

Ameliorative Effects of Prolotherapy on Histomorphology of Tibial Articular Cartilage of Chemically Induced Osteoarthritic Knee Joint in a Rat Model

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ABSTRACT

Objective: To determine the ameliorative effects of prolotherapy on monosodium iodoacetate (MIA) induced and histomorphological changes in the articular cartilage of tibial condyles at rat knee joint.

Study Design: An experimental study.

Place and Duration of the Study: Department of Anatomy, Army Medical College Rawalpindi, NUMS, Rawalpindi, from August to November 2021.

Methodology: Thirty adult male Sprague Dawley rats were divided into three groups, each having 10 rats. Group A was control. Group B was injected with single dose of 1mg MIA intraarticularly in the right knee to induce osteoarthritic changes. Group C was injected with single dose of 1mg MIA intraarticularly, in right knee was followed by 0.1ml Prolotherapy (3ml of 25% dextrose, 2ml of 2% xylocaine, 1ml of injection neurobion, and 1ml of injection methecobal) as intra articular injection at week 2, 6 and 10 in right knee. Rats were sacrificed after one month of the last dose of Prolotherapy. Articular cartilage was collected for gross and histological examination and compared among the groups.

Results: Articular cartilage belonging to control group A was normal. While group B showed statistically significant deterioration in gross appearance ($p = 0.001^{**}$), reduction in number of chondrocytes ($p = 0.005^{*}$) and thickness of articular cartilage ($p = 0.001^{**}$) in comparison to group A. In group C due to prolotherapy statistically significant improvement in gross appearance ($p = 0.034^{*}$), increase in number of chondrocytes ($p = 0.003^{*}$), and thickness of articular cartilage ($p = 0.001^{**}$) was observed as compared to group B.

Conclusion: Prolotherapy significantly ameliorates histomorphology of tibial articular cartilage against MIA induced osteoarthritic changes in rat knee joint.

Key Words: Articular cartilage, Knee joint, Monosodium iodoacetate, Osteoarthritis, Prolotherapy.

How to cite this article: Zahid A, Qamar K, Tabassum A, Abaid M, Kiani MRB, Aslam M. Ameliorative Effects of Prolotherapy on Histomorphology of Tibial Articular Cartilage of Chemically Induced Osteoarthritic Knee Joint in a Rat Model. *J Coll Physicians Surg Pak* 2023; **33(08)**:836-841.

INTRODUCTION

A degenerative joint disease osteoarthritis (OA) causes poor health-related quality of life.¹ More than 250 million people are thought to be affected globally.² Obesity, age, joint injuries, and genetic characteristics are all major risk factors of OA.³ The most effective surrogate for human research of OA is an animal model.⁴ Intraarticular Injection of MIA induce osteoarthritic changes similar to human degenerative OA in terms of histopathology and behaviours related to pain.³ Regenerative medicines have revolutionised the treatment of OA and have potentially eliminated the need for invasive surgery.⁵

Prolotherapy aids healing and pain relief by promoting tissue growth.⁶ Techniques and injectable solutions for prolotherapy differ depending on the disease, clinical severity, and practitioner preferences.⁷ Prolotherapy is a simple and economical procedure with a high range of safety and can be easily administered in a primary care setting, and worthy of consideration.⁸ The objective of this study was to determine the ameliorative effects of prolotherapy on MIA induced histomorphological changes in the articular cartilage of tibial condyles at rat knee joint.

METHODOLOGY

It was an experimental study conducted from August to November 2021 at the Department of Anatomy, Army Medical College/ NUMS, Rawalpindi in cooperation with the National Institute of Health (NIH), and Armed Forces Institute of Pathology, Rawalpindi. Ethical approval was taken from the ethics committee of Army Medical College / NUMS, Rawalpindi (ERC/ID/129). The inclusion criteria were healthy male rats

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Received: December 09, 2022; Revised: June 24, 2023;

Accepted: July 17, 2023

DOI: <https://doi.org/10.29271/jcpsp.2023.08.836>

weighing 250 ± 50 grams, aged 3-4 months. Exclusion criteria were rats having any gross joint deformity.

Thirty male Sprague Dawley rats were obtained from NIH, Islamabad. Rats were kept at the animal house of NIH under standard conditions. Rats were allowed free access to a standard lab diet and clean drinking water. No animal death was reported throughout the study period.

Rats were randomly divided into three groups, each having 10 rats with 5 rats per cage. Sampling technique was non-probability convenience sampling. Group A was control group. Group B (experimental group) single dose of 1mg MIA was injected intraarticularly in the right knee to induce osteoarthritic changes. Group C (experimental group) single dose of 1mg MIA was injected intraarticularly, in the right knee was followed by 0.1ml prolotherapy (3ml of 25% dextrose, 2ml of 2% xylocaine, 1ml of injection neurobion, and 1ml of injection methecobal) as intraarticular injection at week 2, 6, and 10 in the right knee. After euthanasia of all groups, rats were placed in a supine position on dissecting board and the right knee joint was identified. The knee joint was exposed after the skin and extracapsular ligaments were dissected. The fibrous capsule and surrounding tissues were carefully removed. A pediatric bone cutter (size 5) was used to separate the femoral and tibial condyles. Gross osteoarthritic change of articular cartilage were noted using a hand lens. Modified outerbridge classification was used for macroscopic scoring of articular cartilage.⁹

After the macroscopic evaluation of articular cartilage, the upper end of the tibia (the specimen) was fixed for 24 hours in an appropriately labelled plastic container containing 10% formalin. Containers were labelled as A, B, and C for group A, B, and C, respectively. Specimen number was labelled as N.¹⁻¹⁰ Specimen were placed in a jar with 5% nitric oxide for decalcification.¹⁰ The bone was examined on the daily basis and decalcified when it became soft and flexible. The decalcification process was completed in four days. A surgical blade was used to cut the specimen into coronal section according to OARSI guidelines.¹¹ Articular cartilage and subchondral bone was present in each section. Tissue was processed in ascending sequence of ethyl alcohol followed by infiltration and embedded in paraffin wax at 58°C. The wax was then allowed to cool and solidify. Two blocks were made per specimen. Rotary microtome was used to obtain 5 µm thick sections. Sections were floated on water bath at 45°C. Extra folds were removed and sections were mounted on clear glass slide. Two slides per block were made to evaluate histopathological changes. For histological study Hematoxylin and eosin and toluidine blue stain was used.

Microscopic quantitative observations were observed and measured by micrometry under 40X objective. Four observations were made for the number of chondrocytes from each slide. Two from the central regions and two from the peripheral regions across the articular cartilage. The mean was calculated for each slide. The final reading was taken after calculating the

mean of the individual slide of each animal. Cells with large nucleus and basophilic cytoplasm surrounded by lacuna and halo surrounding lacuna were labelled as normal chondrocytes.¹²

At four sites thickness of articular cartilage in µm was assessed, two from the central regions and two from the peripheral regions across the articular cartilage from surface to the tide mark.¹³ The mean value was calculated for each slide. The final reading was taken after calculating the mean of the individual slide of each animal.

Toluidine blue stained slides were graded according to Mankin's histological histochemical grading system. The grade for OA was calculated after counting the score for each parameter. The normal number of chondrocytes was determined using the range of cells in the control group. Hypocellular and hypercellular were labeled for cell count that was above or below this range. Clones were identified as clusters of four or more chondrocytes.¹⁴ A decrease in matrix staining was noted in experimental groups as adopted by Pauli *et al.*¹⁵ A tidemark criterion was excluded, as calcified cartilage and subchondral bone was not included, resulting in the Mankin score being modified to a total score of 11.¹⁶ Mild articular cartilage changes were shown as low scores whereas severe OA was indicated as a high score. Osteoarthritis was classified as (1-3 points) low, (4-7 points) moderate, or (>8 points) high.¹³

IBM SPSS version 22 was used for statistical analysis. Quantitative variables, were shown as means \pm SD whereas qualitative variables were expressed as frequency and percentage. One-way analysis of variance (ANOVA) was applied followed by Post Hoc Tukey's test to compare the difference in quantitative variables, whereas Chi-square test was used to analyse qualitative variables. A p-value equal to or less than 0.05 was considered significant.

RESULTS

Comparison of frequencies and percentage of gross evaluation of articular cartilage according to modified-outer-bridge classification between control group A, group B, and C is shown in Table I. Significant deterioration in articular surface of group B was observed in comparison with group A ($p = <0.001^{**}$) whereas in group C significant improvement in articular cartilage surface was observed in comparison with group B ($p = 0.034^{*}$). There was significant difference in articular surface of group A and group C ($p = 0.01^{*}$).

The comparison of number of chondrocytes and thickness of cartilage among three groups is shown in Table II and Figures 1 and 2. In group B significant decrease in number of chondrocytes ($p = 0.005^{*}$) and thickness of cartilage ($p = 0.001^{**}$) was observed as compared to group A. In group C, significant increase in number of chondrocytes ($p = 0.003^{*}$) and thickness of cartilage ($p = 0.001^{**}$) were observed as compared to group B. Statistically there was no significant difference in number of chondrocytes between group A and group C (p -value 0.986). While significant results were observed when thickness of cartilage was observed between group A and group C ($p = 0.001^{**}$).

Table I: Comparison of frequencies and percentage of gross evaluation of articular cartilage between control group A, group B, and group C.

Gross evaluation of cartilage surface	Grade	Groups (n=10)			p-values for intergroup comparison		
		A	B	C	Group A vs. B	Group B vs. C	Group A vs. C
Normal cartilage	0	10(100%)	0	4(40%)	<0.001**	0.034*	0.01*
Chondral lesions (soft and swell)	1	0	2(20%)	4(40%)			
Partial-thickness defect with fissures reach the subchondral bone	2	0	4(40%)	2(20%)			
Fissuring of the cartilage with an area reaching the subchondral bone	3	0	1(10%)	0			
Erosion of the subchondral bone	4	0	3(30%)	0			

Fisher's exact test was applied for intergroup comparison.

Table II: Comparison of mean values of Number of chondrocytes and thickness of cartilage between control group A, group B, and group C.

Parameters	Group A	Group B	Group C	p-values for intergroup comparison		
				Group A vs B	Group B vs C	Group A vs C
Number of chondrocytes	63.40 ± 13.18	48.30 ± 6.12	64.10 ± 8.26	0.005*	0.003*	0.986
Thickness of cartilage	181.60 ± 28.03	91.10 ± 8.88	139.30 ± 11.77	<0.001**	<0.001**	<0.001**

Post Hoc Tukey's test was applied for intergroup comparison.

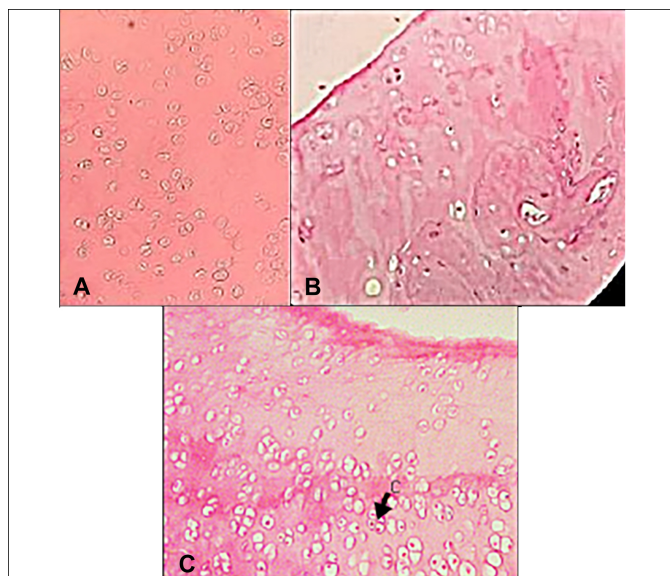


Figure 1: Photomicrograph of a histological section of articular cartilage showing the comparison of a number of chondrocytes in control group A, experimental group B, and experimental group C. H&E 400X. (A) Normal number of chondrocytes in A 10 rat of control group A. (B) Decreased number of chondrocytes in B 10 rat of experimental group B. (C) Increased number of chondrocytes and cloning of chondrocytes in C 10 rat of experimental group C. Key: Cloning (C).

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The severity of osteoarthritis was assessed by using Mankin's histological histochemical grading system shown in Figure 3. Mean Mankin's score for group A, B, and C was (0.000±0.000), (8.80±1.93), and (4.40±2.22), respectively. Intergroup comparison showed highly significant results when group A was compared with group B, group B was compared with group C and group A was compared with group C with a p-value of 0.001**.

DISCUSSION

OA is currently the main cause of disability in elderly people, affecting over 250 million people worldwide and posing a significant financial strain on the healthcare system.¹⁷ Regenerative medicine products had adopted a new approach for treating knee OA in recent years.

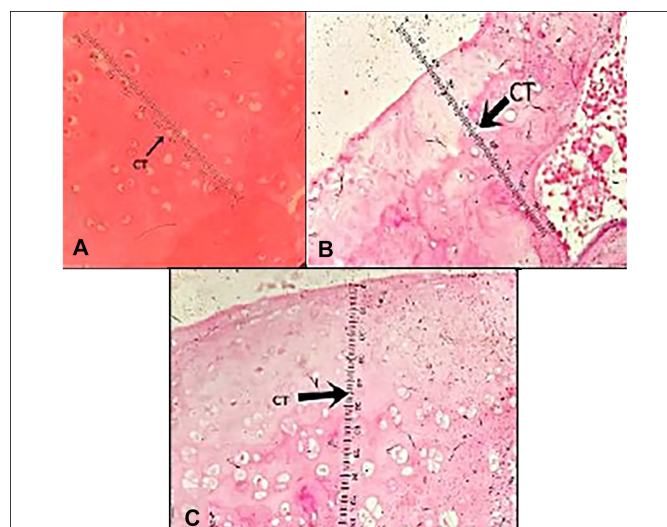


Figure 2: Photomicrograph of a histological section of articular cartilage showing the comparison of the thickness of cartilage (µm) in control group A, experimental group B, and experimental group C. H&E 400X. (A) Thickness of cartilage (80µm) in A10 rat of control group A. (B) Thickness of cartilage (60µm) in B10 rat of experimental group B. (C) Thickness of cartilage (100 µm) in C10 rat of experimental group C. Key: Cartilage thickness (CT).

It allowed the joint to restructure and heal on its own, resulting in less pain, increased mobility, and avoiding the need for invasive surgery.¹⁸ Prolotherapy is regarded as a valuable procedure and is usually reserved for patients who have failed other treatments or who are not surgical candidates. The effectiveness of Prolotherapy was documented in previous studies based on clinical outcomes and radiographic images.¹⁹

Regarding the gross appearance, articular cartilage belonging to control group A were smooth, glistening, and intact, reflecting features of normal cartilage. While group B showed significant deterioration in the gross appearance of articular cartilage in the form of chondral lesions, fissure, and erosion of articular cartilage and fibrillation when compared with group A and group C. According to Takahashi's research, 1-mg of MIA produce fissuring and fibrillation in the patellofemoral joint after 2 weeks, this is similar to this study.¹¹ The prolotherapy

treated group C showed less damage to articular cartilage that was statistically significant. Cartilage appeared normal and intact in most of the specimens while few specimens showed surface lesions and fissures extending to the mid-zone. These findings were in agreement with previous studies in which regenerative PRP was used for the treatment of knee OA.²⁰

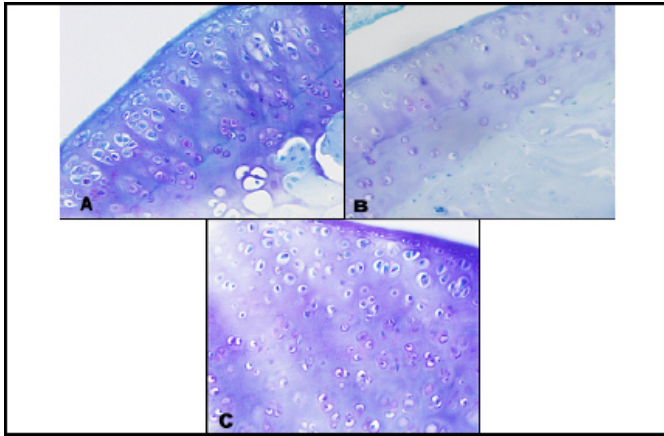


Figure 3: Photomicrograph of a histological section of articular cartilage showing the comparison of matrix staining in control group A, experimental group B, and experimental group C. Toluidine blue 400X. (A) Normal staining of a matrix in A10 rat of control group A. (B) Severe reduction of matrix staining in B10 rat of experimental group B. (C) Light reduction of matrix staining in C10 rat of experimental group C.

When group B was compared with control group A and group C, the intergroup comparison showed highly significant results with a significant decrease in the number of chondrocytes and significant decreases in thickness of cartilage. The reason was high oxidative stress and chondrocytes apoptosis as a result of osteoarthritis progression. As MIA inhibits glyceraldehyde 3 phosphates, an enzyme involved in glycolysis of chondrocytes results in apoptosis and death of chondrocytes.²¹ The hallmark of osteoarthritis progression is decrease in the number of chondrocytes and extracellular matrix loss.²² In the present study, the regenerative capacity of prolotherapy on the histological basis was observed as an increase in the number of chondrocytes, decrease in the size of chondrocytes as compared to control group A, with cluster formation (cloning), and increase in thickness of cartilage. Prolotherapy stimulates the activity of fibroblast, cause proliferation of vessels, deposition of collagen and cartilage growth as supported by prolotherapy (10% dextrose) resulted in pain relief,¹ decreased knee swelling, and improvement in the knee range of motion, with betterment in OA severity, based on radiographic images.²³ In the present study, the improvement in OA severity was observed on a histological basis. Fulltern also conducted a randomised clinical trial that found prolotherapy treatment improved radiological grades.²⁴

The severity of osteoarthritis was graded by using Mankin's histological histochemical grading system. In the present

study, the mean mankins' score for control group A was 0.00 ± 0.00 (range 0-0) revealed no OA. While mean Mankins score for group B was 8.80 ± 1.93 (range 4-7, high-grade OA). Monosodium iodoacetate-induced osteoarthritis was evident on 2 weeks, and the non-treated experimental group B was assessed on fourteenth weeks in the present study had a definitive effect on cartilage and graded as high, as shown in previous studies.²⁵ The mean Mankins score was 4.40 ± 2.22 (range 1-3) in group C revealed moderate grade OA. Prolotherapy had limited the progression of osteoarthritis in this group. In patients with knee osteoarthritis, Dextrose prolotherapy was found to be effective in lowering pain, enhancing function, and improving quality of life.¹

The authors recommend prolotherapy to patients with established osteoarthritic changes after the confirmation of regenerative histological changes seen in the present study. Prolotherapy will be a promising method for the treatment of knee OA in the future due to its ease of use, low cost, safety, and outpatient treatment.

The limiting factor of this study was that histopathological changes were not investigated in conjunction with biochemical markers and radiological imaging to have a better understanding of the ameliorative effects of prolotherapy. Duration of study should be widened in the future study and histopathological changes should be investigated in conjunction with radiological imaging to have a better understanding of the ameliorative effects of prolotherapy.

CONCLUSION

Prolotherapy significantly ameliorates the histomorphology of tibial articular cartilage by improving gross morphology and with corresponding increase in number of chondrocytes and thickness of cartilage hence improving grade of OA against MIA induced osteoarthritic changes in the rat knee joint.

DISCLOSURE:

The article is extracted from a thesis project.

FUNDING:

This study was funded by the National University of Medical Sciences (NUMS), Rawalpindi, Pakistan.

ETHICAL APPROVAL:

Ethical approval was taken from the ethics committee of the Army Medical College / NUMS, Rawalpindi (ERC/ID/129) prior to the initiation of the research work.

PATIENTS' CONSENT:

Not applicable.

COMPETING INTEREST:

The authors declared no competing interest.

AUTHORS' CONTRIBUTION:

AZ, KQ: Substantial contribution to the concept and drafting the work.

AT: Interpretation of the data and drafting of the work.

MA: Substantial contribution to the interpretation of the data and drafting of the work.

MRBK: Drafting and revising the final version.

MA: Drafting the histopathological study.

All the authors have approved the final version of the manuscript to be published.

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