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Histopathological Assessment of the Regenerative Effect of Acellular Bovine Menisci Powder on Healing in Large osteochondral Defects in Rabbit Models

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ABSTRACT

BACKGROUND: Articular cartilage defect repair presents a significant clinical challenge, primarily due to the tissue's inherently low cell density, limited nutrient availability, and the restricted capacity of bone marrow-derived stem cells and resident chondroprogenitor cells to regenerate true hyaline cartilage.

OBJECTIVES: The purpose of the present study was to evaluate the use of bovine menisci powder implants for the regeneration of cartilage defects in a rabbit model.

MATERIALS AND METHODS: Under general anesthesia and aseptic conditions were established, twenty-four healthy male rabbits were used. All rabbits will be carrying out to arthrotomy on the right-hand limbs, a full-thickness cartilage defect (4 mm in diameter, 3 mm in depth) was created in the center of the middle of the trochlear groove, immediately after drilling the defect was rinsed with a saline solution. Depending on the way of the treatment, the animals were divided randomly into three main equal groups (12 rabbits/group). The cartilage defect was treated with 1ml phosphate buffer saline (PBS) as control group, in bovine menisci powder group was treated with press fitted bovine menisci powder implants.

RESULTS: The outcome was assessed macroscopically at 4th and 8th weeks post-surgery in control group showed (ICRS) scores which indicated fibrocartilaginous or fibrous repair in control defects that were improved in the bovine

menisci powder group and further confirmed by higher Goebel scores. Although demonstrated at 8th weeks, significant differences ($P \leq 0.05$) in bovine menisci powder group compare than those in control groups.

CONCLUSIONS: We developed bovine menisci powder with minimal deteriorative effects on the composition and functional characteristics and potential effective in advancing the formation of cartilage-like tissue for repairing cartilage defect through adhere, proliferate, and secrete extracellular matrix. Histopathological examinations demonstrated the gap filled with mature fibrous connective tissue and no obvious cartilaginous extracellular matrix was identified by safranin O staining in control group at 8th weeks after surgery. However, that better filling with hyaline like cartilage of the defect relative to the surface of normal adjacent cartilage, better integration of repair tissue with surrounding articular cartilage and matrix staining with safranin O fast green in the bovine menisci powder groups than in those with control group.

Keywords: Rabbits, osteochondral defect, Transplantation, BMP(Bovine Menisci Powder).

INTRODUCTION

Articular cartilage injuries present a unique and challenging medical problem due to the tissues lack of regenerative ability [1,2]. The reduced vascularity, limited cell population, and dense extracellular matrix (ECM) inhibit cartilage regeneration. However, untreated cartilage defects due to osteoarthritis or injury can lead to swelling, joint pain, and further degeneration of the tissue and eventually the need for a total joint replacement [3,4]. Bovine menisci powder, which can be easily prepared from bovine meniscal tissue, has been used in experimental and clinical practice [5,6]. Bovine menisci powder is rich in bioactive components, including those that promote proliferation of chondrogenic cells and secretion of cartilaginous matrix, such as transforming growth factor (TGF-b), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), basic fibroblast growth factor (b-FGF) and vascular endothelial growth factor (VEGF) [7,8]. As a result of these potential, an alternative approach based on bovine menisci powder as scaffold and the menisci efficacy on large osteochondral defects was used in the current study.

MATERIALS AND METHODS

Ethical approval

The design of the present study was approved by the Animal Care and Use Committee at the College of Veterinary Medicine, Al-Qasim Green University. Babylon, Iraq.

Experimental design

The study utilized 24 clinically healthy adult New Zealand White rabbits, each weighing between 2.0 and 2.3 kg. The physical exam and lab tests (complete blood cell count, blood biochemistry profiles, and urinalysis) showed that they were healthy. We carefully looked at each animal's stifle joint to make sure it wasn't unstable. All of the animals were kept in separate cages for three weeks to become used to their new surroundings. They were fed commercial rabbit pellets and provided water whenever they wanted. Before the trial started, broad-spectrum prophylactic antibiotics (Pencillin 20,000 IU Streptomycine 20 mg/kg) and subcutaneous antihelminth injections of 0.2 mg/kg ivermectin (Ivomec, Holland) were provided. Osteochondral defects were created on the femoral condyles of the stifle joint, as previously documented [9]. The flaws were randomly assigned to two groups; the control group did not receive implantation. The scaffold group was put into the area of the lesion. At the fourth- and eighth-weeks

following operation, twelve rabbits from each group were killed. We looked at the repair quality in three ways: gross morphology, and histology.

***In vitro* study**

Preparation of Scaffold

The protocol of xenogeneic bovine menisci powder preparation was performed according to the protocol followed by [10] (Fig. 1), which included the collection of fresh bovine menisci obtained from the knee joints of healthy bovine animals immediately after slaughter under aseptic conditions. The menisci were carefully dissected and cleaned from surrounding soft tissues, synovial membrane, and any adhering fat. The cleaned menisci were then washed three times with sterile phosphate-buffered saline (PBS) containing 1% antibiotic-antimycotic solution to remove blood and tissue debris. The menisci were subsequently frozen at −80°C for 24 hours and then lyophilized (freeze-dried) for 48 hours to completely remove moisture content. The dried meniscal tissue was mechanically ground using a sterile tissue grinder and passed through a 100 µm sterile mesh sieve to obtain a uniform fine powder. The resulting bovine menisci powder was then subjected to decellularization by immersion in 1% sodium dodecyl sulfate (SDS) solution for 4 hours at 4°C, followed by washing with sterile PBS for 1 hour, and digestion with 200 U/ml DNase I solution for 4 hours to ensure complete removal of cellular components while preserving the extracellular matrix (ECM) bioactive components.

The decellularized powder was washed again three times with sterile PBS and then lyophilized for a second time to obtain the final dry sterile bovine menisci powder. The final powder was weighed, reconstituted with sterile PBS at a concentration of 50 mg/ml, and activated by mixing with 10% calcium chloride ($CaCl_2$) at a ratio of 0.1 ml $CaCl_2$: 1 ml powder suspension to form a gel-like scaffold, then reserved for surgical use.

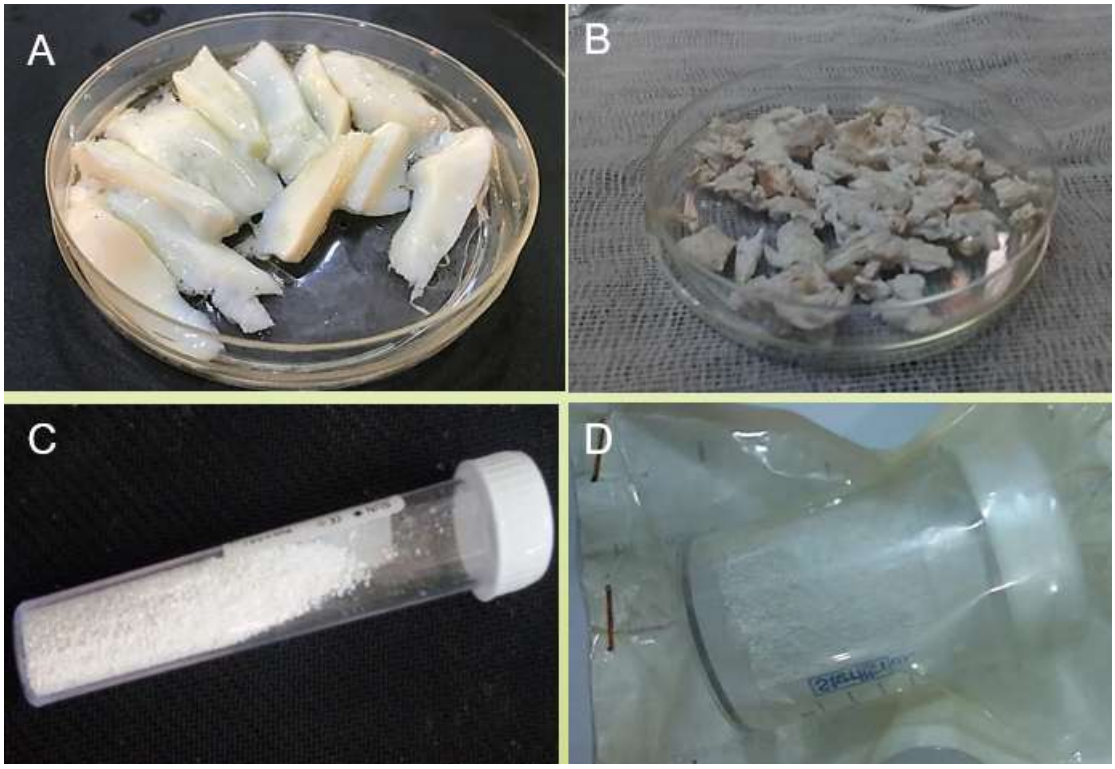

Figure-1: Photograph appearance the steps of acellular menisci matrix powder, **(A)** Cartilage a slice was dissected, **(B)** lyophilization of acellular cartilage slices for 24 hours at -56°C under 5 mm Hg, **(C)** Fabrication powder, **D.** sample was sterilized by Ultraviolet (UV) exposure 2 hrs.

Surgical procedure and creation of Osteochondral Defects

The experimental animals were tranquillized by intramuscular injection of 1mg/kg of Acepromazine (Vietnam). After 10 minutes a mixture of 5mg/ kg of 2% Xylazine Hydrochloride (VMD-Belgium) and 35 mg/ kg of 10% Ketamine (Holland) were injected intramuscularly. Arthrotomy of the stifle joint was performed described by [11] (Fig. 2). An approximately 2-4 cm incision was made in the skin at medial surface over the stifle joint in parallel with patella. The subcutaneous fascias were bluntly separated, incise joint capsule and synovial sac, then lateral patellar luxation after its separation from surrounding tissue with keeping stifle joint at flexion state then, exposed the femoral trochlea. A full-thickness cartilage defect (4 mm in diameter, 3 mm in depth) was created in the center of the middle of the trochlear groove, immediately after drilling the defect was rinsed with a saline solution. The (control group) was filled by 1ml of PBS. however, (BMP- group) by manually press-fitting it into place of defect, then sealed the defect with facial flap by 3.0 vicryl in interrupted pattern. Finally, the patella was relocated and the joint capsule tightly sutured with resorbable 3-0 Vicryl, then subcutaneous tissue and skin was sutured routinely by using 3-0 silk and a slight compression bandage was applied. The external self-adhesive bandage will be applied for the operative limbs for 7 days post-surgery. During first five days post-implantation, the animals were administrated daily intramuscularly with a combination of penicillin and streptomycin in a dose of 10.000 IU and 10mg/kg B.W., respectively. All animals were euthanized after 4,8- and 12-weeks post-operation; the specimens were analyzed for the macroscopical and

histopathological examination to notice the healing of cartilage defect.

Post-operative treatment

After second surgery, the animals have been left free in their fencings without any immobilization of the operated limb. Full weight-bearing was allowed as tolerated. No specific exercise regimen was adopted. General health and weight-bearing status were monitored during recovery by a veterinary .

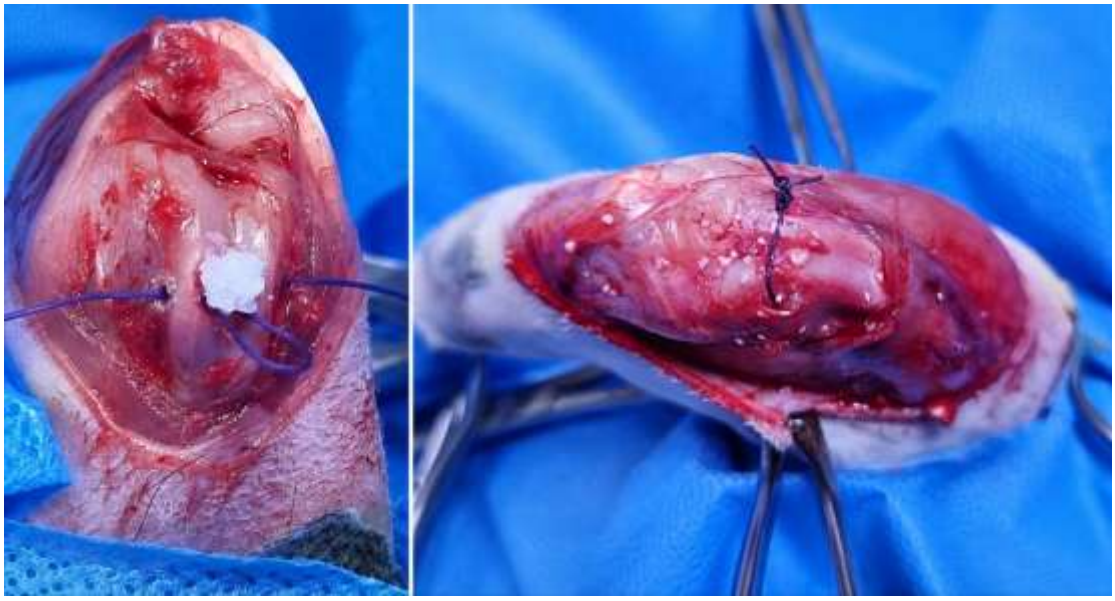

Figure-2: *Intraoperative view of osteochondral defect filled with the BMP*

Histological Investigation of Cartilage Repair

After all samples were washed twice with PBS (PH 7.4), they were fixed in 4.0% paraformaldehyde for one week at (25–30°C). And then they were decalcified in 10% formic acid for 3 weeks. After decalcification, the femoral condyles were divided into three segments from the lateral to the medial condyle along the sagittal plane. After that, all of the samples were put into paraffin and sliced into 5μm pieces. The prepared sections were recolored using H&E and safranin O stain. Cell shape, matrix color, surface smoothness, cartilage thickness, and how well it worked with nearby host cartilage.

RESULTS

Macroscopical Evaluation

Four weeks post-implantation, the results in control group indicated that the defect area was clearly lower than the native cartilage tissue, filled partially with fibrous tissue which had dark red color, and there was irregular tissue surface with depression in the central areas were visibly concave, and the margin of the defect was sharply differentiated from surrounding normal cartilage (Fig. 3). In contrast in BMP groups, the defect was filled with a glistening white tissue which resembled articular cartilage which superior integration with defect edges compared with control group, and there was little depression in the defect and the margin with normal cartilage was indistinct (Fig. 3). **Eight weeks post-implantation,** the results in control group showed the defects area was filled partially with regenerative tissue which had dark red color with large deep central depression, rough uneven tissue, surface irregularity and the defect margins were still identified from surrounding normal cartilage with poor integration between repair cartilage and native cartilage limits the success in articular cartilage regeneration and restoration after articular

cartilage defect (Fig. 3). However, in BMP groups at same period the defect area had filled regenerative white color tissue visually the graft appeared integrated well with the adjacent cartilage, but the defect was filled with a glistening white tissue was very similar to normal in scaffold group (Fig. 3). According to the International Cartilage Repair Society (ICRS) scores from macroscopic showed in (Tab. 1) and (Fig. 3). which indicated fibrocartilaginous or fibrous repair in control defects that were improved in the BMP group was further confirmed by higher Goebel scores. Although demonstrated at 4th weeks, higher significant differences($P \leq 0.05$) in treatment compare than those in the control groups. Appearance of BMP group (Mean score 7.50 ±0.22), and in control group (Mean score 1.67 ±0.21). At 8th weeks, mean macroscopic ICRS score was higher significant differences($P \leq 0.05$) in defects treated with BMP (Mean score 9.67 ±0.61), than control (Mean score 2.83 ±0.17).

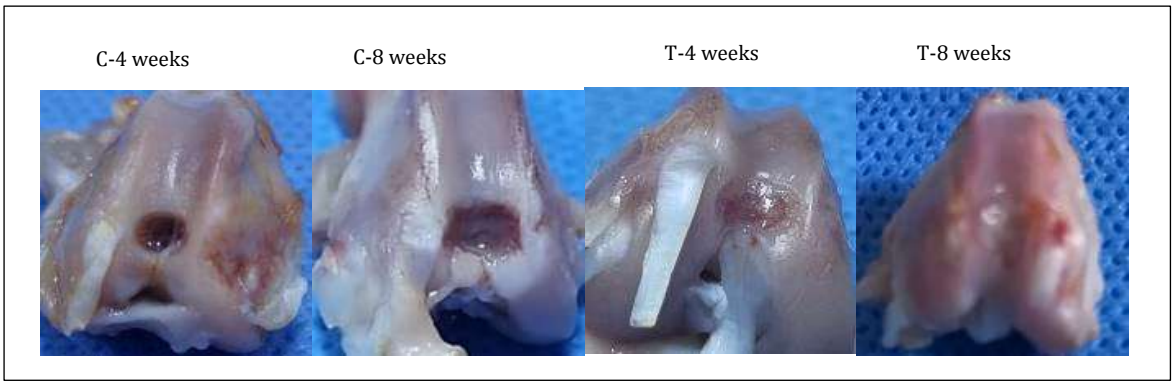

Figure-3: *Gross morphology of repaired cartilage of the control group (C), and the scaffold group (T) at 4 and 8 weeks*

Table 1: The mean values of Macroscopic scores for the control, and PRP groups.

Groups	Mean ±SE of Macroscopic scores		L.S.D.
	4-Week	8-Week	
Control	B 1.67 ±0.21 b	C 2.83 ±0.17 a	0.705 *
BMP	A 7.50 ±0.22 b	B 9.67 ±0.61 a	0.882 *
L.S.D.	0.789 *	1.230 *	---

*Values (Mean ± SE) having with the different big letters in same column and small letters in same row differed significantly. * (P≤0.05).*

Four weeks post-implantation with (H&E) stain, the results in control group revealed the presence of degenerative changes of the cartilage and gap filled by highly cellular fibrous connective tissue attachment to subchondral bone and infiltrated by mononuclear cells (Fig.4). While, safranin-O-fast stain showed that severe loss of safranine O staining, the no obvious cartilaginous extracellular matrix and irregular surface was observed, reaching beyond the level of the adjacent cartilage associated with appearance of loss of articular cartilage and multifocal decrease in chondrocytes (Fig.5). In BMP group, the histopathological sections with (H&E) stain from the site defects treated by implantation of BMP at this time revealed the appearance of that the gap filled with fibrocartilage containing numerous fibroblasts and blood vessels (Fig.6). While, safranin-O-fast stain showed that positive safranin O proliferation of extracellular matrix extended into granulation tissue filled the gap (Fig.7). **Eight Weeks Post-implantation** with (H&E) stain, the results in control group were indicated the gap filled with mature fibrous

connective tissue extended from intact cartilage and gap filled by vascular fibrous connective tissue and surrounded trabecular bone (Fig.8). While, safranin-O-fast stain showed that gap filled by fibrous connective tissue attachment to subchondral bone deep green in color (Fig.9). In BMP group, the histopathological sections with (H&E) stain from the site defects reveled hyaline like cartilage filled the gap, mature fibrous connective tissue contacts two part of the cartilage with initiated converted fibroblast to chondrocytes like cells (Fig.10). In addition, safranin-O-fast green stain positive safranin O, proliferation of extracellular matrix extended into granulation tissue filled the gap (Fig.11).

Figure-4: *Section of cartilage (4-week post-injury in control group) shows degenerative changes cartilage and gap filled by highly cellular fibrous connective tissue attachment to subchondral bone and infiltrated by mononuclear cells (arrows) (H&E stain, X10).*

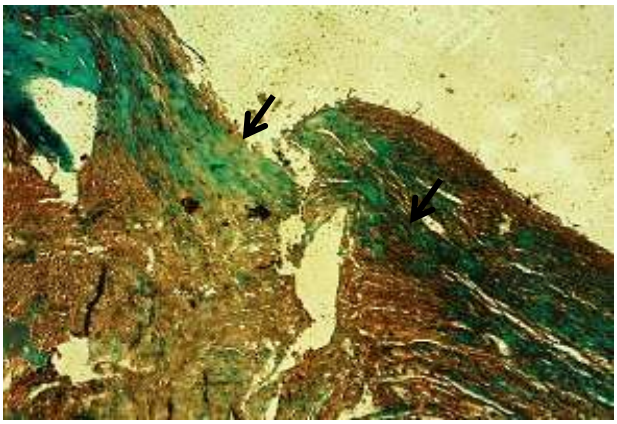

Figure-5: *Section of cartilage (4-week post-injury in control group) shows no obvious cartilaginous extracellular matrix and irregular surface was observed, reaching beyond the level of the adjacent cartilage (arrows) (Safranin O stain, X10).*

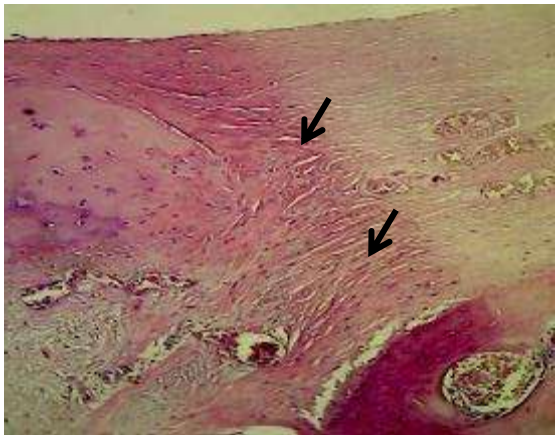

Figure-6: Section of cartilage (4-week post-injury in BMP group) shows the defect was mostly filled with fibrocartilage with individual, scattered chondrocytes and the surface was irregular (arrows) (H&E stain,

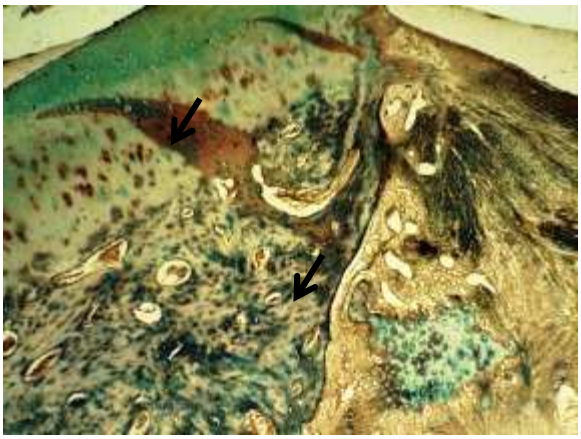

Figure-7: Section of cartilage (4-week post-injury in BMP group) shows positive safranin O proliferation of extracellular matrix extended into granulation tissue filled the gap (arrows) (Safranin O stain, X10).

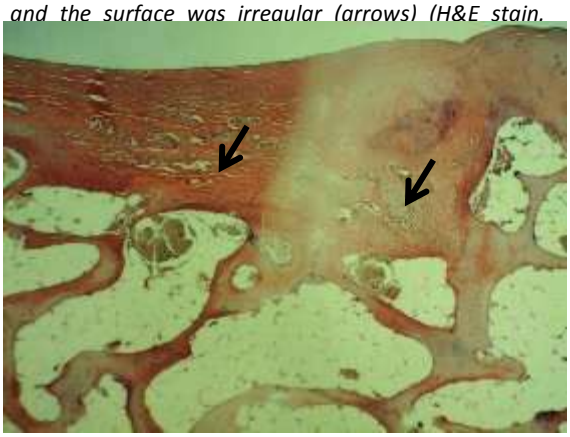

Figure-8: Section of cartilage (8-week post-injury in control group) shows gap filled with vascular fibrous connective tissue extended to subchondral bone and surrounded trabecular bone (arrows) (H&E stain, X10).

Figure-9: Section of cartilage (8-week post-injury in control group) shows gap filled by fibrous connective tissue attachment to subchondral bone deep green in color (arrows) (Safranin O stain, X10).

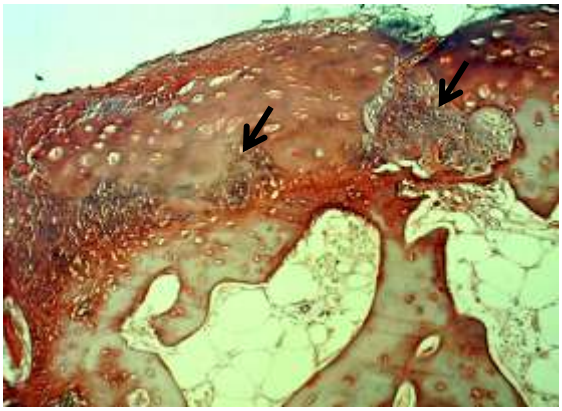

Figure-10: Section of cartilage (8-week post-injury in BMP group) shows the hyaline like cartilage in their center (arrows) (H&E stain, X10).

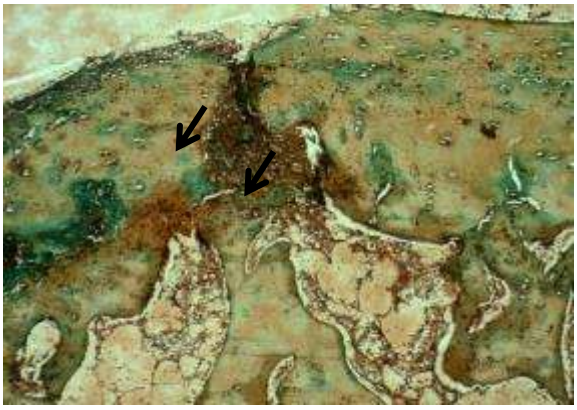

Figure-11: Section of cartilage (8-week post-injury in BMP group) shows positive strain O stain collagen fiber bridge between native cartilages parts (arrows) (Safranin O stain, X10).

DISCUSSION

In the present study demonstrated, there were minor complications during the surgical procedure. There was a suture dehiscence one wound with no signs of infection in the control group, which was resolved by serial dressings, there was one wound infection in the scaffold group that was debrided and treated with antibiotics. The infection was resolved in 2 weeks. The rest of the animals survived and exhibited good clinical evolution without complications. In the control group at four weeks, H&E-stained sections revealed an absence of cartilaginous extracellular matrix at the defect site, with the gap occupied by disorganized fibrous tissue extending toward the subchondral bone. Safranin O staining demonstrated a severe loss of staining intensity in the defect area, accompanied by a multifocal reduction in chondrocyte density in the adjacent native cartilage. These findings are consistent with early osteochondral wound healing in the absence of therapeutic intervention, and have been well documented in the rabbit model [12,13]. By week eight, control defects remained largely filled with mature fibrous connective tissue with no evidence of cartilage regeneration, and mononuclear cell infiltration was observed at the defect margins, likely reflecting a low-grade chronic inflammatory response to the persisting tissue void [14].

The BMP scaffold group yielded the most compelling histological findings among all three groups. At four weeks, defects treated with the BMP scaffold exhibited fibrocartilage filling with scattered chondrocytes distributed throughout the repair tissue, and H&E staining revealed early hyaline-like cartilage formation at the defect margins with adjacent mature fibrous connective tissue. Although fissuring was noted at this stage — likely reflecting the early remodeling phase during which the scaffold undergoes progressive replacement by host tissue — the presence of rounded chondrocyte lacunae and early ECM organization was notably more advanced than in the other groups. This accelerated cellularization is consistent with the capacity of decellularized ECM scaffolds to bind and concentrate endogenous growth factors, including bone morphogenetic proteins and FGF-2, and present them to invading host cells [15,16].

By eight weeks, the scaffold group demonstrated the most mature repair tissue architecture of all groups. Safranin O staining revealed a collagen fiber bridge between native cartilage segments, with positive staining indicating active glycosaminoglycan deposition across the defect zone. High-magnification sections showed that the centrally located fibrous connective tissue had undergone progressive conversion toward hyaline-like cartilage, consistent with chondrogenic differentiation of host mesenchymal progenitor cells within the scaffold matrix. This transformation — from fibrocartilage at week four to hyaline-like cartilage at week eight — mirrors patterns reported in studies employing decellularized meniscal and articular cartilage scaffolds, and reflects the capacity of

such scaffolds to provide both spatial and biochemical instruction to infiltrating progenitor cells over time [17,18].

CONCLUSION

This study shows the efficacy of BMP proved by macroscopical and histopathological examination indicated an improvement in the healing process. this strategy had extensive pertinence to the treatment of and gives the premise for the development of a repair technology that is qualified for regenerating large areas of articular cartilage.

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CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

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