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Analysis of microscopic characteristics of cartilage, synovial membrane, and subchondral bone in collagenase induction model of knee osteoarthritis *Rattus Norvegicus*

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Abstract

Osteoarthritis (OA) is a degenerative arthritis disease of the synovial joints and is one of the most common causes of disability in adults. A study requires experimental animal models to be well understood. Collagenase was found to be able to induce OA in experimental animals. This study was conducted with the aim of analyzing the microscopic characteristics of cartilage tissue, synovial membrane, and subchondral bone in the knee of *Rattus norvegicus* rats after collagenase injection. In-vivo experiment using experimental animals *Rattus norvegicus* which was injected with collagenase was conducted. The sample will be divided into 3 groups: evaluation time at 7 days (group 1), 14 days (group 2), and 21 days (group 3). Synovial and cartilage will be evaluated based on OARSI while subchondral bone will be evaluated based on Subchondral Bone Score (SBS). It was found that cartilage erosion and synovial membrane damage were the most severe in group 3. The level of damage based on OARSI and SBS scoring was also found to be progressive and worsened over time. Subchondral bone values increased in both control and treatment with the control peak at 0.87 and the treatment peak at 3.2. It was found that there was a clear and microscopically progressive difference 3 weeks after collagenase injection. Induction of OA using collagenase injection in experimental animals *Rattus norvegicus* can be a good model for making secondary OA.

Keywords: osteoarthritis, knee, collagenase, cartilage, synovial membrane, subchondral bone

1. Introduction

Osteoarthritis (OA) is a very complex degenerative arthritis disease of the synovial joints and is one of the most common causes of disability in older adults. According to the United Nations, by 2050 people over the age of 60 will make up more than 20% of the world's population, which means it is estimated that by 2050, 130 million people will suffer from OA worldwide, of which 40 million will be severe disability due to the disease (1). Patients with OA in the United States alone have reached 27 million people in 2008 (2).

Synovial membrane inflammation has emerged as another major feature of the pathophysiology of OA. Synovial histological changes include synovial hypertrophy and hyperplasia with an increased number of lining cells often accompanied by sublining tissue infiltration with scattered lymphocyte foci. Activated synovium can produce proteases and cytokines that accelerate damage to the area around the cartilage (3).

In addition to cartilage tissue and synovial membrane, various characteristic changes in subchondral bone are also found which are also considered to be very related in the pathogenesis of OA, thus implying a strong relationship between OA and subchondral bone. Other researchers have published findings that an increase in subchondral bone mass that is rigid (stiff) is an important factor in the pathogenesis of OA, because it contributes to cartilage degeneration, in addition to inflammation of the synovial membrane (4,5).

Research on the pathogenesis of OA is a challenge in itself considering the very complex pathogenesis of OA. The main obstacle faced by researchers in the study of OA is that the pathogenesis of OA is slow and difficult to predict, and clinical symptoms that appear only in advanced OA so that they cannot describe the various structural changes in the joint cavity (6). Therefore, a research model is needed that can be carried out in a shorter time, in this case using an experimental animal model (7).

The small animal trial model is the model of choice as an initial study of the pathogenesis of OA considering the practicality and financing aspects, as well as a foothold before being implemented in a larger model. In addition to the types of experimental animals, the model for making OA is also divided into primary and secondary OA models. Secondary models, both surgical and chemical induction models, have become an option in consideration of making models that are shorter and more practical. Chemical induction has recently

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become the choice of many researchers given its practicality. Chemical induction can use monosodium iodoacetate (MIA) and collagenase, but because MIA is much faster than collagenase, collagenase is considered more capable of representing OA itself (8).

Given the importance of a good model to support future studies for OA, this study was conducted with the aim of analyzing the microscopic characteristics of cartilage, synovial membrane, and subchondral bone in a collagenase-induced model of knee osteoarthritis of *Rattus norvegicus*. This study is expected to be the basis for modeling OA in mice.

2. Material and Methods

This type of research is an in-vivo experiment using *Rattus norvegicus* experimental animals with an analytical research design in the form of a post-test only design. This study has been approved by Animal Care and Use Committee of Universitas Airlangga, Surabaya, Indonesia with the number of 2.KEH.029.03.2022.

Experimental animals used in research must meet the following criteria: (1) experimental animals are *Rattus norvegicus*; (2) rats aged 2 months; (3) rats weighed 200-300 grams; (4) male rats; and (5) in an healthy condition with signs of active movement and solid stools. In the event that *Rattus norvegicus* fell ill and/or rats died during the acclimatization period or during the study, the sample was not included in the data for this study. All institutional and national guidelines for the care and use of laboratory animals were followed.

The sample will be divided into 3 groups. Grouping is based on the time of evaluation: 7 days (group 1), 14 days (group 2), and 21 days (group 3). Each group will be subdivided into 2 subgroups: control group and treatment group. Treatment group will receive injection of collagenase type 7 as much as 1 ml and control group will receive injection of only 1 ml of NaCl. At each time, the sample will be terminated and the knee tissue sample taken for microscopic evaluation: cartilage, synovial membrane, and subchondral bone. Synovial and cartilage will be evaluated based on OARSI (9) while subchondral bone based on Subchondral Bone Score (10).

In brief, OARSI score is calculated based on 4 parameters: hematoxylin eosin staining, cartilage structure, chondrocyte density, and cluster formation with a total score ranging from 0-24. On the other hand, subchondral bone score is based on 3 parameters: subchondral plate condition, bone volume (based on formula), and observable osteophytes. Maximum score of 12 can be achieved with higher score indicating worse subchondral bone.

Based on the calculation of the sample formula, it was found that the total number of experimental *Rattus norvegicus* rats for this study should be 5 for each group. Therefore minimum required sample would be 30 samples (3 treatment groups and 3 control groups).

All research was carried out at the Laboratory of the Faculty of Veterinary Medicine (FKH) Universitas Airlangga, Surabaya, Indonesia from March 2022 to April 2022.

All rats will go through the acclimatization stage for one week. Experimental animals were placed in groups placed in cages, within 12 hours on a light-dark cycle at 24°C and had access to water and food in a veterinary laboratory facility.

OA was induced unilaterally in one knee of each *Rattus norvegicus* by 2 intra-articular injections of 3 collagenase type VII units (Sigma-Aldrich) on the first day of observation (day 0). All intra-articular injections were applied with an injection volume of 6 L using a 50 L glass syringe (Hamilton Company, Ghiroda, Romania) and a 30 G needle. The contralateral knee control was injected intraarticularly using 1 ml NaCl.

All specimens were fixed with 10% buffered formalin for 24 hours, decalcified with 5% formic acid for 5 days and then put into paraffin blocks. A series of 3 sagittal sections (4 m thick) were created for each of the 4 compartments, through the large diameter of the cartilage lesion. Hematoxylin-eosin staining was used to evaluate synovial, cartilage, and subchondral bone scores. Evaluation was done by two anatomic pathology specialist and mean score from the two will be used as the final score on this patient. All pathology anatomist were blinded by the sample's group.

The data collected will be analyzed descriptively by totaling each scoring parameter and averaging the results of the sample used. Calculations were carried out using the Microsoft Excel 2016 program. The data will be presented in tabular form.

3. Results

From the results of the study it was found that cartilage erosion was the most severe in group 3 and the lightest in group 1 (Fig. 1 and fig. 3). The signs of inflammation were most severe in group 1 and mild in group 3. From the results of immunohistochemical analysis, it was also found that cartilage and synovial tissue damage occurred mainly in the area of collagenase injection (Fig. 2). This suggests that collagenase may need to be administered in larger volumes or injected more than once to create a more evenly-spread breakdown.

The average results of the assessment of the synovial membrane for all samples are described in table 1. Overall, the cumulative total OARSI value of the synovial membrane in the control group on average is the same, ranging from 0.6-0.9. Compared with the group treated with collagenase injection, there was a large increase of up to 7.53 on day 21.

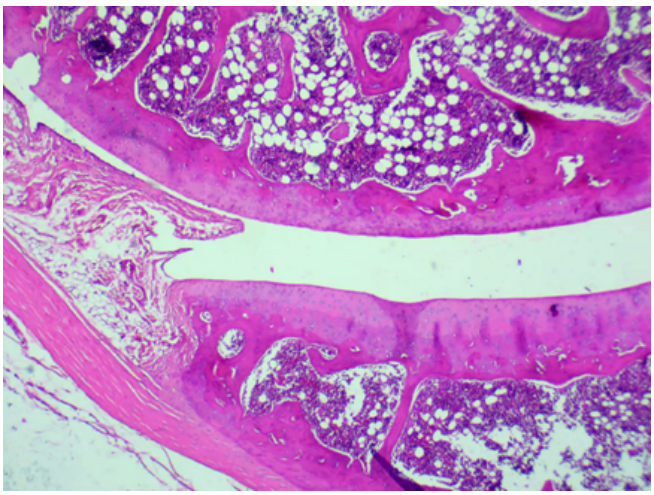

Fig. 1. Microscopic image of the control group 3. A normal picture was found. No erosions or leukocytes were seen in the joint space. There was also no inflammatory cell infiltration in the synovial membrane. This image is used as a reference for detecting pathology in other sections

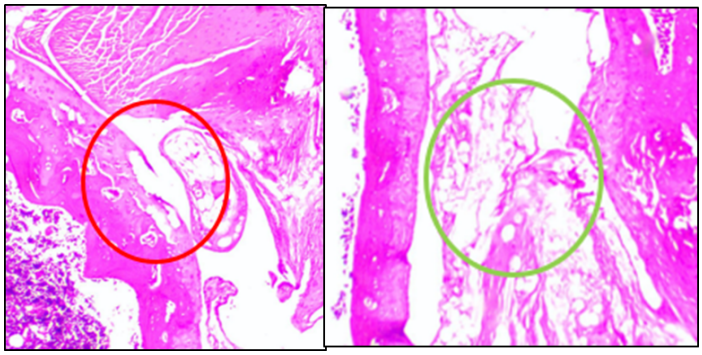

Fig. 2. Microscopic image of treatment group 1 sections. The left image shows an irregularity of the cartilage surface. In the image on the right, there are inflammatory cells infiltrating the synovial membrane. This picture is an early symptom of mild OA

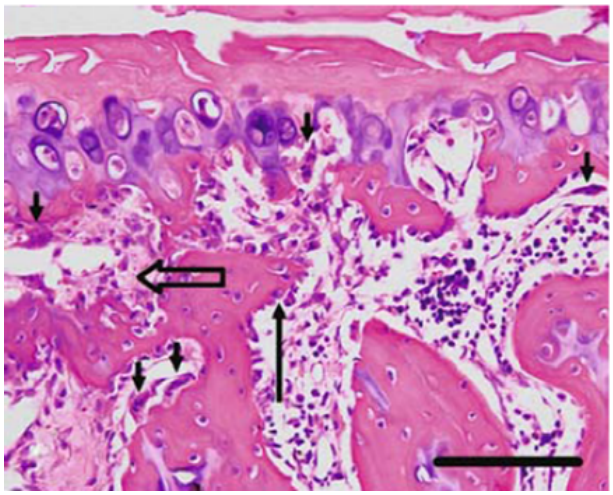

Fig. 3. Subchondral bone changes in treatment group 3. Focal fibrous tissue proliferation (blank fill arrow), increased osteoclasts along the junction between damaged cartilage and subchondral bone (short arrow), and several adjacent trabeculae lined with osteoblasts (long arrow) are seen.

In terms of cartilage microscopy, it was found that the various parameters of cartilage tissue had increased quite a lot from day to day and the difference was large with the control, especially on day 21 (control vs. treatment: 5.13 and 0.6).

Subchondral bone values increased in both control and treatment with the control peak at 0.87 and the treatment peak at 3.2. Although both experienced improvement, there was a significant difference between the two from day 7 to day 21. This indicates the progression of subchondral bone damage, especially in the treatment group.

Table 1. Results of OARSI assessment on synovial membrane, cartilage tissue, and subchondral bone of *Rattus norvegicus* rat after treatment

		Day of Observation		
Group	**Scoring Criteria**	Group 1 (7 Days) Mean Score	Group 2(14 Days) Mean Score	Group 3 (21 Days) Mean Score
Control	Synoviocyte proliferation	0	0.07	0
	Hypertrophy	0	0.13	0
	Inflammatory infiltrate	0.2	0.13	0.07
	Fibrinous exudate	0.07	0	0.2
	Lymphoplasmacytic infiltrate	0	0	0.07
	Lymphoplasmacytic aggregates	0	0	0.13
	Synovial stroma villous hyperplasia	0.07	0	0.07
	Proliferation of fibroblasts/fibrocytes	0.07	0.13	0.07
	Proliferation of blood vessels	0.13	0.07	0.2
	Cartilage/bone detritus	0	0	0.07
	Hemosiderosis	0.07	0.13	0
	Cumulative Microscopic OARSI Synovial Membrane Score	0.6	0.67	0.87
	Hematoxylin-eosin Staining	0.2	0.2	0.13
	Structure	0.47	0.4	0.27
	Chondrocyte Dencity	0.13	0.13	0.13
	Cluster formation	0.07	0.07	0.07
	Cumulative Microscopic OARSI Cartilage Score	0.87	0.8	0.6
	Subchondral Bone Plate	0.13	0.13	0.47
	Bone Volume	0.13	0	0.33
	Osteophyte	0.07	0.13	0.07
	Cumulative Microscopic Cartilage Score	0.33	0.27	0.87

Perlakuan: Injeksi Kolagenase 1x	Synoviocyte proliferation	0.33	0.47	0.67
	Hypertrophy	0.53	0.6	0.47
	Inflammatory infiltrate	0.47	0.73	0.4
	Fibrinous exudate	0.53	0.87	0.87
	Lymphoplasmacytic infiltrate	0.47	0.67	0.67
	Lymphoplasmacytic aggregates	0.47	0.6	0.53
	Synovial stroma villous hyperplasia	0.47	0.67	0.8
	Proliferation of fibroblasts/fibrocytes	0.6	0.53	0.87
	Proliferation of blood vessels	0.53	0.6	0.67
	Cartilage/bone detritus	0.47	0.67	0.93
	Hemosiderosis	0.53	0.87	0.67
	Cumulative Microscopic OARSI Synovial Membrane Score	5.4	7.27	7.53
	Hematoxylin-eosin Staining	0.47	0.87	1.27
	Structure	0.53	1.27	2.27
	Chondrocyte Dencity	0.4	0.6	0.8
	Cluster formation	0.47	0.47	0.8
	Cumulative Microscopic OARSI Cartilage Score	1.87	3.2	5.13
	Subchondral Bone Plate	0.6	0.8	1.47
	Bone Volume	0.47	0.87	0.93
	Osteophyte	0.53	0.73	0.8
	Cumulative Microscopic Cartilage Score	1.6	2.4	3.2

4. Discussion

Collagenase is an enzyme that will damage most joint structures such as tendons, ligaments, and meniscus. Intra-articular injection of collagenase to induce osteoarthritis has been known since 1990. In the previous literature it was found that one injection of collagenase was able to cause significant and progressive osteoarthritis for 2 weeks. Maximum effect occurs at 10 weeks, after which repair will begin to return to normal when collagenase is applied to young *Rattus norvegicus*. The success rate in inducing OA is 100% with cartilage fibrillation occurring within 7 days, cartilage erosion within 21 days and osteophyte formation within 42 days.(11,12)

Apart from the knee joint, collagenase administration was also found to cause OA of the facet joints in the lumbar spine. At all doses (1U, 10U, and 50U), collagenase resulted in cartilage fibrillation and cartilage calcification within 1 week. In addition, hypertrophy and inflammation of the subsynovial tissue and changes in the subchondral bone occur within 1 week as well. Osteoclasts were also found to be elevated, indicating the role of collagenases not only in joint structure but also in bone(13).

When assessed based on OARSI, collagenase administration had a progressive difference over time. This indicates that the progression of OA caused by collagenase is significant every week (13). The effect of giving collagenase in this study was similar to the description of previous studies. Where there are changes in the synovial structure, cartilage, and subchondral bone in *Rattus norvegicus* compared to those not given collagenase.(14)

Another thing that is interesting in this study is that the administration of collagenase, which is small compared to previous studies, has proven to be successful in inducing the occurrence of osteoarthritis. Previous studies generally used 10 collagenase units compared to the 3 units used in this study. This shows that the effectiveness of collagenase is indeed quite large(11,14,15).

It is interesting to note that the collagenase injection technique must be of the right volume, because as previously stated. It was found that the distribution of joint destruction was uneven. This suggests that perhaps collagenase needs to be administered in larger volumes or injected more than once to create a more even breakdown.

It was found that there was a clear and microscopically progressive difference 3 weeks after collagenase injection. Induction of OA using collagenase injection in experimental animals *Rattus norvegicus* can be a good model for making secondary OA because various microscopic changes in cartilage, synovial membrane, and subchondral bone can be clearly observed.

Conflict of interest

The authors declare no potential conflict of interest with respect to the research, authorship and/or publication of this article.

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Authors' contributions

Concept: C.H.B, D.N.U, L.W Design : C.H.B, L.W, A.R.H Data Collection : C.H.B, A.R.H Literature search : C.H.B, A.R.H Writing : C.H.B, D.N.U

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