Efficacy Test of Polycan, a Beta-Glucan Originated from *Aureobasidium pullulans* SM-2001, on Anterior Cruciate Ligament Transection and Partial Medial Meniscectomy-Induced-Osteoarthritis Rats

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The object of this study was to assess the efficacy of Polycan from *Aureobasidium pullulans* SM-2001, which is composed mostly of beta-1,3-1,6-glucan, on osteoarthritis (OA)-induced by anterior cruciate ligament transection and partial medial meniscectomy (ACLT&PMM). Three different dosages of Polycan (85, 42.5, and 21.25 mg/kg) were orally administered once a day for 84 days to male rats a week after ACLT&PMM surgery. Changes in the circumference and maximum extension angle of each knee, and in cartilage histopathology were assessed using Mankin scores 12 weeks after Polycan administration. In addition, cartilage proliferation was evaluated using bromodeoxyuridine (BrdU). As the result of ACLT&PMM, classic OA was induced with increases in maximum extension angles, edematous knees changes, and capsule thickness, as well as decreases in chondrocyte proliferation, cartilages degenerative changes, and loss of articular cartilage. However, these changes (except for capsule thickness) were markedly inhibited in all Polycan- and diclofenac sodium-treated groups compared with OA control. Although diclofenac sodium did not influence BrdU uptake, BrdU-immunoreactive cells were increased with all dosages of Polycan, which means that Polycan treatment induced proliferation of chondrocytes in the surface articular cartilage of the tibia and femur. The results obtained in this study suggest that 84 days of continuous oral treatment of three different dosages of Polycan led to lesser degrees of articular stiffness and histological cartilage damage compared with OA controls 91 days after OA inducement, suggesting that the optimal Polycan dosage to treat OA is 42.5 mg/kg based on the present study.

Keywords: Anterior cruciate ligament transaction, *Aureobasidium pullulans*, β-glucan, osteoarthritis

Osteoarthritis (OA) is the most prevalent articular disease in the elderly [10]. The process is characterized by changes in the structure and function of the articulation, mainly due to a degenerative process that takes place in the articular cartilage [42]. An understanding of OA etiopathology, however, has proven to be elusive [13]. Owing to the fact that OA affects nearly 70% of all people at some point in their lives, it has a major economic and social impact on patients and health care systems [4, 5, 12]. Consequently, there is a pressing need to develop disease-modifying OA drugs [1].

Before a disease-modifying OA drug can reach clinical trials, it must first be successful in preclinical trials. This requires animal models of OA in which specific aspects of drug efficacy in articular cartilage, subchondral bone, and other affected tissues can be examined, as well as potential side effects in other organs [18].

Rodent models of OA were first developed in the late 1970s in mice and rats [11, 38, 45, 49]. Initially, experiments employed models in which OA was induced in the temporomandibular joint [9, 35, 50], but subsequently models were developed in synovial joints, including the knee [38], using either a chemical method (intra-articular injection of, for instance, papain [33] or sodium iodoacetate [28]) or a surgical method (structural alteration to the tendons, muscle, or ligaments [3, 34, 37]). A review by Schwartz [46] in 1987 summarized these early developments. Other models developed since then rely on genetic predisposition or engineering to stimulate OA pathology. However, genetic models may require a long time for OA to develop, and there is often considerable variability between animals (e.g., in the temporal dynamics of OA progression). Disease progression in surgical models is faster and more consistent. Moreover, these models reflect post-traumatic (secondary) OA, because they rely on changes in weight bearing and unnatural joint articulation for OA etiopathology [6, 55].

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It is advantageous to develop surgical models in rats or mice because genetic studies are also possible in these animals [21, 26, 29]. Rat models are of interest because their larger size compared with mice provides more tissue for biochemical and gene expression analysis, and permits cross-disciplinary studies (e.g., genomics, cell biology, electrophysiology, and in vivo small animal imaging) [22]. Models developed in the rat include anterior cruciate ligament transection (ACLT) [15, 20, 52] and partial medial meniscectomy (PMM) [14, 40], or a combination of both [44]. Although disease progression in surgical models is faster and more consistent [1], peak symptoms (mainly stiffness) generally develop within 3 months post-surgery owing to fibrosis [43]. Management of OA has changed as the result of current knowledge based on the physiology and pharmacology of articular cartilage, as well as the use of specific new drugs [43]. Polycan is purified β-glucan from *Aureobasidium pullulans* SM-2001 composed mostly of β-1,3-1,6-glucan and other organic materials, such as amino acids, mono- or di-unsaturated fatty acids (linoleic and linolenic acids), and fibrous polysaccharide [47]. Recently, we found that Polycan has anti-osteoporotic effects [48, 51], inhibiting bone loss and accelerating bone formation, which led us to hypothesize that Polycan would show favorable effects on OA. Therefore, in the present study, the efficacy of Polycan on OA induced by ACLT&PMM was evaluated.

**Materials and Methods**

**Animals**

Female, six-week-old Sprague-Dawley rats (N=120; SLC, Japan) were used after seven days of acclimatization. Animals were housed four or five per polycarbonate cage in a temperature (20–25°C) and humidity (40–45%) controlled room with a 12 h:12 h light:dark cycle. Feed (Samyang, Korea) and water were supplied *ad libitum*. Prior to euthanasia, all animals were fasted overnight, and treatment was in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, Washington DC, USA, 1996). Eight rats per group (total 48 rats) were selected based on body weights and knee thickness seven days post-surgery.

**Preparation and Administration of Polycan or Diclofenac Sodium**

Three dosages of Polycan (85, 42.5, and 21.25 mg/kg; Glucan Co. Korea) were dissolved in distilled water and orally administered, once a day for 84 days in a volume of 1 ml/kg. In sham and OA control groups, only 1 ml/kg of distilled water was orally administered. As a reference control, 2 mg/kg of diclofenac sodium (Sigma, MO, USA) dissolved in saline in a volume of 1 ml/kg was subcutaneously injected once a day for 84 days from one week after OA induction by ACLT&PMM surgery (Table 1). In the present study, diclofenac sodium, a well-known cyclooxygenase inhibitor nonsteroidal anti-inflammatory drug (NSAID), was used as a comparable reference drug for OA [14, 17, 23].

**Induction of OA**

Rats were anesthetized with a 25 mg/kg intraperitoneal injection of Zoletile (Zoletile 50; Virbac Lab., France). The OA treatment group underwent surgery involving ACLT&PMM via an incision on the medial aspect of the joint capsule of the left knee, anterior to the medial collateral ligament. Following surgery, the incision was closed in two layers. The joint capsule was sutured independently from peripheral tissues using dissolvable 5-0 Vicryl suture, and the skin was closed with interrupted silk sutures. This procedure was used to induce OA pathogenesis and referred to as the operated-induced side. Conversely, the right knee joint (non-operated, intact side) was used for contralateral treatment. The sham group of rats underwent an operation in which a similar incision in the joint capsule was made but ACLT&PMM were not performed. Only the left knees of sham animals were used as controls for disease progression.

**Body Weight Changes**

Body weights were measured weekly from the start of treatment to euthanasia, using an automatic electronic balance (Precisa Instruments AG, Switzerland). Body weight gains during the 12 weeks of observation were calculated as follows to reduce the individual differences in body weights at the start of the study: Body weight gain (g) = body weight 1 day before euthanasia – body weight at treatment initiation.

**Knee Thickness Measurement**

The thickness of OA operated right knees was measured using an electronic digital caliper (Mytutoyo, Japan) and recorded weekly. In addition, knee thickness was also measured after joint capsule

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**Table 1. Experimental design.**

<table>
<thead>
<tr>
<th>Group</th>
<th>OA</th>
<th>Dose (mg/kg/day)</th>
<th>Animal No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham-operated</td>
<td>Vehicle 1 mg/kg (0.2 ml/head)</td>
<td>Rat-01 ~ Rat-08</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>Vehicle 1 mg/kg (0.2 ml/head)</td>
<td>Rat-09 ~ Rat-16</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>Diclofenac sodium 2 mg/kg</td>
<td>Rat-17 ~ Rat-24</td>
<td></td>
</tr>
<tr>
<td><strong>Experimental group</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>Polycan 85 mg/ (orally)</td>
<td>Rat-25 ~ Rat-32</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>Polycan 42.5 mg/ (orally)</td>
<td>Rat-33 ~ Rat-40</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>Polycan 21.25 mg/ (orally)</td>
<td>Rat-41 ~ Rat-48</td>
<td></td>
</tr>
</tbody>
</table>

(n=8), OA, osteoarthritis; All three dosages of Polycan were orally administered once a day for 84 days, dissolved in distilled water, from 1 week after ACLT&PMM surgery in a volume of 1 ml/kg; Diclofenac sodium was subcutaneously administered, once a day for 84 days from 1 week after ACLT&PMM surgery.
exposure at euthanasia using the same method to reduce differences due to surrounding tissues.

**Maximum Extensor Angle Measurement**
OA operated knees in all animals were dissected from the coxofemoral region to the ankle, leaving the articular capsule intact. After the dissection, the maximum extension angle of each knee was measured according to previous methods [43], with 0 degrees corresponding to the maximum possible extension. In order to minimize any possible bias, all operations and extension measurements were performed by the same veterinarian.

**Histopathology**
Knee joints were excised with the joint capsule intact and fixed in 10% neutral buffered formalin. After five days of fixation, samples were decalcified using 24.4% formic acid, and 0.5 N sodium hydroxide for five days (decalcifying solution was exchanged once a day for five days), after which median joint parts were longitudinally trimmed and embedded in paraffin, sectioned (3 µm), and stained with hematoxylin and eosin (H&E) or Safranin O for cartilage as previously described [7, 27, 54]. Histological profiles of the knee joints were observed and compared with intact controls.

Mankin scoring: Articular cartilage injuries were evaluated using the Mankin score [2,36] with H&E and Safranin O staining. In this system, the higher the score, the higher the level of OA. The entire histological evaluation was performed by the same pathologist.

**Histomorphometry - The thickness of tibia and femur articular cartilage was measured by histomorphometrical analyses of prepared longitudinally trimmed samples at µm levels using a digital image analyzer (DMI-300, DMI, Korea).**

**BrdU-Uptake Measurement**
To assess the effects of Polycan on the proliferation of cells within the rat knee joints, proliferating cells were labeled by an intraperitoneal injection of BrdU. One hour prior to the injection of diclofenac sodium or oral administration of Polycan (on day 82 of treatment), rats were given intraperitoneal injections of BrdU (MP Biomedicals, OH, USA) 50 mg/kg, in a volume of 2 ml/kg dissolved in saline, and euthanized 72 h later as previously described [24]. BrdU uptake was detected with an anti-BrdU antibody as described by Moore et al. [39]. Fixed tissues were prepared, embedded in paraffin, and sectioned as described previously. Tissues were de-paraffinized through a series of washes with xylene and graded alcohol.

After epitope retrieval by pretreatment of 2 N HCl as previously described [25, 53], sections were immunostained as follows:

1. Incubate sections with methanol and 0.3% H2O2 for 30 min for blocking endogenous peroxidase activity at room temperature. Rinse in 0.01 M phosphate-buffered saline (PBS; pH 7.2) three times.
2. Incubate sections with normal horse serum blocking solution (Vector Lab. Inc., CA, USA) at a 1:100 dilution for 1 h to block nonspecific binding of immunoglobulin at room temperature in a humidified chamber. Rinse in 0.01M PBS three times.
3. Incubate sections with primary antisera [Anti-BrdU (HRP) polyclonal antibody; code Ab2285; Abcam, UK] overnight at 4°C in a humidified chamber. Rinse in 0.01 M PBS three times.
4. Incubate sections with biotinylated universal secondary antibody (Vector Lab. Inc.) at a dilution of 1:50 for 1 h at room temperature in a humidified chamber. Rinse in 0.01 M PBS three times.
5. Incubate sections with ABC reagents (Vectastain Elite ABC Kit; code PK-6200; Vector Lab. Inc.) at a 1:50 dilution for 1 h at room temperature in a humidified chamber. Rinse in 0.01 M PBS three times.
6. Incubate sections in peroxidase substrate (code SK-4100; Vector Lab. Inc.) for 30 s at room temperature. Rinse in 0.01 M PBS three times.
7. Counterstain with Mayer’s hematoxylin solution. Rinse in tap water for 30 min.
8. Dehydrate in 95% ethanol for 2 min and 100% ethanol three times. Clear in xylene twice.
9. Add coverslip with permanent mounting medium and observe under a light microscope (Nikon, Japan).

Among 100 chondrocytes, the number of cells occupied by over 10% BrdU immunoreactivity was detected in both the femur and tibia surface articular cartilage with an automated digital image analyzer (DMI-300, DMI, Korea) and calculated as percentages.

**Statistical Analysis**
All data are expressed as means±standard deviation (SD), and statistical analysis was conducted using the Mann–Whitney U- Wilcoxon Rank Sum W test with SPSS for Windows (Release 14; SPSS Inc., USA).

**RESULTS AND DISCUSSION**
In the present study, to observe the long-term efficacy of Polycan on OA, three different dosages were orally administered once a day for 84 days to rats with OA induced by ACLT&PMM surgery starting one week after the operation. A vehicle control and a sham control group were also included for comparison purposes, as well as a group that received diclofenac sodium. Diclofenac sodium is a well-known cyclooxygenase inhibitor NSAID used as a reference drug for OA [14, 17, 23]. It has been shown to preserve the joint cartilage relatively well at a subcutaneous dose of 2 mg/kg in OA rats [23]. Therefore, 2 mg/kg of diclofenac sodium was subcutaneously administered once a day for 84 days from one week after OA induction as a reference control in the present study.

**Body Weight Changes**
In the present study, no meaningful changes in body weight were detected in all Polycan-treated groups compared with sham or OA control, and the body weights and gains of all rats used in this study were within the range of normal age-matched rats [32].

**Changes on the Knee Thicknesses**
OA, also known as a degenerative joint disease, is a chronic inflammatory disease. The cartilage damage in OA leads to edematous changes in the surrounding tissues, and
the thicknesses of affected joints show marked increases [19]. Significant (p<0.01) increases of OA operated knee thickness were detected in OA controls compared with sham controls from treatment initiation throughout 84 days of experimental period. However, knee thickness of all three doses of Polycan and diclofenac sodium was significantly decreased (P<0.01 or P<0.05) compared with OA controls from day 21 of treatment.

The thickness of OA-induced knees at euthanasia in controls (12.96±0.38 mm) changed significantly (P<0.01) compared with sham controls (10.92±0.56 mm). Diclofenac sodium, and polycan 85, 42.5, and 21.25 mg/kg treatment groups significantly changed (P<0.01 or 0.05) as 12.21±0.46, 12.23±0.45, 12.40±0.32, and 12.21±0.58 mm, respectively, compared with OA controls (Table 2).

These favorable effects of Polycan are considered to be the result of anti-inflammatory effects as previously described [30, 31]. No marked changes were detected in the thicknesses of knee joints after joint capsule exposure, which means that no hyperplasia (overproliferation) of chondrocytes was induced by treatment with Polycan or diclofenac sodium.

**Changes in Knee Thickness After Capsule Exposure**

The fibrosis that occurs in OA from the chronic inflammatory process limits joint motion, and the resultant stiffness of joints is one of major symptoms of OA, which reaches a peak approximately three months after OA inducement. Stiffness of joints has been evaluated by the maximum extension angle of the joint, considering 0 degree as maximum extension; therefore, the lower the value, the better the knee function [43]. The thickness of OA operated knees after joint capsule exposure was significantly increased (p<0.01) in all OA-induced groups compared with sham controls. Quite similar thicknesses were detected with all three dosages of Polycan and diclofenac sodium compared to OA controls.

The thickness of OA-induced control knees (9.24±0.30 mm) after joint capsule exposure at euthanasia changed significantly (P<0.01) compared with sham controls (8.00±0.18 mm). Furthermore diclofenac sodium, and Polycan 85, 42.5, and 21.25 mg/kg treatment groups significantly changed (P<0.01) as 9.14±0.23, 8.98±0.53, 9.14±0.43, and 9.04±0.48 mm, respectively, compared with sham controls. There were no significant changes between diclofenac, Polycan, and OA controls (Table 3). Decreases of maximum extension angles in OA-induced knees are considered as direct evidence that Polycan ameliorated OA in this study.

**Changes in Knee Maximum Extension Angles**

The maximum extensor angle of OA-induced knees was significantly increased (p<0.01) in OA controls compared with sham controls. However, these angles were significantly decreased (p<0.01) with diclofenac sodium and all three doses of Polycan compared with OA controls.

The maximum extensor angles of OA-induced knees at euthanasia in OA controls (74.25±4.13°) changed significantly (P<0.01) compared with sham controls (28.63±3.20°). Diclofenac sodium, and polycan 85, 42.5, and 21.25 mg/kg treatment groups significantly changed (P<0.01) as 60.75±6.78°, 60.50±3.30°, 52.75±6.82°, and 56.38±4.31°, respectively, compared with OA controls (Table 3).

**Table 2.** Changes on the OA-induced knee thickness during 84 days continuous oral treatment of Polycan or subcutaneous treatment of diclofenac sodium in OA rats.

<table>
<thead>
<tr>
<th>Items</th>
<th>D0</th>
<th>D7</th>
<th>D14</th>
<th>D21</th>
<th>D28</th>
<th>D35</th>
<th>D42</th>
<th>D49</th>
<th>D56</th>
<th>D63</th>
<th>D70</th>
<th>D77</th>
<th>D83</th>
<th>euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>11.26±0.62c</td>
<td>11.11±0.24a</td>
<td>11.62±0.16a</td>
<td>11.70±0.24a</td>
<td>10.81±0.28a</td>
<td>10.78±0.28a</td>
<td>11.15±0.18a</td>
<td>10.95±0.41a</td>
<td>10.79±0.54a</td>
<td>10.38±0.54a</td>
<td>10.54±0.54a</td>
<td>11.18±0.54a</td>
<td>12.10±0.54a</td>
<td>11.18±0.37a</td>
</tr>
<tr>
<td>OA</td>
<td>13.75±0.28ab</td>
<td>13.64±0.37a</td>
<td>13.16±0.52a</td>
<td>13.13±0.40a</td>
<td>13.04±0.43a</td>
<td>13.30±0.52a</td>
<td>13.41±0.34a</td>
<td>12.82±0.43a</td>
<td>12.52±0.51a</td>
<td>12.74±0.44a</td>
<td>12.82±0.55a</td>
<td>13.00±0.48a</td>
<td>12.98±0.46a</td>
<td>12.96±0.38a</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>13.75±0.33a</td>
<td>13.26±0.51a</td>
<td>12.64±0.32a</td>
<td>12.43±0.71ab</td>
<td>12.13±0.59ab</td>
<td>12.42±0.80ab</td>
<td>12.39±0.28ab</td>
<td>12.28±0.24ab</td>
<td>12.15±0.25ab</td>
<td>11.57±0.59ab</td>
<td>12.04±0.60ab</td>
<td>12.26±0.45ab</td>
<td>12.34±0.43ab</td>
<td>12.21±0.46ab</td>
</tr>
<tr>
<td>Polycan treated</td>
<td>85 mg/kg</td>
<td>13.73±0.20a</td>
<td>13.29±0.40a</td>
<td>12.79±0.26a</td>
<td>12.63±0.62a</td>
<td>12.46±0.56a</td>
<td>12.45±0.52a</td>
<td>12.43±0.54ab</td>
<td>12.40±0.39a</td>
<td>12.25±0.34a</td>
<td>12.15±0.29ab</td>
<td>11.99±0.71ab</td>
<td>12.33±0.20b</td>
<td>12.21±0.45ab</td>
</tr>
<tr>
<td></td>
<td>42.5 mg/kg</td>
<td>13.87±0.14a</td>
<td>13.33±0.42a</td>
<td>12.83±0.39a</td>
<td>12.51±0.49a</td>
<td>12.43±0.24a</td>
<td>12.56±0.47ab</td>
<td>12.43±0.27ab</td>
<td>12.37±0.22a</td>
<td>12.39±0.29a</td>
<td>12.10±0.57ab</td>
<td>12.32±0.38ab</td>
<td>12.50±0.28a</td>
<td>12.40±0.28a</td>
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<tr>
<td></td>
<td>21.25 mg/kg</td>
<td>13.62±0.35a</td>
<td>13.29±0.42a</td>
<td>12.95±0.57a</td>
<td>12.53±0.27a</td>
<td>12.26±0.35ab</td>
<td>12.71±0.32ab</td>
<td>12.57±0.31ab</td>
<td>12.50±0.28ab</td>
<td>12.36±0.26a</td>
<td>11.98±0.37ab</td>
<td>12.18±0.80ab</td>
<td>12.41±0.54ab</td>
<td>12.47±0.58ab</td>
</tr>
</tbody>
</table>

Values are expressed as Means ± SD (n = 8); unit, mm; OA, osteoarthritis; 1day of Polycan administration; 2p<0.01 compared with sham control; 3p<0.01 p<0.05 compared with OA control.
Changes in the Mankin Scores

The Mankin scoring system is generally used for histopathological evaluation to detect articular cartilage injuries. In this system, the higher the score, the higher the level of OA [2, 43]. We found favorable decreases in Mankin scores with diclofenac sodium treatment and all three dosages of Polycan for both the tibia and femur. This is also considered as direct evidence that Polycan ameliorated OA. Marked decreases of articular cartilage thickness occur in OA [8, 39, 41]. In the present study, Polycan effectively inhibited the decreases in articular cartilage thickness.

Various degrees of articular cartilage surface damages, hypocellularity, clones, and stain intensity for Safranin O were detected in all OA-induced groups. The total Mankin scores in tibia and femur of OA controls were significantly increased (p<0.01) compared with sham controls. Although individual scores varied in all groups,

<table>
<thead>
<tr>
<th>Groups</th>
<th>Knee thicknesses after joint capsule exposure at sacrifice (mm)</th>
<th>Maximum extensor angles (degrees, °)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>8.00±0.18</td>
<td>28.63±3.20</td>
</tr>
<tr>
<td>OA</td>
<td>9.24±0.30</td>
<td>74.25±4.13</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>9.14±0.23</td>
<td>60.75±6.78</td>
</tr>
<tr>
<td>Polycan treated</td>
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<tr>
<td>85 mg/kg</td>
<td>8.98±0.53</td>
<td>60.50±3.30</td>
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<tr>
<td>42.5 mg/kg</td>
<td>9.14±0.43</td>
<td>52.75±6.82</td>
</tr>
<tr>
<td>21.25 mg/kg</td>
<td>9.04±0.48</td>
<td>56.38±4.31</td>
</tr>
</tbody>
</table>

Values are expressed as Means ± SD (n = 8); OA, osteoarthritis; *p<0.01 compared with sham control; †p<0.01 compared with OA control.

Table 4. Mankin scores detected in femur at sacrifice.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Surface</th>
<th>Hypocellularity</th>
<th>Clones</th>
<th>Safranin O</th>
<th>Totals1)</th>
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<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.25±0.46</td>
<td>0.25±0.46</td>
<td>0.00±0.00</td>
<td>0.25±0.46</td>
<td>0.75±0.89</td>
</tr>
<tr>
<td>OA</td>
<td>2.75±0.46</td>
<td>2.38±0.52</td>
<td>2.25±1.16</td>
<td>2.50±0.53</td>
<td>9.88±1.55</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>2.13±0.35</td>
<td>1.38±0.52</td>
<td>2.38±0.92</td>
<td>1.25±0.46</td>
<td>7.13±1.36</td>
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<tr>
<td>Polycan treated</td>
<td></td>
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</tr>
<tr>
<td>85 mg/kg</td>
<td>2.13±0.64</td>
<td>1.13±0.83</td>
<td>1.88±0.83</td>
<td>1.13±0.83</td>
<td>6.25±2.49</td>
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<tr>
<td>42.5 mg/kg</td>
<td>1.88±0.35</td>
<td>0.88±0.64</td>
<td>1.38±0.92</td>
<td>1.00±0.76</td>
<td>5.13±1.46</td>
</tr>
<tr>
<td>21.25 mg/kg</td>
<td>2.13±0.64</td>
<td>1.63±0.74</td>
<td>1.88±0.99</td>
<td>1.13±0.99</td>
<td>6.75±2.66</td>
</tr>
</tbody>
</table>

Values are expressed as Means ± SD (n = 8); OA, osteoarthritis; *p<0.01 and †p<0.05 compared with sham control; *p<0.01 and †p<0.05 compared with OA control.

Table 5. Mankin scores detected in tibia.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Surface</th>
<th>Hypocellularity</th>
<th>Clones</th>
<th>Safranin O</th>
<th>Totals1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>0.25±0.46</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.13±0.35</td>
<td>0.38±0.74</td>
</tr>
<tr>
<td>OA</td>
<td>2.63±0.52</td>
<td>2.00±0.53</td>
<td>2.38±0.52</td>
<td>1.50±0.93</td>
<td>8.50±1.93</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>2.00±0.53</td>
<td>1.63±0.52</td>
<td>1.75±0.71</td>
<td>1.38±0.74</td>
<td>6.75±1.16</td>
</tr>
<tr>
<td>Polycan treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85 mg/kg</td>
<td>2.00±0.76</td>
<td>1.13±0.83</td>
<td>1.75±0.89</td>
<td>1.13±1.13</td>
<td>6.00±2.39</td>
</tr>
<tr>
<td>42.5 mg/kg</td>
<td>2.25±0.71</td>
<td>1.38±0.74</td>
<td>1.50±0.93</td>
<td>1.00±0.93</td>
<td>6.13±2.42</td>
</tr>
<tr>
<td>21.25 mg/kg</td>
<td>2.13±0.64</td>
<td>1.63±0.74</td>
<td>1.88±0.99</td>
<td>1.13±0.99</td>
<td>6.75±2.66</td>
</tr>
</tbody>
</table>

Values are expressed as Means ± SD (n = 8); OA, osteoarthritis; *p<0.01 compared with sham control; †p<0.05 compared with OA control.
the total Mankin scores in both tibia and femur with diclofenac sodium and all three doses of Polycan were dramatically decreased as compared with OA controls.

The total Mankin scores of femur articular cartilage in OA controls (9.88±1.55) changed significantly compared with sham controls (0.75±0.89). Diclofenac sodium, and polycan 85, 42.5, and 21.25 mg/kg treatment groups significantly changed (P<0.01) as 7.13±1.36, 6.25±2.49, 5.13±1.46, and 6.75±2.66, respectively compared with OA controls.

The total Mankin scores of tibia articular cartilage in OA controls (8.50±1.93) changed significantly compared with sham controls (0.38±0.74). Diclofenac sodium, and polycan 85, 42.5, and 21.25 mg/kg treatment groups significantly changed (P<0.01 or 0.05) as 6.75±1.16, 6.00±2.39, 6.13±2.42, and 6.75±2.66, respectively, compared with OA controls (Tables 4–6 and Fig. 1).

### Changes in the Articular Cartilage Thickness

Significant decreases (p<0.01) in articular cartilage thickness were detected in OA controls compared with sham controls in both the tibia and femur. However, these decreases in cartilage thickness were significantly inhibited (p<0.01) by treatment with diclofenac sodium and all three dosages of Polycan in both the tibia and femur, except for the Polycan 21.25 mg/kg treatment group in which the tibia articular cartilage thickness was also significantly increased (p<0.01) compared with OA controls, and femur articular cartilage thickness was nonsignificantly increased.

The OA-induced femur articular cartilage thickness in OA controls (303.07±91.40 µm) changed significantly (P<0.01) compared with sham controls (569.43±95.77 µm). Diclofenac sodium, and Polycan 85, 42.5, and 21.25 mg/kg treatment groups significantly changed (P<0.01 or 0.05) as 409.48±69.80, 520.81±173.05, 566.34±203.02, and 513.34±162.18 µm, respectively, compared with OA controls.

The OA-induced tibia articular cartilage thickness in OA controls (296.51±73.69 µm) changed significantly (P<0.01) compared with sham controls (780.16±145.83 µm). Diclofenac sodium, and Polycan 85, 42.5, and 21.25 mg/kg treatment groups significantly changed (P<0.01 or 0.05) as 500.53±196.69, 571.85±137.17, 532.26±177.22, and 352.08±105.69 µm, respectively, compared with OA controls (Table 6 and Fig. 1).

### Changes in BrdU Uptake

Among the methods for the detection of cell proliferation in histological sections, immunohistochemistry using BrdU...
is the most preferred [16, 39]. BrdU staining is easier to read and reflects cell proliferations more specifically than other staining methods [24]. In addition, BrdU uptake is also prevalently used to detect chondrocyte proliferation in OA cartilage [39]. Cells containing BrdU indicate proliferated or proliferating cells.

Significant decreases (p<0.01) in BrdU-immunoreactive cells were detected in both the tibia and femur articular cartilage of OA controls compared with sham controls, which indicates that proliferation of chondrocytes was markedly inhibited. However, these decreases in BrdU-immunoreactive cells were significantly inhibited (p<0.01) by treatment with all three dosages of Polycan, except for Polycan 21.25 mg/kg, which produced nonsignificant increases in BrdU-immunoreactive cells in the tibia articular cartilage but a similar number of immunoreactive cells in the femur articular cartilage compared with OA controls. Similar numbers of BrdU-immunoreactive cells were detected in both the femur and tibia of diclofenac sodium-treated rats compared with OA control.

BrdU-immunoreactive cell numbers in femur articular cartilage OA controls (7.88±3.56/100 chondrocytes) changed significantly (P<0.01) compared with sham controls (45.50±9.49/100 chondrocytes). Diclofenac sodium, and Polycan 85 and 42.5 mg/kg treatment groups significantly changed (P<0.01) as 8.63±4.03, 26.75±8.37, and 24.25±6.71/100 chondrocytes, respectively, compared with OA controls.

The BrdU-immunoreactive cell numbers in tibia articular cartilage of OA controls (8.00±4.24/100 chondrocytes) changed significantly (P<0.01) compared with sham controls (40.88±8.43/100 chondrocytes). Diclofenac sodium, and Polycan 85 and 42.5 mg/kg treatment groups significantly changed (P<0.01) as 8.13±3.52, 28.75±7.57, and 27.63±3.66/100 chondrocytes, respectively, compared with OA controls.

These results are considered as direct evidence that Polycan induced proliferation of chondrocytes that differed from diclofenac sodium (Table 7 and Fig. 2).

Table 7. BrdU-immunoreactive cell numbers.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of BrdU-immunoreactive cells</th>
<th>Femur</th>
<th>Tibia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>45.50±9.49</td>
<td>40.88±8.43</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>7.88±3.56</td>
<td>8.00±4.24*</td>
<td></td>
</tr>
<tr>
<td>Diclofenac</td>
<td>8.63±4.03</td>
<td>8.13±3.52*</td>
<td></td>
</tr>
<tr>
<td>Polycan treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85 mg/kg</td>
<td>26.75±8.37*</td>
<td>28.75±7.57*</td>
<td></td>
</tr>
<tr>
<td>42.5 mg/kg</td>
<td>24.25±6.71*</td>
<td>27.63±3.66*</td>
<td></td>
</tr>
<tr>
<td>21.25 mg/kg</td>
<td>8.38±4.03*</td>
<td>12.63±6.63*</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as Means ± SD (n=8), percentages; OA, osteoarthritis; *p<0.01 compared with sham control; **p<0.01 compared with OA control.

A period of 84 days after initiation of treatment was assumed sufficient for the development of fibrotic stiffness of OA joints based upon a previous study [43]. Changes in the thickness and maximum extension angle of each knee, thickness of capsular joint regions, and cartilage histopathology were measured using the Mankin score (histomorphometry included the thickness of surface articular cartilages of femur and tibia in individual rats) at
necropsy. In addition, the proliferation of cartilage was also assessed using BrdU. As a result of ACLT&PMI, classic OA was induced, identified by changes in maximum extension angles, limited extension values, edematous changes, capsule thickness increases, decreases in chondrocyte proliferation (detected by BrdU uptake), cartilages degenerative changes, Mankin score differences, and loss of articular cartilage. However, these OA changes were markedly inhibited by 84 days of diclofenac sodium treatment and all three dosages of Polycan compared with OA controls, with the exception of capsule thickness in that similar thickness was detected regardless of treatment. Whereas diclofenac sodium did not influence BrdU uptake, BrdU-immunoreactive cells were increased by treatment at all three dosages of Polycan, which means that Polycan induced proliferation of chondrocytes in tibia and femur surface articular cartilage.

Similar or more favorable effects on stiffness and cartilage losses induced by OA were detected with all three oral dosages of Polycan compared with diclofenac sodium 2 mg/kg subcutaneously treated rats. These overall effects of Polycan showed clear dosage dependencies between 42.5 and 21.25 mg/kg treatment groups, but slightly lower or similar effects were detected between the 85 and 42.5 mg/kg treatment groups. Therefore, based upon these results, we suggest that the optimal concentration to treat OA is 42.5 mg/kg in the present study. This dosage can be converted to 425 mg/head/day in a 60 kg human, based on the body surface differences of rats being 1/6 that of humans.

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REFERENCES


