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# Histopathological evaluation of Utilizing decellularized xenograft cartilage and adipose tissue mesenchymal stem cells (AT-MSCs) with Ozone therapy for treating articular cartilage defect of stifle joint in Dogs

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**Abstract: The articular cartilage is a connective tissue with significant mechanical durability and elasticity function. Cartilage has limited self-repair capabilities due to its intrinsic low vascularity, which leads to a diminished replicative capability of chondrocytes. Cartilage defect is a debilitating joint disorder that affects the dog. In this study, we investigated the utilization of decellularized cartilage and Adipose tissue mesenchymal stem cells associated with medical ozone for treating full-thickness cartilage defect in dogs. Methods: Twenty-five dogs were used. A full-thickness 8 mm diameter by (4) mm deep cartilage defect was performed in the left stifle joint of all dogs. They were divided into five equal groups. The induced defects of the first group were left without treatment. The defects of second group were filled with decellularized cartilage (DC). The defects of the third group were filled with (DC) then treated with Medical Ozone. The defects of the fourth group were filled with (DC) + (AT-MSCs). While the defects of the fifth group were filled with (DC) and (AT-MSCs) then treated with medical ozone. The dogs were euthanized at 56-day post operation. Results: The cartilage healing process of fifth group showed a new cartilage tissue formation in the peripheral and extended to the centre of the cartilage defect. The middle and superficial areas of the cartilage defect tend to be compact. Conclusion: Decellularized cartilage with both (AT-MSCs) and medical ozone which used in this present study as a treatment can effectively accelerate and promote the healing of cartilage defect of stifle joint in dogs.**

**Key word: Cartilage defect, xenograft cartilage, mesenchymal stem cells, ozone therapy.**

## INTRODUCTION

Cartilage defect (CD) is a debilitating disease in dogs, mostly affecting the stifle joint and inflicting damage to joint structures. Its currently intractable and induced relapsing damage to the joint. It's most common in case of ruptured cranial cruciate ligament and patellar luxation. CD causes serious pain and substantial restrictions on a dog's life quality because It's prolonged and progressive nature (1). There are a variety of treatment options available to reduce the pain associated with Cartilage defect, ranging from surgical procedures to a variety of drug therapies (2) Various strategies have been used to repair articular cartilage defects. Cartilage xenograft from animal donors have several significant advantages compared to other biomaterials. Their greatest benefit is that they can maintain enlarged cartilage for several years after surgical implantation. Unlike allografts, which tend to resorb over time, xenografts maintain their volume due to their non-resorbable properties (3). Various surgery procedures have been adopted to take advantage of these low replacement rate materials. A desirable scaffold for articular cartilage would possess a structure meaningful of natural cartilage, substantial mechanical resilience to uphold the defect's contour and high porosity. To promote cell biocompatibility and positive ingrowth, it must be degradable; Autologous, allogeneic (from individuals of the same species), and xenogeneic (from different species) materials are optimal for cartilage regeneration due to their ability to preserve the innate structure and Extra-cellular matrix composition found in natural cartilage tissue. Adipose mesenchymal stem cells is the mixture of cells originating from adipose tissue, through mechanical or enzyme digestion, centrifugation and filtration. This solution lacks adipocytes but encompasses diverse cells such as (MSCs) stromal cells, pericytes, endothelial progenitor cell (EPC) populations, and immune components. Compared to other cell therapies, (AT-MSCs) is less restrictive and relatively safe than

mesenchymal stem cells as it can be obtained directly from adipose tissue. It synergises immunomodulation, anti-inflammation, and angiogenesis and may be more effective than single-cell therapy (4). Three oxygen atoms combine in a ring structure to create ozone. In nature, ozone can be discovered in the stratosphere. However, diatomic O2 may be exposed to a high-voltage electrical discharge to create man-made O3 (5). Cartilage problems are frequently treated using ozone therapy. Its efficacy is supported by empirical data, but there are few research that offer histopathological and biochemical evidence. Ozone is now a viable alternative for intra-articular injection when treating stifle cartilage defects. It has been demonstrated that ozone alleviates the pain and inflammation associated with stifle cartilage lesions. As a bioregulation element, O3 promotes the release of diverse chemicals from endothelial cells and restores the redox balance of the cells when it interacts with bodily fluids (6).

The aim of this study evaluation of the efficacy of decellularize cartilage and both (AT-MSCs) and medical ozone on healing of degeneration articular cartilage in dogs.

## MATERIALS AND METHODS

**Ethics Statement:**

The Animal Ethics Committee approved this study at the College of Veterinary Medicine, Basrah University according to the approval number (83 at 2023/3/20). All procedures mentioned in the study were done with the animal's public guidelines for experiments established by the institution. Each dog had a complete blood count (CBC) test and a clinical assessment to detect systemic disease, and the dogs received the rabies vaccine. The animals were housed in typical conditions. In addition, anti-parasitic medications (1% Ivermectin 0.4 mg/kg BW SC) (7).

**Experimental Design:**

Twenty-five adult male mongrel dogs (Crossbreed dogs (heritage of Boxer, American Pit Bull, Great Dane, and others)) aged 1-1,5 years and the body weights of the dogs extended from 18 to 22 kg at a mean of 20 kg were used in this study. A full-thickness cartilage defect 8mm diameter, 3-4 mm deep by manual drill pointed by index was induced at the trochlear groove of the distal femoral bone of each dog.

**Decellularization Cartilage:**

Bovine chondral graft flake decellularization was performed with some modifications. Briefly, the samples were washed 5 times with PBS solution supplemented with (pen-strept). then, stored in PBS frozen in liquid nitrogen at − 196 °C, then quickly thawed at 37 °C. The freeze-thaw procedure was repeated three times. The freeze-thawed graft flakes were immersed in protease enzyme and incubated 2 days at 4 °C. Then, cartilage was transported to 1 % Triton X-100 with stirred at 4 °C for 1 h. Then, the cartilage immersing with PBS containing (DNAse/RNAse) for one day. The cartilage was then incubated with 0.02% Tris/EDTA solution for 48 hours and the procedure was repeated 5 times. After that, washing with PBS, the cartilage was preserved in saline at 4 °C even use. The flakes were particulate aseptically in small 1–2 mm fragments (figure 1). Then, the cartilage was washed with normal saline and evaluated histopathological using HandE stain' (8).

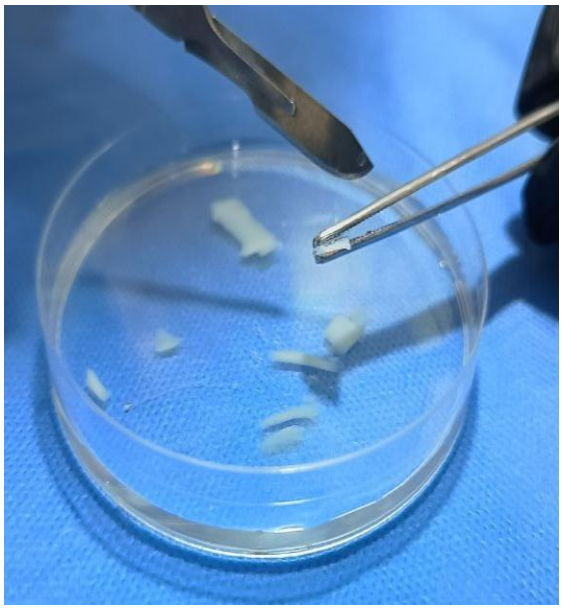

**Figure 1.** Showed the particulate of the cartilage flakes to small particles

**Prepare of Adipose Tissue-Mesenchymal Stem Cells:**

Procedure: The xyphoid region of each dog were aseptically prepared for surgery. Dogs were anaesthetised with xylazine (2%) 5mg/kg body weight and ketamine (10%) 15mg/kg body weight respectively under sterile conditions (9). An incision 6 cm in length was made in xyphoid region. Falciform adipose tissue (10) g were collected with sharp dissection. The incision was closed routinely. The adipose tissue was Chopping into small fragments by Metzenbaum scissor, and the adipose tissue transferred to a petri dish containing minimal essential media (MEM), supplemented with (pen-strept). In the hood, the adipose tissue was washed 4 times with phosphate puffer solution (PBS) and incubated with 0.1% collagenase type II at 37 ºC with shaking. then, added fetal bovine serum (FBS) 10% and minimal essential media (MEM) to the cell suspension to stop the enzymatic reaction. A suspension filtered by a 100-µm mesh to eliminate debris, then, centrifugation was done at 1000 rpm 10 min. Pellets were cultured in a growth medium comprised of minimal essential media with 20% fetal bovine serum supplemented with (pen-strept). The cells were cultured in 25cm culture flasks and incubated with 95 % humidified air at 37 ºC (10).

**Cell Culture:**

The suspension of cells was cultured for 4 days in minimal essential media supplemented with 10% fetal bovine serum supplement with (pen-strept). Then, incubated in a humidified condition at 37 °C, then, examined under inverted microscope, cell account (about 5.106-6.106 million cells / ml). (11).

**Cell Staining:**

Dissolve ½ g crystal violet in 80 mL DW and add 20 mL methanol to solution. Then keep in the dark at room temperature. Then, Aspirate the medium (MEM and FBS) from cells, and wash the cells twice with PBS. Used 5 ml of stain to staining the cells for (20 min), then aspirate the stain and wash with PBS, the cells examined with inverted microscope, crystal violet staining for identification of dell growth issues and observing unused and nonuniform patterns of cell proliferation, staining make it easy to detect (12). Under microscope show mainly mature adipocytes, and (adipose tissue-mesenchymal stem cells (AT-MSCs).

**Surgical Procedure:**

The surgery was performed under sterile conditions. All animals were withheld from food for 12 h and water 3 h pre-surgery. All dogs were given prophylactic antibiotic (pen-strept) (10,000 IU and 20 mg/kg B.W) before one hour of the surgical procedure. The dogs were anaesthetised with a xylazine 2% and ketamine 10% at dose 5 mg /kg,15 mg/kg respectively (Landry and Maza, 2020). The left hind leg of each dog was aseptically prepared after being put in lateral recumbency on the surgical table. Using a #11 scalpel blade, Metzenbaum scissors, and a combination of blunt and sharp dissection, a normal lateral para-patellar approach was made through the skin, subcutaneous tissue, lateral fascia, and joint capsule. Using hemostatic forceps to stop the bleeding, the incision ran longitudinally from the proximal end of the patella to the insertion of the patellar ligament on the tibial tuberosity. To access the distal femoral joint surface, the patella and joint tissues were medially luxated, and the stifle was flexed. The trochlear groove was exposed, and the defect point was selected in the centre of the trochlear groove. A circular chondral defect (cylindrical defect) (8mm diameter by ~ 3-4mm deep) was induced by using a manual drill pointed by index (figure 3-20). The surgical site was then washed with standard saline solution (0.9% NaCl) to remove debris. Dogs were randomly divided into five equal groups:

1. G1: first group was left without treatment. 2. G2: second group was treated with decellularize cartilage (5 g). 3. G3: third group was treated with Xenograft decellularize cartilage (5 g) and Ozone (10 ml 30 micro g/ml (13),] once times). 4. G4: fourth group was treated with Xenograft decellularized cartilage and adipose tissue mesenchymal stem cells (about 5.106-6.106 million cells/ml).] 5. G5: fifth group was treated with Xenograft decellularize cartilage (5g) with ozone (30 micro g/ml) and adipose tissue. The surgical wound was closed as Routinely using Polydioxanone (PDS II) for close approximation of joint capsule and lateral fascia. Finally, the skin was closed. External immobilisation (cast) was used for one week. All animals were confined to individual cages throughout the Time of experiment. Following surgery, dogs received daily medical care as well as a 5-day intramuscular of (pen-strept) doses of 10,000 IU and 20 mg/kg B.W. Then dogs were allowed to move freely. The wound sutures were removed 14 days post-operatively. All dogs underwent histopathological examinations (14). After 56 days post operation, dogs were euthanised and joints harvested to evaluate cartilage healing process.

**Ozone Production:**

Ozone was freshly obtained from an ozone generator (Model NO: AOT-MD 520).

Procedure: The dogs were readied for ozone injection. The ozone generator is attached to the oxygen tank, and the oxygen regulator is adjusted to ½ L/min. Next, the ozone generator is calibrated to a concentration of 30 micro g/ml, left for 30 seconds, and the 10 ml syringe is connected to the gas outlet. The gas is injected into the trochlear groove after confirming the proper location by withdrawing a sample of synovial fluid (13).

**Histopathological Evaluation:**

Immediately following euthanization, the stifle joint was opened, and chondral samples from the operation site were collected. Samples were immersed in PBS during the entire testing duration. They were preserved in 10% neutral buffered formalin. Subsequently, the specimens underwent de-calcification and were filled with paraffin. The sagittal sections were then cut and stained using haematoxylin and eosin. A pathologist assessed the sections microscopically (15).

**Scoring of Cartilage Healing:** Healing of the cartilage was scored according to (ICRS) scoring system (16), which includes the criteria shown in the (Table 1).

**Table 1:** shows the scoring of cartilage healing (16)

| S | parameter | Ratio | Score |
|---|---|---|---|
| 1 | Inflammation | No inflammation | 0 |
| | | ≥ 25 | 1 |
| | | 26- 50 | 2 |
| | | 51- 75 | 3 |
| | | Above 75% | 4 |
| 2 | Angiogenesis | No angiogenesis | 0 |
| | | ≥ 25 | 1 |
| | | 26- 50 | 2 |
| | | 51- 75 | 3 |
| | | Above 75% | 4 |
| 3 | Fibrous tissue | No fibrous tissue | 0 |
| | | ≥ 25 | 1 |
| | | 26- 50 | 2 |
| | | 51- 75 | 3 |
| | | Above 75% | 4 |
| 4 | Regenerated cartilage | No cartilage regeneration | 0 |
| | | ≥ 25 | 1 |
| | | 26- 50 | 2 |
| | | 51- 75 | 3 |
| | | Above 75% | 4 |

## RESULT & DISCUSSION

**Histopathological Evaluation**

Control group revealed that the operation site was occupied by collagen fibers (score 4), with a considerable presence of newly formed blood vessels (score 3). Nevertheless, this group lacks cartilage regeneration (score 0), as depicted in figures (3-1). The decellularization cartilage group indicated the presence of a cartilage graft at the operation site. It is encapsulated by

extensive collagen deposition (score 3). The surrounding area of the cartilage graft showcases numerous newly formed blood vessels (score 4). (Figures 2) illustrate that there was no statistically significant ($p \geq 0.05$) difference in the changes related to fibrous tissue amount and angiogenic process between this group and the control group. The ozone group exhibited noticeable cartilage regeneration at the operation site (score 3); however, the regenerated cartilage is not well differentiated. Additionally, there remains fibrous tissue in the area surrounding the operation site between the regenerated and original cartilage; the fibrous tissue in this group is scored (1), and the newly generated blood vessels display moderate development (score of 1), (figures 3). These changes represented a significant ($p \leq 0.05$) difference compared to the (control and DC) groups. The AT-MSCs group showed the operation site containing structureless material, with significant fibrous deposition around this material (score 4) (figure 4); the newly formed blood vessels received a score of (1). 'Statistically, there were no significant ($p \geq 0.05$) differences in the changes between this group and the (control and DC) groups. The (AT-MSCs) with ozone group demonstrated favorable outcomes relative to the other study groups, exhibiting marked cartilage regeneration that filled the entire operation sites (score 4), with no fibrous tissue remaining at the site (score 0), (figures 5). The area is occupied by fully mature cartilage. This group exhibited significant differences with all other treated groups ($p \leq 0.01$); (figure 6).

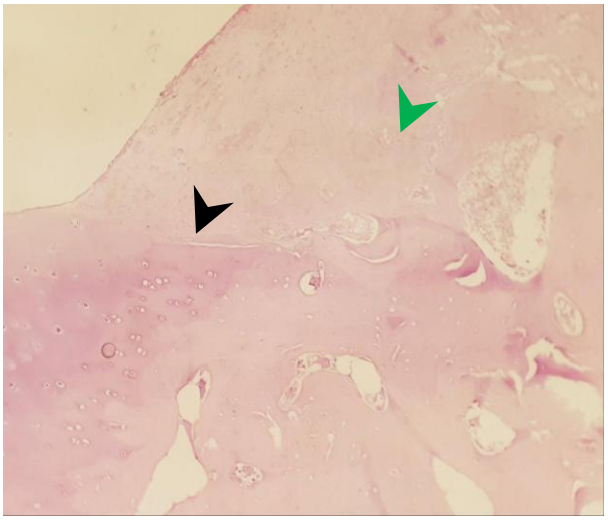

**Figure** (2): (A) articular surface the site of cartilage damage (control group) showed well demarcated line between the original cartilage and the operation site (black arrow) chondrocyte differentiation in the operation site (green arrow) HandE 4X.

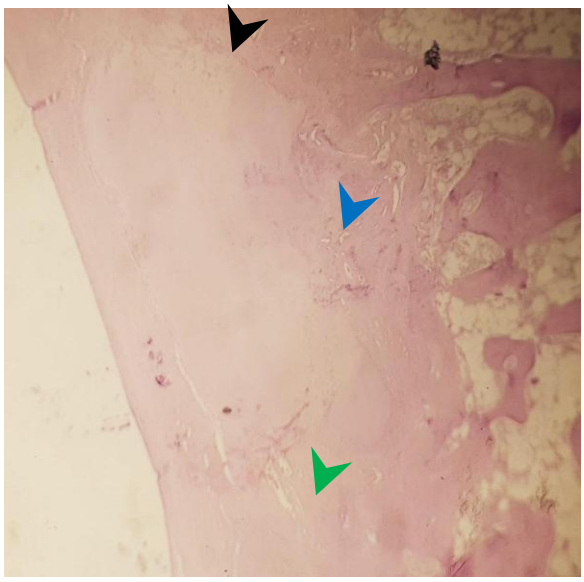

**Figure** (3) (A) articular surface-the site of cartilage damage (DC group) showed well demarcated line between the original cartilage and the operation site (black arrow) intensive collagen deposition around the cartilaginous graft (green arrow) and newly generated blood vessels in the operation site (blue arrow). HandE 4X

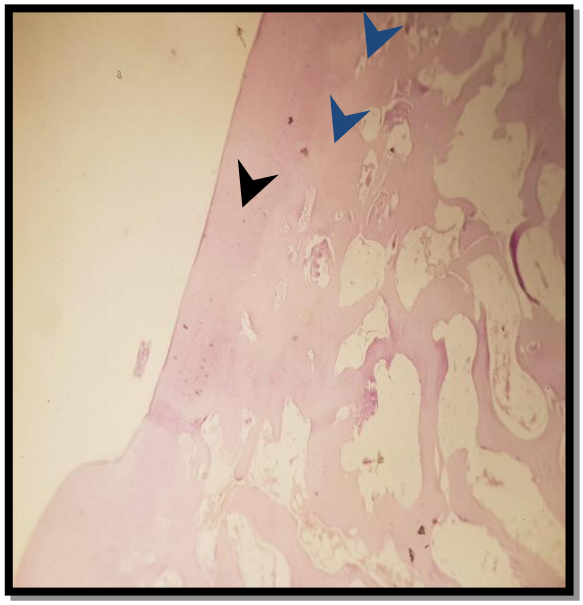

**Figure** (4) (A) articular surface-the site of cartilage damage (AT-MSCs and ozone group) showed marked cartilage regeneration in the operation site (black arrow), demarcation line between the new cartilage and the original one (blue arrow). HandE 4X

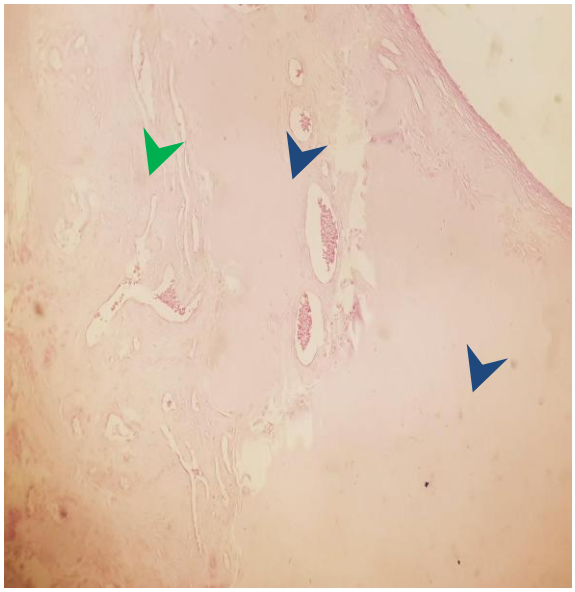

**Figure** (5) (A) articular surface-the site of cartilage damage (AT-MSCs group) showed marked fibrous tissue deposition in the operation site (green arrow) area of structureless material (blue arrow). HandE 4X

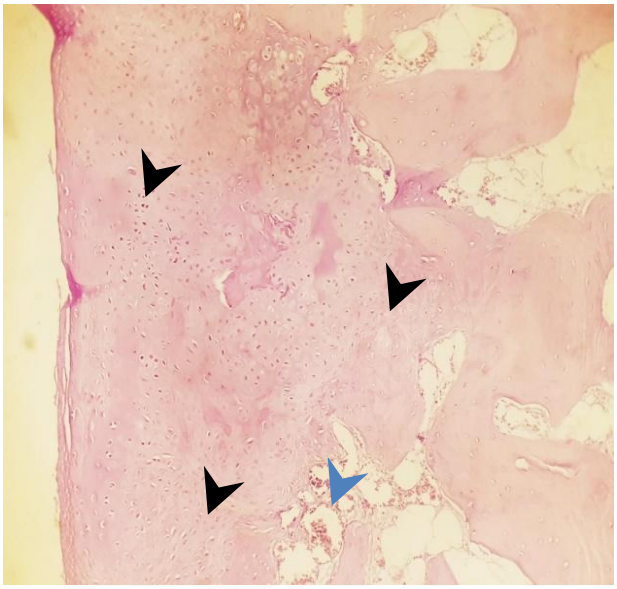

**Figure** (6) (A) articular surface-the site of cartilage damage (ozone group) showed marked cartilage regeneration in the grafting site (green arrow) and newly generated blood vessels around the new cartilage (blue arrow). HandE 4X

## Discussions

The control group showedhe minimum healing process where the defect cavity is still apparent in the site of operation indicating no or minimum healing due to cartilage has reduced healing capabilities, its intrinsic low vascularity, which leads to a diminished replicative capability of chondrocytes, compare with other group that observed by (1). The Decellularization cartilage group and the ozone group showed more advanced healing in which the fibrous tissue appeared to fill the gap completely. The (DC) an excellent option for tissue-engineered cartilage scaffold, as they maintain the original structure and ECM elements of cartilage tissue for regeneration purposes. This observation agreed with (17). The medical ozone increases the tissue oxygen supply by a hemorheological action based on vasodilatation and angiogenesis stimulation observed by (18). Showed an obvious difference in the healing process between the study groups, ranging from a mild healing process in the grafting site to well-developed cartilage containing some areas of mineralisation. The control group showed that the graft site contained an intensive inflammatory process with angiogenesis and mild collagen fibre deposition. This indicates that the healing process is still at the beginning where the inflammatory process is still active the newly formed vasculature occurs in response to the inflammation. The (DC) group: The section revealed pieces of decellularized cartilage in the grafting site surrounded by an area of more intense newly formed vasculature and more intense collagen. The newly generated blood vessels are massive in the area surrounding the cartilage graft. At the same time, the inflammatory process is reduced dramatically, indicating the inflammation's subsite to permit the healing process to complete gradually and over time. As mentioned earlier, the changes were overwhelming in the decellularization cartilage and ozone, decellularization cartilage and (AT-MSCs), respectively. These two groups showed marked cartilage development, which appears to be more fixed in the grafting site, surrounded by an area of intensive collage deposition; the newly generated cartilage was more in the third group than in the fourth one. The combination of (AT-MSCs) and ozone demonstrated better outcomes in cartilage formation and diminishing inflammation at the grafting site, with the site being occupied by cartilage. Total cartilage regeneration is evident in all surgical sites with no presence of fibrous tissue. The region is packed with completely developed cartilage; hence, assessing the blood vessels would be pointless. These results matched those of (19), who proved that fat tissue contains collagens (I, III, IV, VI, and VI), glycosaminoglycans (GAG), laminin, elastin, fibronectin, vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF). It also enhances the formation of fat cells from mesenchymal cells in adipose tissue and can attract and boost the development of stem cells into different cells. (20) confirmed the findings that adipose tissue has high levels of bioactive ECM components and progenitor cells, which can stimulate the development of fat cells and blood vessels. Alternatively, the outcomes discussed could be linked to the impacts of ozone, as shown by (21).

## Conclusions

Combination of decellularized xenograft articular cartilage, (AT-MSCs), and ozone can dramatically accelerate the cartilage regeneration and promote healing.

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