Regenerative Medicine: Effect of Treatment with Biphasic Cross-Linked Hyaluronic Acid in Osteochondral Lesions

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ABSTRACT

Objective: To demonstrate whether treatment with biphasic cross-linked hyaluronic acid in osteochondral lesions promotes the regeneration of cartilage tissue. **Materials and Methods:** Fifteen adult female rabbits were randomly assigned to three groups. G1 was the control group, whereas G2 and G3 underwent surgery to treat an osteochondral injury in the right knee (4mm diameter, 5mm depth). G3 received treatment with 0.2 ml of hyaluronic acid intrarticularly after surgery. Clinical, biochemical, histopathological controls and imaging studies were performed. **Results:** Clinically, G3 exhibited less pain on palpation than G2 after 45 days. In G3, almost all samples showed evidence of cartilage tissue regeneration at the injury site, with neither bone edema or considerable joint effusion detected on MRI. The histological tests of G3 samples revealed an increase in the extracellular matrix of cartilaginous tissue when compared to G2, with hypercellularity caused by chondrocytes that formed axial and coronal isogenic groups. **Conclusions:** This study provides evidence that treatment with biphasic cross-linked hyaluronic acid in experimental units of rabbits with osteochondral injuries did not cause pain in the early stages of the injury. In turn, imaging and histopathological studies revealed that the injured tissue had been repaired.

Keywords: Hyaluronic acid; cartilage regeneration; osteochondral injury.

Medicina regenerativa de cartílago: efecto del tratamiento con ácido hialurónico reticulado bifásico en lesiones osteocondrales

RESUMEN

Objetivo: Evaluar si el tratamiento con ácido hialurónico reticulado bifásico de lesiones osteocondrales promovería la regeneración del tejido cartilaginoso, favoreciendo así la reparación de la lesión. **Materiales y Métodos:** Quince conejos hembra adultos fueron divididos aleatoriamente en tres grupos: grupo 1, de control; grupo 2 y grupo 3, sometidos a una estrategia quirúrgica de lesión osteocondral en la rodilla derecha (4 mm de diámetro, 5 mm de profundidad), el grupo 3 recibió tratamiento con 0,2 ml de ácido hialurónico por vía intrarticular después de la cirugía. Se realizaron controles clínicos, bioquímicos, histopatológicos y estudios por imágenes. **Resultados:** Se detectaron menos casos de dolor a la palpación en el grupo 3 que en el grupo 2 a partir de los 45 días. En la resonancia magnética, casi todas las muestras del grupo 3 tenían signos de regeneración del tejido cartilaginoso en el sitio de la lesión, sin edema óseo, ni derrame articular significativo. Los estudios histopatológicos de las muestras del grupo 3 indicaron un aumento de la matriz extracelular propia de tejido cartilaginoso, comparada con la del grupo 2, con hipercelularidad, dada por condrocitos, los que formaban grupos isogénicos axiales y coronales. **Conclusiones:** Este estudio brinda evidencias de que el tratamiento con ácido hialurónico reticulado bifásico en unidades experimentales de conejos con lesión osteocondral no tuvieron dolor en etapas tempranas después de la lesión, a diferencia de las unidades intervenidas y sin dicho tratamiento. A su vez, los estudios por imágenes e histopatológicos mostraron la reparación del tejido dañado.

Palabras clave: Ácido hialurónico; regeneración de cartílago; lesión osteocondral.

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INTRODUCTION

Osteochondral tissue is composed of articular cartilage and subchondral bone, and it has a low regenerative capacity. Its chemical nature and histoarchitecture allow it to support the high loads it endures. Osteochondral lesions involve the hyaline cartilage and subchondral bone of the joints, with effects ranging from minor discomfort to disabling conditions.[3] These lesions are acquired pathological entities of the subchondral bone that also involve the associated articular cartilage.[4] Regarding etiology, they are linked to mechanical factors, recurrent trauma, overload, infections, and vascular compromise, among others. These injuries commonly occur in the knee, but the articular surfaces of the elbow, ankle, hip, and shoulder can also be affected. The injury can result in delamination and potential sequestration of the affected bone, compromising joint function. Such injuries can lead to progressive joint destruction and cause moderate to severe pain, resulting in disabling conditions.[5-7] Their management and treatment remain a challenge in Traumatology and Orthopedics, as these entities behave differently depending on the joint affected. Despite promising clinical outcomes in the short and medium term, bone marrow stimulation often results in fibrocartilaginous tissue with mechanical and biological properties that differ from those of normal cartilage.[8]

Among the strategies that have shown improvement for patients with joint injuries, treatments with hyaluronic acid (HA) stand out. HA is a biologically active molecule, naturally secreted by chondrocytes, the cells of joint tissue.[9] It was first isolated in 1934.[10] HA is a natural, non-sulfated glycosaminoglycan, consisting of repeating units of D-glucuronic acid and N-acetylglucosamine, forming a central part of the proteoglycans in the extracellular matrix of the tissue. These complex macromolecules are responsible for the compressive strength of cartilage, playing a fundamental structural role in the tissue. HA can also interact with cells through receptors on the plasma membrane, such as the CD44 receptor, which activates several biological effects, including increased motility and amplified cell proliferation. [11-13]

The initial proposal to treat patients with osteochondral lesions using HA was based on promoting viscosupplementation, reducing pain, and improving the viscoelasticity of joint tissue. [14-17]

Numerous studies on HA treatment for joint injuries have been published, with encouraging clinical results.[15-18] In our country, a biphasic cross-linked HA (XLHA) with a longer half-life has been developed. However, there are no studies specifically considering the potential action of XLHA in osteochondral lesions in an in vivo model, examining clinical, biochemical, imaging, and histological aspects, and characterizing the potential changes in the injured tissue area after treatment.

Our working hypothesis is that in situ treatment with this product for osteochondral lesions would promote cartilage tissue repair and de novo cartilage tissue regeneration, thereby favoring the repair of the lesion with tissue that properly fulfills its functions.

MATERIALS AND METHODS

The *in vivo* studies were conducted on female New Zealand line rabbits (3-4 months old) in accordance with the Bone and/or Cartilage Tissue Engineering III project of the Faculty of Medical Sciences at the Universidad Nacional de Rosario. This project was approved by the Ethics Committee and CICUAL (Institutional Commission for the Care and Use of Animals in Laboratories), Code 80020220700113UR, Resolution No. 4888/2022. The rabbits were housed in individual cages with food and water provided *ad libitum* until the day before surgery and from the day after.

Fifteen experimental units were randomly divided into three groups (5 in each): group 1 (without osteochondral lesion: 1a, 1b, 1c, 1d, 1e) was used for control clinical and biochemical studies; group 2 (with osteochondral lesion, without receiving treatment: 2a, 2b, 2c, 2d, 2e); and group 3 (with osteochondral lesion treated with XLHA [Cientific Sinovial 60 A.F., Lab. Futerman International Products - Allanmar International Company S.R.L.]: 3a, 3b, 3c, 3d, 3e). Preliminary studies confirmed that this treatment did not clinically or biochemically affect healthy rabbits.

Preparation for the surgical procedure

A sterile surgical environment was established using ultraviolet irradiation. The right knee was shaved, and skin asepsis was performed with an iodine solution. Cefazolin (50 mg/kg/day) was administered intramuscularly. Anesthesia-sedation was achieved by administering a combination of ketamine hydrochloride (35 mg/kg), xylazine hydrochloride (2.0%, 18 mg/kg), and acepromazine maleate (1.0%, 1 mg/kg) intramuscularly.

Surgical procedure

Each experimental unit was placed in a supine position on the operating table, on sterile fields. A fenestrated sterile drape was placed centered on the knee, covering the torso while leaving the head uncovered. A 5 cm long midline skin incision was made on the right knee, with dissection through the skin layers. The medial approach was followed by arthrotomy via a subvastus approach, followed by lateral dislocation of the patella. An osteochondral lesion 4 mm in diameter and 5 mm deep was created in the trochlear groove at the level of the epicondyles using a motorized drill as an anatomical reference. The patella was reduced, and the joint capsule and medial retinaculum were sutured with Vicryl #2.0. A continuous subdermal suture was placed with Vicryl #4.0, and the skin was closed with Nylon 3.0. XLHA was then infiltrated into the knee.

Treatment with XLHA

After surgery, each experimental unit in group 3 received a single intra-articular injection of 0.2 ml of XLHA into the right knee.

Treatment of postoperative pain

Tramadol (100 mg/ml, 0.12 ml every 12 hours, 6 mg/kg/day) was administered intramuscularly for three days to manage postoperative pain.

Clinical monitoring

Clinical monitoring included assessments of body temperature, general condition, and response to light stimuli (using a flashlight with an intensity of 2-4 lux, each eye of each experimental unit was shined at a distance of 30 cm, and it was observed whether the rabbits squinted, as all healthy control units typically do). The presence or absence of associated inflammatory states in the operated area was also noted macroscopically. These evaluations were performed daily during the first week, and then every 15 days until the end of the experiment. From day 15 onwards, knee palpation was conducted every 15 days to detect pain (when experiencing pain, the experimental units would retract the leg and emit complaining sounds). This process involved lightly compressing the knee with the palm of the hand, first thing in the morning, on each knee of all experimental units. This procedure was carried out by a single operator in the presence of two other observers to verify the results.

Sampling for biochemical studies

Blood samples were taken before the procedure and at 1, 7, and 120 days after implantation. After homogenization, the samples were placed in EDTA tubes for hemograms. Red blood cell and white blood cell counts, hemoglobin levels, and platelet counts were determined to assess post-surgical status.

Statistical analysis

Non-parametric tests were conducted to determine if there were intergroup differences in the biochemical variables tested (Kruskal-Wallis test at 1, 7, and 120 days after implantation).

Imaging studies

At 120 days post-procedure, the anesthetized animals underwent magnetic resonance imaging (1.5 TESLA, General Electric, Signa HDxt with microcoil). MRI images were obtained in 1 mm slices in axial, sagittal, and coronal planes. The sequence used was proton density with fat suppression, which is suitable for assessing the articular cartilage. At the lesion site, the following were evaluated based on previous research:

a) the presence of cartilage,[19-21]

b) the possible presence of bone edema,[22,23].

c) joint effusion. [24,25]

Animal euthanasia protocol

The animals were euthanized immediately after the MRI scans. While still sedated, they were placed in a CO2 chamber, following international standards approved by CICUAL.

Protocol for sample collection, preservation and procedures for histopathological studies.

A longitudinal incision was made on the medial aspect of the thigh, following the direction of the femur. Two transverse incisions were made, one proximal to the coxofemoral junction and the other distal to the end of the knee joint. Dissection was performed in layers. The knee was disarticulated by incising the articular ligaments, separating the thigh from the leg. The femur was then disarticulated from the hip by incising the capsule and coxofemoral ligaments, and each piece was obtained for study.

Histopathological studies

The samples were preserved in 10% formaldehyde (48 hours), followed by decalcification processes. Each sample was immersed in stabilized decalcifying Biopur solution (EDTA 0.5M), with gentle periodic shaking, and the solutions were exchanged weekly until the absence of minerals in the samples was verified. The samples were then processed using conventional histological methods until paraffin-embedded plugs were obtained. These plugs were sectioned with a microtome (Leica SM2010 R) into 3 µm longitudinal sections, placed on slides, and stained with hematoxylin-eosin (Biopack). All samples were processed simultaneously during this final stage. The samples were observed under an optical microscope at 100x and 400x magnifications and evaluated using a double-blind strategy. For cell counting, 10 fields were observed at higher magnification for each sample.

RESULTS

Clinical studies

All animals in groups 2 and 3 responded adequately to the anesthetic and surgical processes. None of the animals experienced a temperature increase above normal values (compared to group 1), indicating a successful surgical process and effective antibiotic prophylaxis (normal temperature for New Zealand rabbits: 38.5°-39.5°C).

After the anesthesia wore off, the animals received normal hydration, and food consumption was enabled four hours later, matching that of group 1 animals from the second post-surgical day (300 ± 20 g/experimental unit/day).

There were no significant intergroup differences in response to light stimuli from day 4 post-procedure. On days 1, 2, and 3, all experimental units that underwent surgery were less responsive to light, which is typical of animals treated with tramadol, causing mild lethargy.

None of the units subjected to the procedure exhibited macroscopically visible inflammatory phenomena during the experiment. Beginning on day 15, knee palpation was performed every 15 days to assess pain. All animals in group 2 showed retraction to palpation with audible signs of pain up to and including day 120. From day 45 until the end of the study, none of the experimental units in group 3 retracted the knee or showed signs of pain upon palpation.

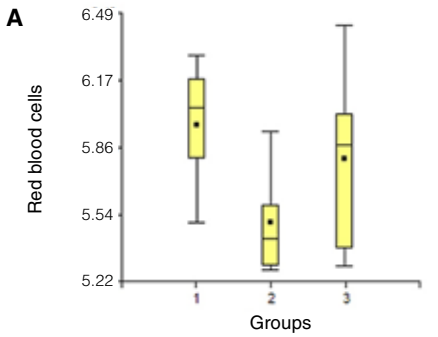
Biochemical results

No significant intergroup differences were found in the levels of red blood cells, white blood cells, hemoglobin, and platelets at any of the times studied (Figure 1).

Results obtained by MRI

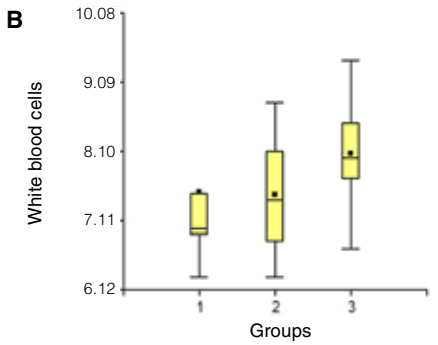
In group 2, no features indicative of cartilage regeneration were observed. A large amount of bone edema, increased joint effusion, and the absence of chondral tissue in the lesion area were noted (Table).

In contrast, group 3 showed the presence of cartilage, with no bone edema or significant joint effusion in the T1 sequence, as reported by other authors[19-21] (Table, Figure 2).



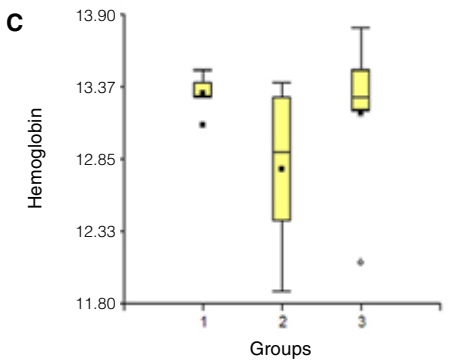
Kruskal–Wallis test

Variable	Groups N	Mean	S.D.	Median	H	p
Red blood cells 1	5	5.96	0.32	6.04	3.82	0.1479
Red blood cells 2	5	5.50	0.27	5.43		
Red blood cells 3	5	5.80	0.47	5.87		



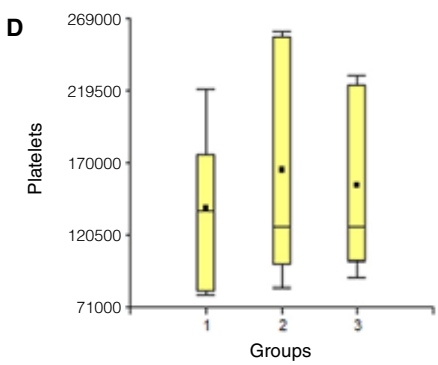
Kruskal–Wallis test

Variable	Groups N	Mean	S.D.	Median	H	p
White blood cells 1	5	7.52	1.40	7.00	-5.22	>0.9999
White blood cells 2	5	7.48	1.00	7.40		
White blood cells 3	5	8.06	1.00	8.00		



Kruskal–Wallis test

Variable	Groups N	Mean	S.D.	Median	H	p
Hemoglobin 1	5	13.32	0.15	13.30	2.18	0.3283
Hemoglobin 2	5	12.78	0.63	12.90		
Hemoglobin 3	5	13.18	0.65	13.30		



Kruskal–Wallis test

Variable	Groups N	Mean	S.D.	Median	H	p
Platelets 1	5	139000.00	60547.50	137000.00	0.76	0.6851
Platelets 2	5	165400.00	85806.76	126000.00		
Platelets 3	5	154400.00	67069.37	126000.00		

Figure 1. Box-and-whisker plots showing the distribution and central tendency of numerical values through their quartiles for different variables in groups 1, 2, and 3. **B.** Values for the variable white blood cell levels. **C.** Values for the variable hemoglobin levels. **D.** Values for the variable platelet levels. The results of the Kruskal-Wallis statistical analysis performed with the Infostat program are displayed alongside each graph.

Table. Results obtained by magnetic resonance imaging.

Sample	Group	Presence of cartilage	Presence of bone edema	Hydrarthrosis
2a	2	No	No	Moderate
2b	2	Data lost due to technical problems		
2c	2	No	No	Moderate
2d	2	No	Yes	Moderate
2e	2	No	Yes	Scarce
3a	3	Slight	No	Scarce
3b	3	Yes	No	Moderate
3c	3	Yes	No	Scarce
3d	3	Yes	No	Moderate
3e	3	Yes	No	Scarce

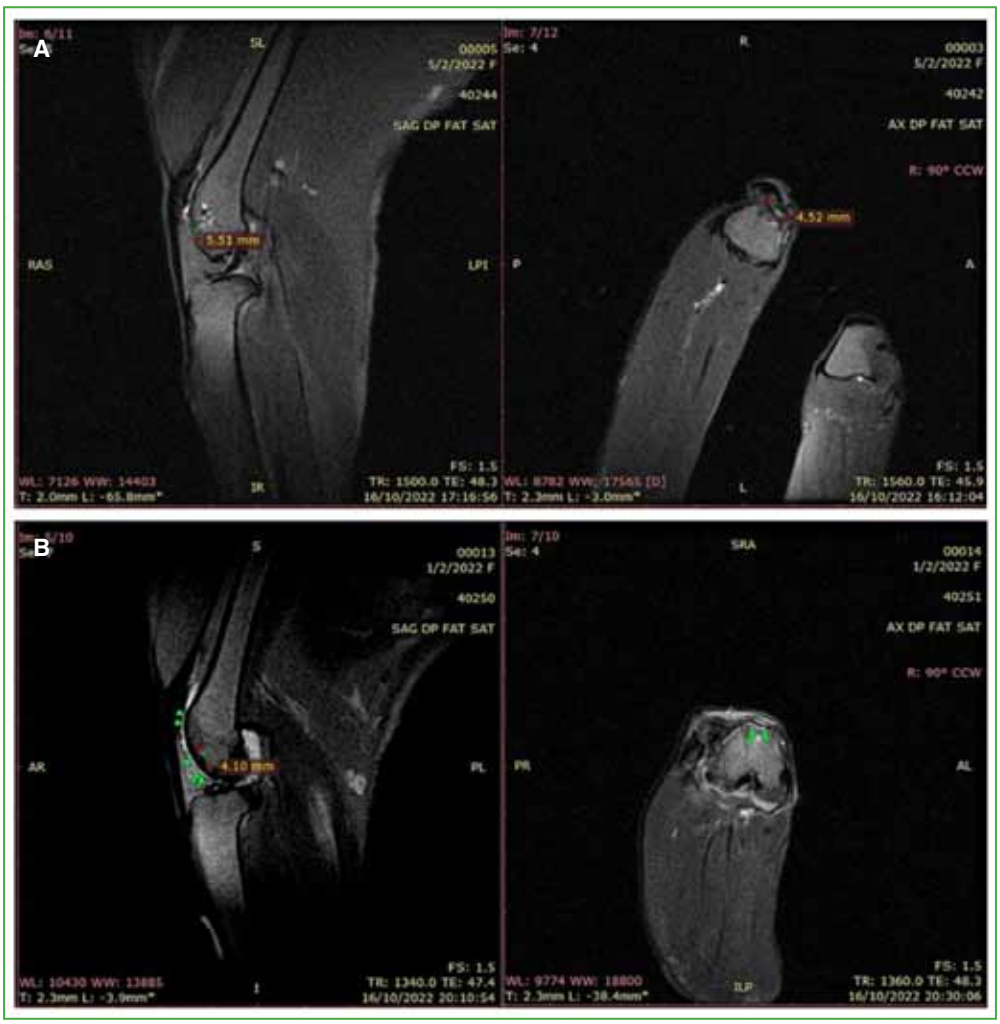


Figure 2. Representative images from each group obtained by magnetic resonance imaging using a General Electric 1.5 TESLA equipment with microcoil, four months after treatment. **A.** Representative image of group 2. **B.** Representative image of group 3.

Results of histopathological studies

Representative images from each experimental unit of each group are shown in Figure 3.

Group 1: Chondrocytes with axial or coronal arrangements were observed in all fields of each unit, within a basophilic extracellular matrix typical of normal cartilaginous tissue.

Group 2: At the injury site, tissue with eosinophilic matrix staining, characteristic of scar connective tissue, was observed. Chondrocytes were few and arranged in a scattered, disorganized manner, as expected in an osteochondral lesion.

Group 3: Generally, the extracellular matrix showed more basophilic staining than in group 2, and more closely resembled that of group 1. Additionally, hypercellularity due to chondrocytes arranged in axial and coronal isogenic groups was observed in many areas.

The chondrocyte cell count was: group 1 > group 2 ($p < 0.0002$; Wilcoxon test), group 2 < group 3 ($p < 0.0002$; Wilcoxon test), with no significant differences between groups 1 and 3. The Kruskal-Wallis test for the three groups showed highly significant differences between groups 1 and 2, but not between groups 1 and 3. Although group 3 exhibited some variation, with one sample showing low cellularity, the rest displayed high cellularity consistent with healthy cartilage tissue. In all samples treated with XLHA, the chondrocyte arrangement was similar to that of healthy cartilage tissue, which is strongly associated with proper tissue functionality (Figure 4).[1,2]

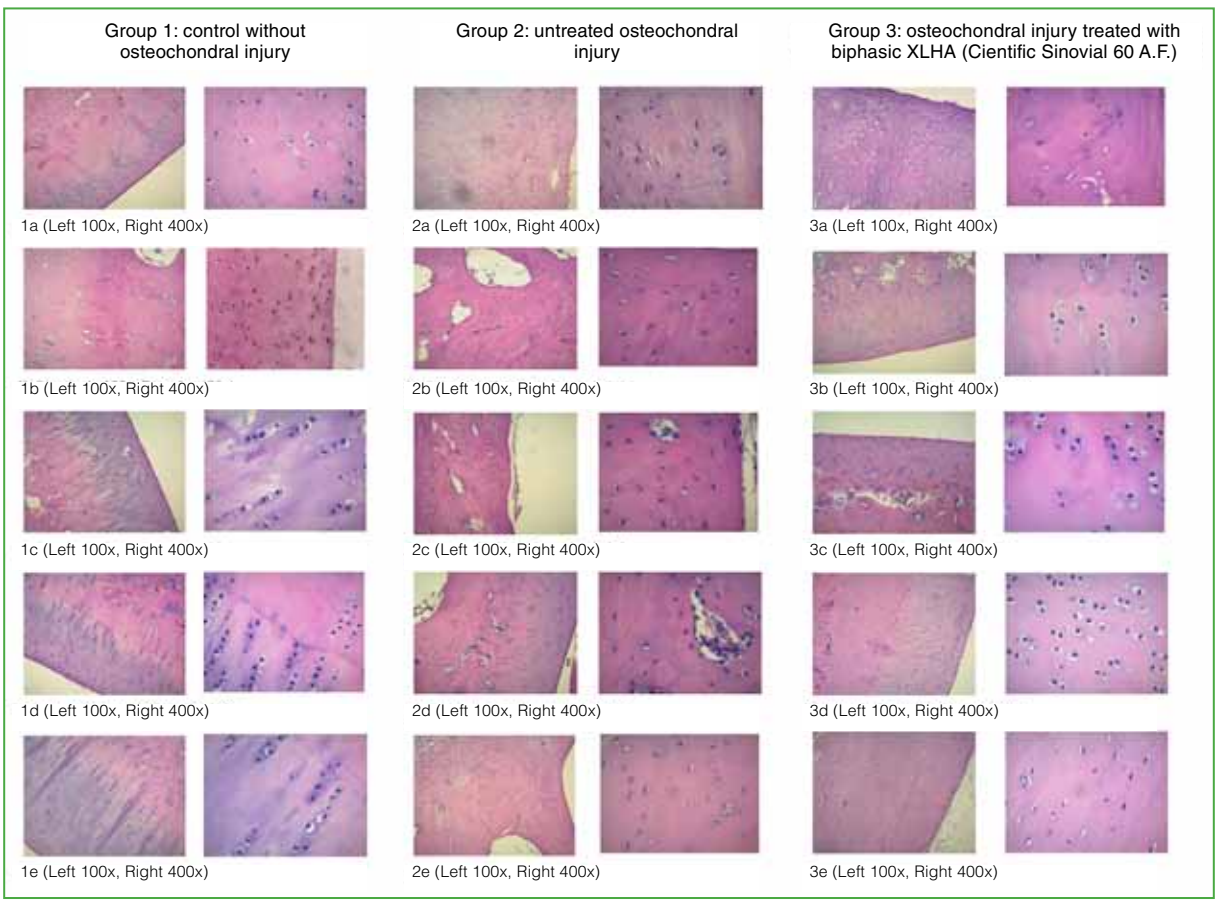


Figure 3. Images obtained through a camera connected to an optical microscope, displaying histological samples from the three groups.

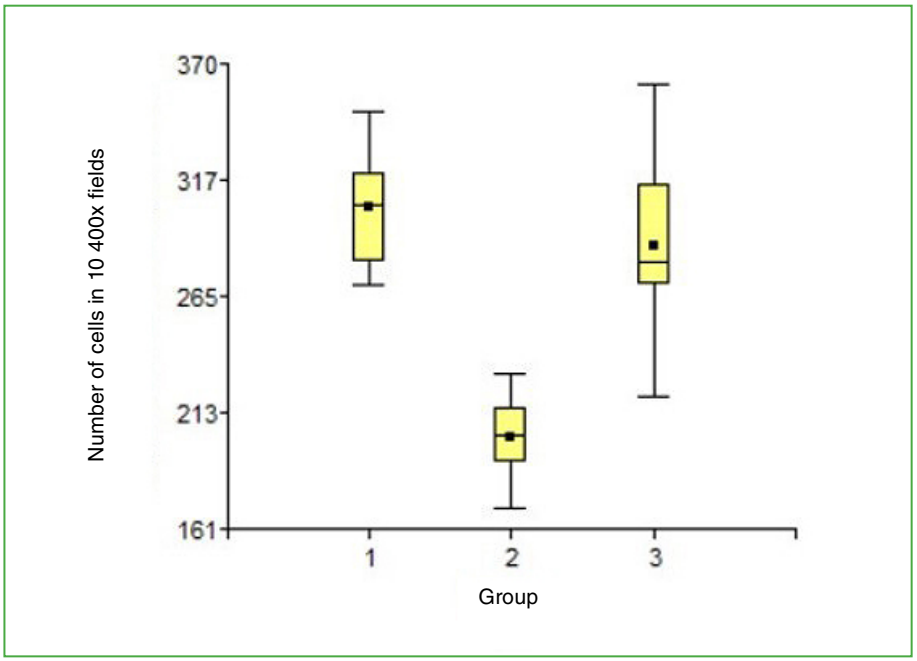


Figure 4. Chondrocyte count.

DISCUSSION

It has been demonstrated that cartilage tissue, when faced with an osteochondral lesion, lacks the capacity to regenerate on its own, meaning it does not form new tissue identical to the original that can fulfill the same functions.[26] When a cartilage defect is left untreated, the joint deteriorates progressively and irreversibly, leading to osteoarthritis and eventually disability.[27]

HA is commonly used via intra-articular injections to increase viscosupplementation as a treatment for osteoarthritis, due to its ease of use and good tolerability.[28] However, there are very few studies that explore the potential regenerative effect of HA, and in those published, HA is usually applied in combination with other molecules. [29-33]

Although some evidence of rabbit ear cartilage regeneration through HA application has been reported,[34] as well as research in rats,[35] these are preventive studies in which HA is injected before inducing an osteoarthritis phenomenon. There is no evidence, based on imaging and histological studies, that exclusive treatment with XLHA promotes cartilage repair in an established osteochondral lesion in the knee. In this study, the cartilage regenerated with an appropriate histological structure to perform its functions as in healthy tissue, which may explain the 100% absence of pain in treated experimental units, compared to untreated animals. While determining the presence of pain may introduce subjective bias, it provided an additional contribution to other investigations, since pain is a common issue in living beings with osteochondral lesions.

This research builds on other studies conducted in our laboratory in the field of tissue engineering,[36-41] which suggest that using certain biomolecules or matrices derived from them can promote the *de novo* repair of damaged tissue with characteristics similar to those of healthy tissue. This approach offers a simple, less invasive treatment compared to conventional methods, with fewer undesirable side effects[42-44] and more cost-effective.[45,46] Future studies on the functional profiles of chondrocytes will further enhance our understanding of joint biology, imaging, and treatment options.[47]

CONCLUSIONS

This study provides preliminary evidence that the experimental units with osteochondral lesions treated with XLHA experienced no pain in the early stages after the lesion, unlike the intervened units that did not receive such treatment. This suggests that cartilaginous tissue repair occurred at the injured site, as confirmed by magnetic resonance and histological studies, without any undesirable side effects. Future studies utilizing cell markers and immunohistochemical techniques will further enhance this regenerative medicine proposal.

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