Establishment of a bioindustry need-osteoarthritis rat model via surgery induction

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Abstract

The degenerative arthritis has been a common knee joint disease in Taiwan. In mild cases of degenerative arthritis, it may just be a little uncomfortable to walk, but severe degenerative arthritis will cause difficulty in walking and even loss of basic mobility. The loss of articular cartilage in degenerative arthritis is irrecoverable. Currently, the degeneration of the knee joint can be easily divided into three levels based on its severity: primary, intermediate, and severe. The choice of treatments for degenerative arthritis will vary in accordance to different levels that is mainly divided into the non-surgical treatment and surgical treatment. Therefore, how to prevent knee joint degeneration is an important issue. In this study, anterior cruciate ligament transection (ACLT) combined with medial meniscectomy (MMx) surgery were used to establish a rat model with degenerative arthritis. According to all data, we have successfully established a rat model with degenerative arthritis via ACLT-MMx surgery. We wish this model will be applied on the research of the degenerative arthritis drugs and the design of therapeutic strategies for degenerative arthritis in the future.

Keywords: Anterior cruciate ligament transection; Degenerative arthritis; Medial meniscectomy; Osteoarthritis; Rat model; Surgery

1. Introduction

Osteoarthritis (OA), also known as degenerative arthritis, is a prevalent knee joint disease in Taiwan, characterized by pain, particularly under weight-bearing conditions. While mild OA may only cause slight discomfort during walking, severe cases can lead to significant mobility issues. The knee joints, being the largest joints in the body, bear substantial weight daily, contributing to the inevitable wear of articular cartilage. As cartilage loss is irreversible in OA, prevention of wear and knee joint degeneration is paramount [1-4].

While many OA patients inquire about dietary strategies to maintain knee joint health, effective prevention primarily involves reducing knee joint burden through measures such as weight control, minimizing weight-bearing activities, and avoiding excessive exercise. Although multiple factors contribute to cartilage degradation, reducing knee joint burden remains a crucial preventive method [4-5].

Currently, knee joint degeneration severity is categorized into primary, intermediate, and severe stages, with treatment options varying accordingly between non-surgical and surgical interventions. While treatment aims to alleviate symptoms, it cannot reverse degeneration. Primary stage treatment typically involves anti-inflammatory drugs and rest,
with surgical interventions like corrective surgery or partial knee replacement for mid-stage OA. Total knee arthroplasty (TKA) is common for severe OA cases, with an increasing number of Taiwanese undergoing TKA annually, particularly among the elderly [6-8].

Advancements in research and medical technology have led to diversified treatment approaches. Detailed evaluations allow for tailored surgical treatments, such as proximal osteotomy corrective surgery, aiming to alleviate OA symptoms without joint replacement. Additionally, the Taiwanese government’s approval of cell therapy offers potential cartilage regeneration options, though success rates vary [9-10].

Despite these advancements, the need for an optimal OA animal model remains urgent for drug research and therapeutic strategy development. While artificial joint replacement yields positive outcomes, exploring alternative treatments before resorting to surgery is essential. Collaboration between physicians, patients, and their families is crucial for devising effective treatment plans [9-10].

In conclusion, while artificial joint replacement remains a reliable treatment, ongoing research aims to improve OA treatment outcomes. An ideal animal model is imperative for advancing drug research and therapeutic strategies in OA management.

2. Materials and Methods

2.1. Experimental Animals

Adult male Sprague Dawley (SD) rats (4 weeks old) with specific pathogen-free conditions were used for this study, were purchased from BioLASCO Taiwan Co., Ltd. (Yilan, Taiwan). These SD rats were fed with standard laboratory diet (No. 5001, LabDiet®; PMI Nutrition International, St. Louis, MO, USA) and distilled water ad libitum during the experimental period. The environment was maintained room temperature (24-27°C) and 60%-70% humidity with a photoperiod of 12-hr light/12-hr dark cycle. The study will begin after a week acclimation. The Institutional Animal Care and Use Committee (IACUC) of Agricultural Technology Research Institute inspected all animal experiments and this study comply with the guidelines of protocol IACUC-112055C1 approved by the IACUC ethics committee.

2.2. Experimental Design

The 11 SD rats were divided into as the positive control (PC) group (n = 3) and the negative control (NC) group (n = 8). The surgical induction in 2 groups was performed. In PC group, all rats were subcutaneously injected with human recombinant growth hormone (20 μg/kg BW) starting from the third week, administered continuously for 10 days. The NC group received daily water intake via gavage feeding. Body weight (BW) and the widths of the knee joints were measured before the surgery as the baseline. At the end of the experiment (9th week), the knee joint section confirmed the progressive deterioration of OA joint with a reactive chondrocytes hypertrophy, and increasing cartilage erosion accompanied by chondrocytes loss.

2.3. Anterior Cruciate Ligament Transection (ACLT) Plus Medial Meniscectomy (MMx)-induced OA Rat Model

Surgery-induced OA rat model was proceeded as described previously [11]. The ACLT-MMx surgery was performed on the left knee joint of rats by a senior veterinarian. Briefly, male SD rats (100 g per rat, 5 weeks ago) were anesthetized with 5% isoflurane and then maintained with 2% isoflurane. Hair was removed around the left knee skin and sterilized with povidone-iodine solution and 70% ethanol. Later, the left knee joint skin was cut with a sterilized scalpel. An incision was made in the medial aspect of the left joint capsule. The anterior cruciate ligament was transected using a sterilized scalpel, and the medial meniscus was removed completely using a sterilized tenotomy scissor. Following ACLT-MMx surgery, the incision joint was irrigated with normal saline/antibiotics. The incision joint capsule was sutured with 4-0 vicryl. The 4-0 monofilament nylon was used for the incision skin closure. Finally, the wound area was sterilized with povidone-iodine solution. Analgesics (3 mg/kg/day ketoprofen) and antibiotics (30 mg/kg/day amoxicillin) were administered intramuscularly for 3 days to prevent pain and infection.

2.4. Measurement Values of Physiological Items

All rats were recorded the changes in BW and food intake during the experiment. Regular measurement and comparison of BW of rats in the PC group and NC group. In addition, the changes in water intake and food intake in the NC group and PC group were also recorded daily.
2.5. Measurement of the Width of Knee Joints

The width of the knee joint was measured with an electronic digital caliper (Mitutoyo America Corporation) once a week after ACLT-MMx surgery until the end of the experiment. The level of actual joint swelling induced by ACLT-MMx surgery was evaluated according the width (mm) of the surgical left knee joints of OA rats minus the width (mm) of the right knee joints of OA rats.

2.6. Animal Specimen Collection and Examination

The experiment lasted for 9 weeks, including a 1-week adaptation period. On day 28 of the experiment, calcein was administered subcutaneously at a dose of 15 mg/kg BW. On day 36 of the experiment, xylenol orange was administered subcutaneously at a dose of 90 mg/kg BW. At the end of the experiment, the experimental animals were euthanized using CO₂, and blood was collected via cardiac puncture to obtain serum for subsequent analysis. The tibiae of all rats were dissected to the femur level, and the lengths of the tibiae and femurs were measured. After measurement, the tibiae and femurs were immersed in 10% neutral buffered formalin for subsequent sectioning, staining, and pathological examination. The left femurs of the rats were fixed in formalin for 1 week, followed by decalcification (Surgipath Decalciﬁer II). After decalcification, standard dehydration procedures were followed, and the specimens were embedded in paraffin blocks. Longitudinal sections were then made and stained with H&E. Following staining, the sections were observed under a light microscope using software for quantitative analysis. Specific regions near the growth plate were selected for measurement of bone tissue morphology parameters, following the criteria used by Bitto et al. [22] for scoring trabecular bone structure and trabecular bone quality.

Table 1 Trabecular bone structure and trabecular bone quality scoring

<table>
<thead>
<tr>
<th>Score</th>
<th>Structure of trabecular bone</th>
<th>Quantity of trabecular bone (% of interest area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Absent</td>
<td>0-30</td>
</tr>
<tr>
<td>1</td>
<td>Markedly reduced</td>
<td>30-60</td>
</tr>
<tr>
<td>2</td>
<td>Partially reduced</td>
<td>60-90</td>
</tr>
<tr>
<td>3</td>
<td>Normal</td>
<td>90-100</td>
</tr>
</tbody>
</table>

2.7. Evaluation of Trabecular Mineral Apposition Rate in Rat Tibia

The right tibia of rats was assessed for trabecular mineral apposition rate (MAR, μm/day) according to the method described by Porter et al. [24]. The tibiae were fixed in formalin for 48 hours and then immersed in 5% KOH solution, shaken at room temperature at 30 rpm for 96 hours to soften the bone tissue. After completion of the process, the bones were rinsed with water for 2 hours and subsequently dehydrated and sectioned using standard procedures. The sections were not stained, and blank slides were observed under a fluorescence microscope to measure the distance between the green fluorescence of calcein and the red fluorescence of xylenol orange in the bone. This distance was used to calculate the trabecular mineral apposition rate.

2.8. Biochemical Index of Osteoblast Metabolism–Bone Alkaline Phosphatase Concentration Measurement

The concentration of bone alkaline phosphatase (BAP) was determined using the Rat Bone Specific Alkphase B ELISA kit (MyBioSource, Cat. No. MBS732296). The measurement method is based on competitive enzyme-linked immunosorbent assay (ELISA) principles to determine the BAP content in serum. According to the instructions provided with the kit, 100 μL of standard solution, test samples, and phosphate-buffered solution (PBS) (as blank) were added to each well. Subsequently, 50 μL of enzyme conjugate was added to each well to mix thoroughly with the standard solution and test samples. The mixture was then incubated at 37°C for 1 hour. After incubation, each well was washed 5 times with 300 μL of 1× wash buffer. Following washing, 50 μL of substrate A and 50 μL of substrate B were added to each well and thoroughly mixed. The mixture was then incubated at 37°C for 15-20 minutes. Finally, 50 μL of stop solution was added to each well and mixed evenly. The absorbance was immediately read at OD 450 nm using an ELISA reader. The absorbance values were then used in the standard curve equation to calculate the BAP content.

2.9. Biochemical Index of Osteoclast Metabolism–Pyridinoline Concentration Measurement

The concentration of pyridinoline (Pyd) was determined using the Rat Pyridinoline ELISA Kit (MyBioSource, Cat. No. MBS720260). The measurement method is based on the competitive ELISA principles to determine the Pyd content in serum. According to the instructions provided with the kit, 100 μL of standard solution, test samples, and PBS (as blank)
were added to each well. Subsequently, 50 μL of Enzyme conjugate was added to each well to mix thoroughly with the standard solution and test samples. The mixture was then incubated at 37°C for 1 hour. After incubation, each well was washed 5 times with 300 μL of 1× wash buffer. Following washing, 50 μL of substrate A and 50 μL of substrate B were added to each well and thoroughly mixed. The mixture was then incubated at 37°C for 15-20 minutes. Finally, 50 μL of stop solution was added to each well and mixed evenly. The absorbance was immediately read at OD 450 nm using an ELISA reader. The absorbance values were then used in the standard curve equation to calculate the Pyd content.

2.10. Statistical Analysis
All values are presented as mean ± SD. One-way analysis of variance was conducted using Graphpad Prism 6 statistical software to analyze differences between different treatments. A significance level of $p < 0.05$ was considered statistically significant. Due to the slow physiological changes in bone, when using bone density and other indicators for inter-group statistical analysis, $p < 0.1$ (slightly significant) can be considered as indicating physiological effects that promote bone growth or slow bone loss.

3. Results

3.1. Measurement of BW and Food Intake of Rats

![Figure 1](image1) Rats’ BW change in the negative control group (NC) and the positive control group (PC) during the experiment. Weekly measurements of BW. BW change rate was calculated using the formula: \[\text{rate} = \left(\frac{\text{BW of the week} - \text{initial BW}}{\text{initial BW}}\right) \times 100\%.\] All values are presented as mean ± SD. A significance level of *$p < 0.05$* was considered statistically significant.

![Figure 2](image2) The average daily feed consumption per week for each group of rats was measured. The feed consumption for the negative control group (NC) and the positive control group (PC) was measured weekly. The average daily feed consumption was calculated using the formula: \[\text{Average daily feed consumption} = \frac{\text{total weekly food consumption}}{\text{the number of rats per cage}} \div 7 \text{ days}.\] All values are presented as mean ± SD.
During the experiment, the changes in BW and food intake of rats in each group are shown in Figures 1-2. During the entire experimental period, the rats in NC group showed that the ACLT-MMx surgery would slow the growth of the rats, but the BW of each group maintained a steady increase with time. To the end of the experiment (9th week), there was significant difference on the 3rd, 5th week BW between groups ($p<0.05$) (Figure 1). In addition, the average daily food intake of each group during the experimental period showed that there was no significant difference between two groups ($p>0.05$) (Figure 2).

3.2. Pathologic Examination of Rats’ Knee Joints

Longitudinal sections of the left femurs of rats were prepared and subjected to trimming, followed by staining with H&E. After staining, specific areas near the growth plate of bone were selected for the measurement of histomorphometric parameters under a light microscope using quantifiable software. The results revealed two significant increase in the trabecular structure and overall loss of bone trabeculae in PC than that in NC ($**p<0.01$) (Figures 3-4).

**Figure 3** Longitudinal sections of rat femurs were prepared and stained with H&E. The black arrows indicate trabecular bone. The histopathologic examination of rat femurs in the negative control group (NC) and the positive control group (PC) was performed.

**Figure 4** The trabecular bone structure, trabecular bone quality score, and total score for rats in the negative control group (NC) and the positive control group (PC) were evaluated. All values are presented as mean ± SD. A significance level of $**p<0.01$ was considered statistically significant.
3.3. Trabecular Mineral Apposition Rate

Blank sections of the femur prepared according to the method of Porter et al. (2017) [24] were observed under a fluorescence microscope to capture images of red and green fluorescence. Subsequently, ImageJ image processing software was used to calculate the distance between the fluorescent markers of calcein and xylenol orange, which was then converted to the trabecular mineral apposition rate (TMA, μm/day). After ANOVA analysis, significant differences were observed between the positive control group (PC) and the negative control group (NC) (**p < 0.001) (Figure 5).

![Figure 5](image)

**Figure 5** Trabecular mineral apposition rate (MAR) in each group. After sacrificing the rats, sections were obtained from the right femurs, and blank slides were prepared. Fluorescence images were captured under a fluorescence microscope, and the distance between red and green fluorescence was measured to calculate the trabecular mineral apposition rate (MAR, μm/day). Upon calculation, significant differences were observed between the negative control group (NC) and the positive control group (PC). All values are presented as mean ± SD. A significance level of ***p < 0.001 was considered statistically significant.

3.4. Bone Alkaline Phosphatase and Pyridinoline Concentration Measurement

The concentrations of bone alkaline phosphatase (BAP) and pyridinoline (Pyd) were determined using the Rat Bone Specific Alkphase B ELISA Kit (MyBioSource, Cat. No. MBS732296) and Rat Pyridinoline ELISA Kit (MyBioSource, Cat. No. MBS720260), respectively. The measurement method is based on the competitive ELISA to determine the BAP or Pyd content in serum. The BAP or Pyd content in the serum of each group was analyzed by ANOVA. The result of Pyd concentration was presented that the significant difference between PC and NC groups (p < 0.05). However, the result of BAP concentration was presented that the slight difference between PC and NC groups (p < 0.1) (Figure 6).

![Figure 6](image)

**Figure 6** The bone alkaline phosphatase and pyridinoline concentrations in the serum of each group were measured. After euthanizing the rats, serum samples were collected and bone alkaline phosphatase (BAP, ng/mL) and pyridinoline (Pyd, nmol/L) concentrations were determined according to the instructions provided with the assay kit. Following calculation, it was found that there were no significant differences in BAP concentrations among the groups. The result of Pyd concentration was presented that the significant difference between PC and NC (p < 0.05). However, the result of BAP concentration was presented that the slight difference between PC and NC (p < 0.1). All values are presented as mean ± SD. *p < 0.05 was considered statistically significant and p < 0.1 was considered statistically slight significant.
4. Discussion

According to the literatures [12-16], ACLT-MMx surgery induced a constant and gradually increasing knee width as a result of progressive knee joint inflammation. The static hindlimb weight-bearing forces test to assess pain behavior during OA progression. Similar to the previous report [15-16], ACLT-MMx surgery-induced OA elicited a constant change in weight-bearing asymmetry compared to that of the NC group, which only presented acute pain in the first few weeks as a result of the surgical procedure.

In addition, according to the literatures [17-21], no significant difference of BW between NC group and OA group. In this study, during the entire experimental period, the rats in OA group showed that the ACLT-MMx surgery would slow the growth of the rats before 4th week ACLT-MMx surgery, but the BW of each group maintained a steady increase with time. From the 4th-week to the end of the experiment (12th week), there was no significant difference on the BW between each group. In addition, the average daily food intake and the average daily water intake in NC and OA groups during the four-week experimental period showed that there was no significant difference between two groups.

According to the literatures [12-16], ACLT-MMx surgery induces a constant and gradually increasing knee width due to progressive knee joint inflammation. The static hindlimb weight-bearing forces test was conducted to assess pain behavior during OA progression. Consistent with previous reports [15-16], ACLT-MMx surgery-induced OA resulted in a constant change in weight-bearing asymmetry compared to the NC group, which only exhibited acute pain in the initial weeks following the surgical procedure.

According to our previous study [25], the using of the incapacitance tester to assess whether the rat’s knee joints was painful or discomfort. It was observed that the pain value increased significantly after the ACLT-MMx surgery in OA group. The pressure between the hindlimbs of the OA group was significantly higher than that of the NC group. Using of an electronic digital caliper to measure the width of the knee joint of the rat's hind limbs. After ACLT-MMx surgery, the width of rat's knee joint in the OA group was significantly wider than that in the NC group and a significant increase in the rat's knee joint swelling was observed. Pathologic examination on knee joints of rats in each group were performed. After ACLT-MMx surgery for 12 weeks, knee joints of rats were sectioned and stained with H&E, toluidine blue, Safranin O/fast green, Masson’s trichrome, respectively. The average scores of lesions in the OA group were significantly higher that than in the NC group. The condition of collagen and bone was observed by Masson’s trichrome staining. The average score of lesion severity in OA group was significantly higher than that of the NC group. Finally, the average score of the total lesion degree of the OA group was significantly higher than that of the NC group based on H&E staining, toluidine blue staining, Safranin O/fast green staining, and Masson’s trichrome staining. According to the above results, an ACLT-MMx surgery-induced OA rat model has been successfully established.

In this study, the experimental period was shorten to 9 weeks. An incapacitance tester was also used to evaluate whether the rat’s knee joints were experiencing pain or discomfort. It was observed that the pain value significantly increased after ACLT-MMx surgery in the NC group, with higher pressure between the hindlimbs in the PC group compared to the NC group. The width of the rat’s knee joint, measured using an electronic digital caliper, was significantly wider in the NC group post-ACLT-MMx surgery, indicating significant knee joint swelling (data not shown).

According to literature [17-21], there was no significant difference in BW between the groups during the experiment. During the experimental period, rats in the NC group exhibited slowed growth post ACLT-MMx surgery, but BW in each group steadily increased until the end of the 9-week experiment, there was no significant difference in BW between the groups. Moreover, average daily food intake during the nine-week experimental period also showed no significant difference between the NC and PC groups.

Additionally, pathological examination of the knee joints from each group of rats was performed [22-25]. After 8 weeks post ACLT-MMx surgery, knee joint sections were stained with H&E. H&E staining was used to observe cartilage damage, with histopathological scores indicating greater severity of articular cartilage damage in the NC group compared to the PC group. Overall, the total lesion degree score based on H&E staining was significantly higher in the NC group than that in the PC group, confirming the successful establishment of an ACLT-MMx surgery-induced OA rat model. On the other evaluation, significant differences were observed between PC and NC groups on the trabecular mineral apposition rate. Pyd concentration was presented that the significant difference between PC and NC groups. However, BAP concentration was presented that the slight difference between PC and NC groups. NC group presented more severity in the molecular expressions of bone metabolism than PC group to confirming the successful establishment of an ACLT-MMx surgery-induced OA rat model.
5. Conclusions

Degenerative arthritis, commonly known as osteoarthritis (OA), is a prevalent knee joint disease in Taiwan. While mild cases may only cause slight discomfort while walking, severe OA can lead to significant difficulty in walking and even loss of basic mobility. Once the articular cartilage is lost, it cannot be recovered. Therefore, preventing knee joint degeneration is a crucial concern. In this study, we utilized ACLT-MMx surgery to establish an OA rat model. Based on our comprehensive data analysis, we have successfully created an OA rat model. We anticipate that this model will be instrumental in advancing research on OA drugs and the development of therapeutic strategies in the future. By leveraging this model, we hope to uncover new insights into the treatment and management of OA, ultimately improving the quality of life for individuals affected by this condition.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of Ethical Approval

The Institutional Animal Care and Use Committee (IACUC) of Agricultural Technology Research Institute inspected all animal experiments and this study comply with the guidelines of protocol IACUC 112055C1 approved by the IACUC ethics committee.

References


