EXPERIMENTAL STUDY

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Effect of low-dose irradiation on structural and mechanical properties of hyaline cartilage-like fibrocartilage

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Objective: The aim of this study was to analyze the effect of low-dose irradiation on fibrous cartilage and to obtain a hyaline cartilage-like fibrocartilage (HCLF) with similar structural and mechanical properties to hyaline cartilage.

Methods: An osteochondral defect was created in 40 knees of 20 rabbits. At the 7th postoperative day, a single knee of each rabbit was irradiated with a total dose of 5.0 Gy in 1.0 Gy fractions for 5 days (radiotherapy group), while the other knee was not irradiated (control group). Rabbits were then divided into four groups of 5 rabbits each. The first three groups were sacrificed at the 4th, 8th and the 12th postoperative weeks and cartilage defects were macroscopically and microscopically evaluated. The remaining group of 5 rabbits was sacrificed at the 12th week and biomechanical compression tests were performed on the cartilage defects.

Results: There was no significant biomechanical difference between the radiotherapy and the control group (p=0.686). There was no significant macroscopic and microscopic difference between groups (p=0.300). Chondrocyte clustering was observed in the irradiated group.

Conclusion: Low-dose irradiation does not affect the mechanical properties of HCLF *in vivo*. However, structural changes such as chondrocyte clustering were observed.

Key words: Hyaline cartilage; hyaline cartilage-like fibrocartilage; low-dose irradiation; mechanical; structural.

Joint cartilage injuries are either partial-thickness or full-thickness injuries and may progress toward the subchondral bone.[1] The response of the human body to cartilage injury and stages of the healing process depends on the injury type. Although a rapid and active healing process begins after superficial chondral injuries, the efficacy of the repair process diminishes over time and the repair activity cannot be sustained for long periods.[2] However, superficial chondral injuries do not cause progressive lesions which lead to clinical osteoarthritis. Full-thickness subchondral injuries heal with hyaline cartilage-like fibrocartilage (HCLF).[3]

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Some treatment techniques for full-thickness chondral defects are based on stimulating the healing process. Techniques that involve the subchondral bone including microfracture, drilling and abrasion stimulate bone marrow.[4-6] These types of interventions to the bone cause a fibrovascular tissue growth at the site of the chondral defect. This fibrovascular tissue contains bone marrow-derived mesenchymal cells with high replication capacity and the potential to transform into the fibrochondrocytes that form HCLF. HCLF contains type-1 collagen instead of type-2 collagen. Due to this structural difference, the mechanical properties of HCLF differ from the original chondral tissue. The resistance of HCLF against mechanical forces is weak and its ability to fill defects and protect structural integrity is not as good as that of hyaline cartilage.[7,8] One potential problem with these stimulating techniques is that the amount of mesenchymal cells supplied from the subchondral bone is insufficient.[9] It has been proposed that if the amount of fibrous tissue can be decreased then the amount of mesenchymal cells and chondrocytes derived from these cells will increase proportionally.

Radiotherapy has been long in use for the treatment of benign disorders. The main aim of radiotherapy in treating benign disorders is to suppress the inflammatory process and the accumulation of unwanted tissues.[10] The high activity level and increased proliferation rate of fibroblasts following tissue trauma make them more susceptible to radiation. Low-dose radiotherapy on fibroblasts is shown to suppress the expression of certain growth factors, alter extracellular matrix deposition and modification and cause growth arrest and paucity of proliferation and neovascularization. In addition, it has a stronger suppressive effect over fibrous tissue than over mesenchymal cells.[11,12] These properties have led to its use in selected cases, such as heterotopic ossification, keloids, macular degeneration, arteriovenous malformations, and Graves' disease.[13] The common rationale in treating these diseases with radiation is to suppress the unwanted aberrant connective tissue. Its effectiveness against fibroblast activity and its non-invasive applicability make it a potential candidate to prevent the formation of fibrous cartilage with high fibrous tissue content.

The aim of this study was to evaluate if irradiation of HCLF changes the ratio of fibrous tissue to mesenchymal cells in favor of mesenchymal cells and therefore increases the strength of HCLF.

Materials and methods

This animal experiment was carried out after institutional approval and in accordance with Medical School Policies and Guidelines for the Care and Use of Laboratory Animals. All measures were taken to minimize animal suffering.

As a preliminary experiment, an osteochondral defect was made in the left knee of a rabbit. The rabbit was sacrificed on Day 10 and the defect in the medial femoral condyle was observed macroscopically. Although the defect could be macroscopically seen, it was filled with healing tissue. In microscopic evaluation, it was demonstrated that this healing tissue was fibrovascular in nature (Fig. 1). Previous studies reported the dominance of undifferentiated mesenchymal cells with fibroblastic and chondrogenic potential at 7 days and the appearance of fibroblasts and chondrocyte-like cells at 10 days.[14] We aimed to use the suppressive effect of radiotherapy on fibroblasts in this critical healing period where undifferentiated cell lineages prepare to transform to specific cell types. Since the mesenchymal stem cells were fibroblastic at 10 days,[14] it was not possible to determine the specific cell types to which they would transform in the healing tissue. Therefore, the rationale of timing of radiotherapy was assumed that radiotherapy performed at the appropriate time would decrease domination of fibrous tissue.

Twenty mature female New Zealand white rabbits weighing between 2,200 and 3,750 g with no significant weight differences (p=0.632) were used. The left knee of each rabbit was included in the study group and the right knee was included in the control group,

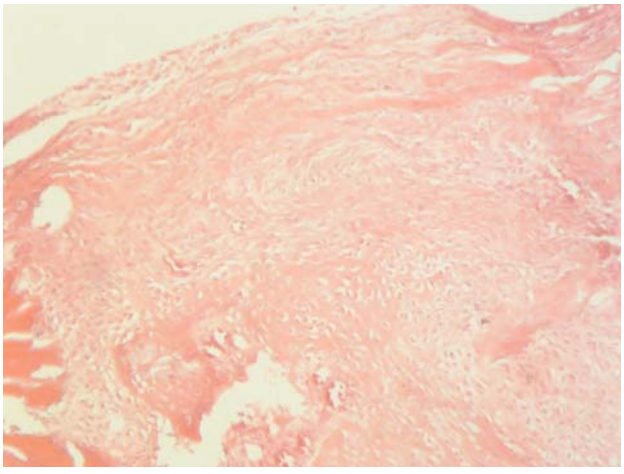

Fig. 1. Dominance of fibrovascular tissue in the healing cartilage (H&E x100). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

for a total of 40 knees. Osteochondral defects were created in both knees. On the following week, the knees in the study group (left knees) were irradiated with 5.0 Gy. Subjects were divided into 4 groups, each including 5 rabbits. Three groups were sacrificed at 4th, 8th, and 12th weeks respectively for histological evaluation. The remaining 5 rabbits were sacrificed at the 12th week for biomechanical tests.

Rabbits were anesthetized with intramuscular ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (4 mg/kg). Each rabbit received intramuscular antibiotic prophylaxis (15 mg/kg cefazolin sodium). The medial femoral condyle was exposed via medial parapatellar arthrotomy. A defect with 3 mm depth was created with a 2.5-mm drill bit. A small hole was drilled in the subchondral bone with a 1-mm Kirschner wire to stimulate the bone marrow. All surgical procedures were carried out by the same surgeon. No sign of infection or locomotion disturbance were noted during the study period. No animals were lost before the time of sacrifice.

Beginning from the postoperative 7th day, a total dose of 5.0 Gy radiation was applied to the knees for five consecutive days in 1.0 Gy fractions per day with a standard linear accelerator using 6 MV photons (Siemens Mevatron-KD2 Linear Accelerator; Siemens AG, Erlangen, Germany), as this dose has been known for its anti-inflammatory and fibroblast suppressing effects in the rabbit and it is well below the lethal dose for fibroblasts and chondrocytes.[15-17] Rabbits were anesthetized in the same way as the surgery. The rabbits were sacrificed in groups of 5 at 4th, 8th and 12th weeks. Chondral defects in the femoral condyles were evaluated macroscopically for filling of the defect and degenerative changes in the surrounding chondral tissue. After macroscopic evaluation, specimens were fixed with 10% formaldehyde solution for 3 days and then decalcified with formic acid. Serial slides were cut and stained with hematoxylin-eosin and safranin-O. Tissue morphology and healing process were evaluated according to the O'Driscoll scale (Table 1).[18,19]

Biomechanical testing was performed on the knees of the fourth group of 5 rabbits sacrificed at the 12th week. Harvested knees were stored at -80°C. The samples were slowly thawed overnight at +4°C and at room temperature on the following day. Specimens were kept moist with 0.9% NaCl-soaked gauzes during the testing period. The diaphyseal parts of the specimens were potted in 1.5×1.5 cm containers using polyurethane resin. The diaphyseal part of the femur was fixed to the lower clamp of the testing machine (Model 4301; Instron, Norwood, MA, USA). Indentation testing was performed using a 2-mm diameter, cylindrical, plane-ended indenter. Gradually increasing force was applied with a speed of 1 mm/min and displacement was recorded continuously for 60

Table 1. Tissue morphology and healing process evaluation with O'Driscoll scale.

Nature of the predominant tissue	Score
Cellular morphology	
Hyaline articular cartilage	4
Incompletely differentiated mesenchyme	2
Fibrous tissue or bone	0
Safranin-O staining of the matrix	
Normal or nearly normal	3
Moderate	2
Slight	1
None	
Structural characteristics	
Surface regularity	
Smooth and intact	3
Superficial horizontal lamination	2
Fissures: 25-100% of the thickness	1
Severe disruption, including fibrillation	0
Structural integrity	
Normal	2
Slight disruption, including cysts	1
Severe disintegration	
Thickness	
100% of normal adjacent cartilage	2
50-100% of normal cartilage	1
<50% of normal cartilage	0
Bonding to the adjacent cartilage	
Bonded at both ends of graft	2
Bonded at one end, or partially at both ends	1
Not bonded	
Cellular changes and evaluation of degeneration	
Hypocellularity	
Normal cellularity	3
Slight hypocellularity	2
Moderate hypocellularity	1
Severe hypocellularity	0
Chondrocyte clustering	
No clusters	2
<25% of the cells	1
5-100% of the cells	0
Absence of degenerative changes in adjacent cartilage	
Normal cellularity, no clusters, normal staining	3
Normal cellularity, mild clusters, moderate staining	2
Mild or moderate hypocellularity, slight staining	1
Severe hypocellularity, poor or no staining	0
Total	24

Fig. 2. Assembly of the samples in the indentation testing device. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

seconds (Fig. 2). Load-deformation curve was drawn and the slopes of the linear parts of the load-deformation curves produced within the first millimeter of compression (Δ Force / Δ Displacement) were used to calculate the stiffness of the specimens.

Statistical analysis of the biomechanical test results and microscopic evaluation scores were performed using the Wilcoxon test. Significance was indicated at $p<0.05$.

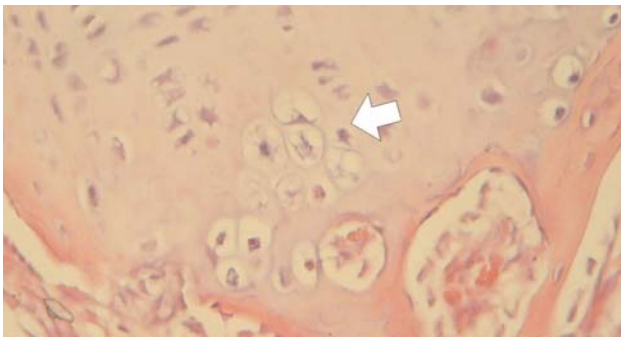

Fig. 3. Clustering of chondrocytes (arrow) occurred in the irradiated group in 4 weeks (H&E x400). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Results

Chondral defects were repaired with a whiter and well-demarcated healing tissue in all knees. No difference was visualized between the control and study groups. No macroscopic evidence of osteoarthritis including osteophyte, chondral erosion or synovial hypertrophy was detected.

HCLF tissue grown from the chondral defects were evaluated according to O'Driscoll scale's cell morphology and with the scores after safranin-O staining (Table 2). There was no statistical difference between the study and the control groups ($p=0.577$ and $p=0.773$, respectively). HCLF was seen in all knees. Clustering of chondrocytes occurred in the study group in the 4th week (Fig. 3).

Mechanical results of the specimens 12 weeks after defect formation are summarized in Table 3. Curves at

Table 2. O'Driscoll scoring of radiotherapy and control groups.

Nature of the predominant tissue	Radiotherapy	Control	p
4 weeks	3.60±1.81	5.20±3.03	0.216
8 weeks	2.40±1.67	2.80±2.77	0.705
12 weeks	4.20±2.48	3.20±2.16	0.500
Structural characteristics	**Radiotherapy**	**Control**	**p**
4 weeks	4.60±2.88	7.20±3.49	0.104
8 weeks	2.20±1.78	2.80±2.48	0.276
12 weeks	4.20±2.16	4.00±2.00	0.785
Cellular changes and evaluation of degeneration	**Radiotherapy**	**Control**	**p**
4 weeks	5.20±1.92	7.40±1.30	0.041
8 weeks	4.00±1.73	4.00±1.73	1.000
12 weeks	5.60±0.54	5.40±0.54	0.317
Total score	**Radiotherapy**	**Control**	**p**
4 weeks	13.40±5.77	19.80±7.80	0.068
8 weeks	8.60±4.39	9.60±6.06	0.892
12 weeks	14.00±4.52	12.60±3.97	0.478

Table 3. Biomechanical results indicated in cartilage stiffness (Δ Force / Δ Displacement).

Specimen	Radiotherapy	Control
1	92.3 N/mm	114.3 N/mm
2	99.6 N/mm	182.7 N/mm
3	180.9 N/mm	257.1 N/mm
4	242.6 N/mm	163.3 N/mm
5	150.7 N/mm	91.9 N/mm

the straight points of the load-deformation curve were measured and chondral stiffness was determined. The results were 161.87±64.55 N/mm and 153.20±61.91 N/mm in the study and control group, respectively. There was no statistically significant difference between the groups.

Discussion

The repair of traumatic defects in joint cartilage has been a challenge for researchers. These traumatic defects can lead to degenerative changes which may eventually result in secondary osteoarthritis.[1,20-23] In this study, we evaluated the effect of radiotherapy on HCLF tissue formed after osteochondral defect and bone marrow stimulation. Our defect model is a focal osteochondral defect usually seen in young individuals.[2]

Radiotherapy can be utilized in non-malignant disorders including arthritis, tendinitis and heterotopic ossification.[24] Several previous studies reported the effects of irradiation on normal chondral tissue[12,25] and the anti-inflammatory effects of low-dose irradiation on arthritis.[15,26,27] However, the effect of irradiation on HCLF has not been documented before.

Radiosynovectomy is an alternative to surgical synovectomy and can be used to suppress synovial inflammation. Its effects in chondrocyte culture, including decrease in collagen production, nitric oxide (NO) production and cell death have been demonstrated *in vitro*.[28] However, *in vivo* animal studies utilizing different substances for β-radiation did not show any evidence of chondral damage caused by radiosynovectomy.[29,30]

Healing of osteochondral defects occurs with HCLF tissue on the articular side and new bone formation on the osseous side.[1] There are various methods for biomechanical testing of HCLF. Wakitani et al. measured the depth of chondral tissue with the needle penetration technique and integrated the results into indentation tests to determine compliance and stiffness of chondral tissue.[14] Messner[31] and Nam et al.[32] described the same technique, which was also used in our study. In both studies, porous and flat surfaces with different radii were used, however, as the healing tissue can form concave and convex surfaces, contact surface areas can be different. In our study, we could not find a significant difference between study and control groups (study group: 153.20±61.91, control group: 161.87±64.55). It is possible that the sample size was too small to rule out beta error. As our study aimed to analyze both histological and biomechanical properties at different time periods, populations of individual groups were relatively small in order to avoid animal sacrifice. A study solely on biomechanical properties with a larger sample size is needed to detect potential alterations on tissue stiffness.

Timing of the radiotherapy was planned to cover the time interval in which multipotent cells transform to chondrocytes. Shapiro et al.[6] evaluated the formation of HCLF tissue after osteochondral defects. They observed chondral extracellular matrix synthesis at Day 10. In our study, we applied radiotherapy within 10 days (Days 7, 8, 9, 10 and 11). One possible approach to modify this experiment could have been to initiate radiotherapy earlier, since fibroblasts express growth factors for some period of time even after receiving radiation in lethal doses.[17] However, early application of radiotherapy may lead to suppression of inflammation and stem cell transport due to decreased blood supply, interrupting the cartilage regeneration process.[11] Because fibrous tissue is shown to be more sensitive to radiotherapy than chondrocytes,[11,12] we aimed to suppress fibrous tissue and promote chondrocyte proliferation in healing tissue. Since 5 Gy of radiation did not alter the mechanical properties of HCLF, effect of higher doses in different fractions or as single dose may be investigated to come up with an ideal dose which can suppress the fibroblast activity without damaging chondral and subchondral tissues.

Irradiation has also been reported to prevent vascularization.[10] Thus, it can be helpful in preventing neovascularization which contribute to the structural damage observed in osteoarthritis. In our study, we did not observe neovascularization in the late degenerative phase following chondral damage.

It is still not clear which type of local and systemic factors affect the differentiation of mesenchymal cells to osteoblasts, chondroblasts or fibroblasts.[6] Some factors important in chondrogenesis include; IGF-1, TGF-β, BMPs, growth/differentiation factor-5, FGF, PDGF, and cartilage-derived growth factor.[33,34] In addition to these anabolic factors, TNF-α, IL-1 and IL-17 have influence on the healing process.[35] Poole emphasized that blocking these cytokines while giving anabolic factors can aid in chondral repair.[23] Although

no detailed study on the effects of empirical radiotherapy over cytokines in inflammatory diseases has been made, few have suggested that it decreases cytokines.[15,26,27,36]

We found more degenerative changes in chondral defects in the study group in the 4th week ($p<0.05$). Degenerative changes in joint cartilage are related to hypertrophic differentiation of chondrocytes. Bone matrix calcifies, leading to a fragile chondral tissue and degenerative changes.[23] In our study, clustering of chondrocytes occurred in the study group in the 4th week and resulted in a poorer O'Driscoll score in which cell clustering is addressed to be a degenerative change. Further detailed studies are necessary to distinguish whether chondrocyte clustering can be considered evidence of new chondrocyte production or degenerative changes.

Shapiro et al. proposed that the formation of HCLF tissue starts at the 4th week after osteochondral defects, reaching maturity at 12th week.[6] For this reason, animals are followed-up for 12 weeks before sacrifice in the majority of studies.[31,32,37-41] In our study, results at the 12th week follow-up showed no significant difference between the study and control groups. However, evaluation scores of the control group showed a decline from 4 weeks to 12 weeks. No difference was found in the study group in terms of scores at weeks 4 and 12. From this, it is thought that longer follow-up would allow for observation of more degenerative changes and a significant statistical difference between two groups.

One limitation of this study is that it did not examine the effect of radiation on the regenerative cells. Instead, we based our experiment on the current knowledge that low-dose radiation has a stronger suppressive effect over fibrous tissue than over mesenchymal cells and hypothesized that it would improve the mechanical properties of the regenerative tissue. Since results of this study did not show a difference between the two groups, it is possible that several steps in the regeneration process may be different than previously stated in other studies. New studies focusing on the migration, proliferation and transformation of regenerative cells to different dosing regimens are necessary as structural properties and responsiveness of these cells may differ in cartilage defect model due to local mediators and environmental conditions.

In conclusion, radiotherapy application to HCLF tissue does not change its mechanical properties *in vivo*. As studies evaluating the effects of irradiation on chondral defect repair have recently gained popularity, its possible role as an adjuvant technique in treatment of chondral tissue repair needs further evaluation. The present study can be accepted as a pioneer regarding the effects of irradiation on chondral healing. Still, comprehensive studies with longer follow-up and a larger sample size are needed to clarify the benefits, adverse effects and appropriate dose and timing for its plausible use in the clinical scene.

Conflicts of Interest: No conflicts declared.

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