterized by the joint space narrowing (arrows) and sclerosis with cortical/articular surface irregularity (star), (D): The CLX-treated group knee joints with minor joint space narrowing (arrow), (E): Represents the SMN-treated group with normal thickness of the cartilage surface (arrow) and (F): The CLX + SMN-received animals with normal joint space.
Fig.2. Photomicrographs of rat’s articular cartilage; (A): control group; articular cartilages with normal thickness and orange red tonality (arrow) without inflammatory cells and additional connective tissue in synovial space. Safranin-O, 40-X (B): sham group; destructed and fibrillated surface of cartilage (black arrow) and cellular debris on it (arrowhead) with increase of connective tissue as villous like formation (asterisk) and presence of multinucleated osteoclasts (small arrows) in
specified region by white arrow, Safranin-O, 40- X (main panel), 400-X (magnified figure) (C): CLX-treated group; slight focal destructive changes in articular cartilages and replacement of mild connective tissue in the same places (asterisk). Safranin-O, 40-X (D): SMN-received group; healing of articular cartilage and regaining of regular thickness with low tonality in new cartilaginous tissue (arrow), Safranin- O, 40-X (E): CLX + SMN-treated group; normal articular cartilages with more tonality in comparison with SMN group with no inflammatory reaction and extra connective tissue, Safranin-O, 40-X.
Table 1

Pathological findings in the articular cartilage following OA induction and various regimens of treatment, data represents mean ± SEM (n = 8 in each group).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Fibrillated surface</th>
<th>Increase of connective tissue</th>
<th>Presence of multinucleated osteoclasts</th>
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<tr>
<td>C</td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>OA&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.6 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8 ± 0.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.4 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>OA&lt;sup&gt;+&lt;/sup&gt;CLX&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.4 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.6 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.2 ± 0.3&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>OA&lt;sup&gt;+&lt;/sup&gt;SMN&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.2 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.2 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.6 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>OA&lt;sup&gt;+&lt;/sup&gt;SMN&lt;sup&gt;+&lt;/sup&gt;CLX</td>
<td>0.4 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.6 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.4 ± 0.2&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>abc</sup> Values in same column with different superscripts differ significantly (p < 0.05).

C: control, OA<sup>+</sup>: animals with osteoarthritis and only were treated with saline normal, OA<sup>+</sup>CLX<sup>+</sup>: the rats which after OA induction were treated with CLX (100 mg/kg), OA<sup>+</sup>SMN<sup>+</sup>: the animals which following OA induction were treated with 50 mg/kg SMN, and OA<sup>+</sup>SMN<sup>+</sup>CLX: indicates the group of rats that after OA induction were administered both CLX (100 mg/kg) and SMN (25 mg/kg).
Table 2

Radiological findings of femur tibia knee joint following OA induction and treatment, all the values are represented in percentage (n=6 in each group).

<table>
<thead>
<tr>
<th>Group</th>
<th>KL0</th>
<th>KL1</th>
<th>KL2</th>
<th>KL3</th>
<th>Sclerosis</th>
<th>Presence of osteophyte</th>
</tr>
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<tbody>
<tr>
<td>C</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>OA^+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>45</td>
<td>50</td>
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<td>OA^+CLX^+</td>
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<td>20</td>
<td>70</td>
<td>0</td>
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<tr>
<td>OA^+SMN^+</td>
<td>50</td>
<td>50</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>OA^+SMN^+CLX</td>
<td>66</td>
<td>34</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
</tbody>
</table>

All the values are in percentage

C: control, OA^+: animals with osteoarthritis and only were treated with saline normal, OA^+CLX^+: the rats which after OA induction were treated with CLX (100 mg/kg), OA^+SMN^+: the animals which following OA induction were treated with 50 mg/kg SMN, and OA^+SMN^+CLX: indicates the group of rats that after OA induction were administered both CLX (100 mg/kg) and SMN (25 mg/kg).