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Original Article

The Healing Effect of Biodegradable Scaffolds Treated with Bone-Marrow Obtained Mesenchymal Stem Cells on Major Tendon Damage in the Dog as a Model

Salman Hamza, A[1]*, Traef Ali, Q[1], Hadi Farman, R[1]

1. Department of Surgery and Obstetrics, College of Veterinary Medicine, University of Al-Qadisiyah, Al-Qadisiyah, Iraq

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Corresponding Author: abdulsatar.hamza@qu.edu.iq

Abstract

The present study aimed to evaluate the implantation of decellularized small intestinal submucosa- extracellular matrix (SIS-ECM) seeded with bone marrow mesenchymal stem cells (BM-MSCs) to repair full-thickness Achilles tendon defect. For this purpose, 20 healthy adult stray dogs aged 8-12 months old (15±3 kg of weight) were enrolled in this study under an aseptic environment and general anesthesia. A 1.5 cm-long segment-based resection was performed in the mid-substance of the Achilles tendon in the control group (n=10) that did not receive treatment. While, in the experimental group (n=10), regarding the defect of the tendon, the stumps were bridged with decellularized SIS seeded with BM-MSCs (5×10^6) cells implanted. Afterward, the stumps of the tendon were sutured using the modified Kessler technique (4-0) polypropylene thread. The biomechanical observations of the tendon defect showed an increase in the tensile strength in the experimental group, compared to the control animals. It should be mentioned that this difference was significant ($P\leq0.05$). Histopathological observations of biopsies harvested after the 4th, 8th, and 12th weeks revealed that the implanted graft had seeded with MSCs enhanced high-quality cellular infiltration and the host tissue healing was improved. Similar to the normal tendon, a dense organized collagenous tissue with high cellularity and vascularity was observed due to the presence of the remodeled ECM. However, the arrangement of collagen-fiber-derived connective tissue appeared to be more dominant than that in the experimental group, with less adhesion in the 12th week post-treatment. These findings suggest that the BM-MSCs inoculated with SIS can be employed to repair a damaged Achilles tendon due to the fact that this combination enhances the regeneration of the affected tendon.

Keywords: Achilles tendon, Bone marrow, Dog, Implantation, Mesenchymal stem cells-Small intestinal submucosa (SIS), Tendon-healing

1. Introduction

Overuse and acute traumas have increased Achilles tendon injuries among elite and recreational sports players (1). An ongoing debate is taking place over the pathophysiology and management of this injury. However, there are advocators of the non-operative approach (2). The repair phases of the Achilles tendon during the healing processes have been investigated extensively in experimental studies (3). Histologically, tendon inflammation subsides before the 7th postoperative day (4), and the fibroplasia phase begins between the 2nd and 3rd weeks of the healing that precedes the longitudinal alignment of the collagen formation (5).

It has been shown that the small intestinal submucosa (SIS) is a biomaterial scaffold for bioengineering applications in the areas of arteries, ligaments, tendons, bones, wall of the abdomen, incomplete and complete

layer cutaneous injuries, the lower urinary tract, and dura mater, which is rich in collagen, glycosaminoglycans, and growth factors (6). Angiogenesis growth factor (VEGF) and other angiogenic factors predominate among the growth factors abundant in SIS indicate that SIS plays a crucial role in promoting angiogenesis (7).

Furthermore, multipotent mesenchymal stem cells (MSC) can be multiplied *in vitro* and are readily obtained by bone marrow aspiration, which makes them a very desirable supply for tissue engineering purposes (8). The MSCs in the tendon tissue are specialized cells that can also develop *in vitro* and retain their phenotypic and collagen type I synthesis throughout the early stages (9).

Scientists have found that autologous tendon tissue can be used to construct tendon tissue (10). This indicates that autologous tenocytes have great promise for tendon tissue design, as shown by the mechanical strength of the produced tendons after 14 weeks (83% close to the normal tendon). According to a previous study, the tensile modulus and maximum stress of the MSC-collagen composite graft of healed tendon tissues were much higher than those of the spontaneously healed tissues (11). This study aimed to evaluate the tissue regeneration ability of biomaterial scaffold composites implanted with MSC in severe complete tendon damage. It was assumed that the bio-scaffold injected with MSC would enhance the biomechanical robustness and histological integrity of the repaired Achilles' tendons.

2. Materials and Methods

2.1. Experimental Animals

This study was conducted on 20 healthy adult stray dogs (8-12 months old and weighing 15-20 kg). They were randomly divided into two groups of experimental (n=10) and control (n=10). The control group was left without treatment, while, in the experimental group, regarding the defect of a tendon, the stumps were bridged with decellularized SIS seeded with BM-MSCs (5×10^6) cells that were implanted to fill the defect with a polypropylene thread (4-0). Subsequently, the dogs were sacrificed in the 4^{th}, 8^{th}, and 12^{th} weeks after the operation for biomechanical and histopathological investigations.

2.2. Bone Marrow Stromal Cells Isolation and Culture

According to recognized standards, the bone marrow stromal cells (BMSCs) of animals were separated and grown for eventual utilization (12). Using a 3-ml syringe with a needle size of 18, 1.5 ml of bone marrow was withdrawn from the ilium between the external curve and the hips after the region was shaved and sterilized. Afterward, 3 ml of Dulbecco's modified Eagle medium (DMEM) and elevated glucose-DMEM were applied with 30% fetal bovine serum (FBS), 10 U/ml of streptomycin, 10 U/ml of penicillin G, 25 mg/ml of amphotericin B, 100 ng/ml of sodium pyruvate, and 1% non-essential amino acid (GIBCO® Invitrogen Corporation).

It took 3 min for 3 mm of FBS to dissolve in a 75 cm^2 flask, and then it was time to add the bone marrow-mixed medium to the flask. Non-adherent cells were removed from the flask 72 h after the incubation at 37 °C in 5% CO_2 in the air, and the media was replenished. After reaching the confluence phase, a 12-day culture was rinsed twice with 2 mL of phosphate-buffered saline (pH 7.2). Subsequently, 2 ml of 0.20%-trypsin-0.02%-EDTA (Sigma, USA) was added to the cultures, and the solution was left to rest on top of the cell layer for 2 min while being monitored under light microscopy till they re-obtained their round form and the trypsin was removed.

For the following three sub-cultures, DMEM containing 10% FBS was poured into the media and lightly tapped to separate cells. The media was removed from the flask by a decanter, the cells were rinsed with PBS, and trypsin was applied. Afterward, 10 ml of DMEM was added to the flask to collect the cells. Later, a centrifugation process was performed at 2,000 rpm for 10 min after the fluid and cells were recovered in sterile tubes. The precipitated pellets were then combined with 1 mL of DMEM. A

hematocytometer was used to verify that at least 1×10^6 MSCs were present in 10 µl of culture media. Each animal received a BMSC transplant after being successfully isolated.

2.3. Harvest and Preparation of Small Intestinal Submucosa Extracellular Matrix

The SIS was set up according to the instruction provided by Tilley, Chaudhury (13) with the reduction of the time for de-epithelialization and decellularization. The small intestine of a goat was acquired from a slaughterhouse in Babylon after the slaughter in Babylon, Iraq. Briefly, the small intestine was submerged in PBS to be shipped to the research center. The tissue was softly flushed with PBS to discard the remaining blood and subsequently scoured to dispose of the tunica muscularis externa and most of the tunica mucosa. The remainder of the tunica submucosa of mucosa and the basilar segment was then sanitized, decellularized in a 0.1% peracetic corrosive and 4% ethanol blend for 2 h, cleaned with PBS, and submerged in deionized water for 15 min. Decellularized scaffold and connected tissue grids were taken care of at 4 °C in PBS containing 1% gentamycin.

2.4. Design of Mesenchymal Stem Cell-Loaded Scaffold

The sterile container of the biomaterial sheet was opened, and the sheet was sliced into small pieces (about 1.5×1.5 cm^2). Moreover, it was rehydrated for each SIS in culture media. Once the culture medium was withdrawn after cultivation for 24 h, the BM-MSCs (1×10^6 cells) were planted on the surface of the SIS. After implantation, patches were grown at 37 ˚C under 5% CO_2 and 95% humidity for 5-7 days (Figure 1a). A 10% PBS-based formalin solution was used to fix the patched layers for two days. Afterward, light microscopes were used to inspect the resulting patches.

2.5. Surgical Procedure

An atropine sulfate (Kepro, Holland) at a dosage of 0.03 mg/kg was intramuscularly administered to the dogs, followed by a combination of Xylazine hydrochloride 5 mg/kg (Xyla, Holland) and ketamine hydrochloride 15 mg/kg (Kepro, Holland) after 10 min. Subsequently, one hind leg of each animal was shaved and sanitized based on the standards. Midline skin and fascia incisions of the Achilles tendon were prepared to expose it 0.5 cm distally from the gastrocnemius muscle and 0.5 cm above its calcaneus. A bilateral 1.5 cm-long segmental resection was made in the mid portion of the Achilles tendon. No therapy was administered to the control group (n=10) (Figure 1a).

Decellularized-MSCs-seeded SIS (5×10^6) cells in constructions that were designed to suit the defects with the polypropylene thread (Figure 1b) were used to link the stumps of the tendon in the experimental group (n=10) according to a modified Kessler technique. Afterward, the skin was stitched with silk no. 1 thread utilizing an interrupted horizontal mattress. During the first 5 days after placement, a mixture of penicillin (10.000 IU) and streptomycin (10 mg/kg bw) was intramuscularly administered to the animals.

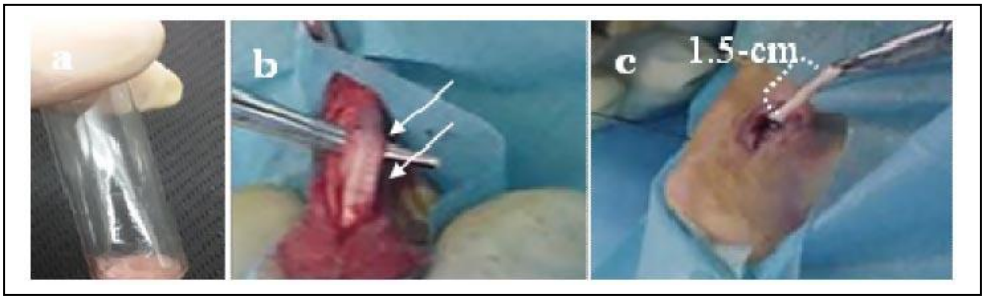

Figure 1. (a) Shows the MSC-loaded scaffold. **(b)** Tendon segment was severed **(c)** Tenorrahphy site is wrapped with Decellularized-SIS seeded with BM-MSCs and fixed using Kessler pattern then fascia and subcutaneously sutured, and interrupted horizontal mattress was used to close the skin

Surgical tendon samples were taken during the fourth, eighth, and twelfth postoperative weeks. The tendon tissues were taken from the mid-metacarpal area of the contralateral limb and used as a primary control length of about 10 cm. At the Directorate of Material Research Laboratory of the Ministry of Higher Education and Scientific Research, biomechanical characteristics of normal and damaged areas were studied. Tensile force analysis was performed on all biopsies within 3 h of tissue collection in PBS. The proximal and distal ends of the tendon were clamped to tensometer clamps on a piece of tensile testing equipment (model H50KT-English by Tinus Olsen). Afterward, the proximal and distal ends of the tendon were clamped to tensometer clamps, and a 20 mm/min tensile load was applied. The force-displacement graph was generated by plotting the load parameters on an X-Y chart recorder.

2.6. Histopathological Evaluation

Histopathological examination of the tendon samples was carried out at 4, 8, and 12 weeks after the operation. Hematoxylin and eosin staining was performed on 5-7 µ slices on a rotary microtome when the formalin solution was replaced after 72 h, evaporated by graded alcohol series, and buried in paraffin. According to the study performed by Kamishina, Deng (14), data was analyzed semi-qualitatively. Table 1 summarizes the use of key metrics to monitor the repair state of the damaged tendon location.

2.7. Statistical Analysis

Adjectival statistical analysis was performed for each factor in this study. The SAS software (15) was utilized to evaluate the impact of various factors (treatment and days) within study parameters. The least significant difference test was utilized to find the relationships between percentages.

3. Results

3.1. Morphological Characteristics of Bone Marrow Mesenchymal Stem Cells

The BMSCs colonies increased after 5 days in culture. After 12 days of culture, in the first passage cells appeared small rounded, spindle-shaped, or large flat (Figure 2A). In the second passage, 2 days post-culture, the cells joined as a spindle-shaped alignment along their longitudinal axis (Figure 2B). In the third passage of subculture, the BMSCs showed large, flat, round spindle-shaped, and polygonal-shaped cells (Figure 2C). In the fourth passage, the cells appeared to be fibroblast-like cells (Figure 2D). The results of phenotypic characterization from the third passage showed that almost all of the BM-MSCs were positive for CD105 and CD90 but negative for CD45.

Table 1. Mean values of tensile force test for experimental groups in the different analyzed time

Groups	Mean±SE of tensile strength		
	4 week	8 week	12 week
Control group	33. 00± 1.69 [b]	140.75± 2.71[a]	185. 25± 2.37[a]
Combination group	98. 00±1.8[b]	165. 00±2.5[b]	232. 37±1.5[a]
LSD value	31.48 *	16.07 *	24. 61 *

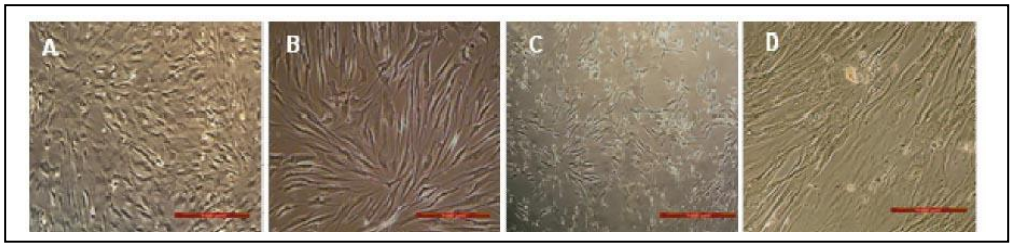

Figure 2. Light micrograph of BMSCs **(A)** Shows polymorphic spindle cells with round and polygonal cells at zero passage **(B)** fibroblastic-like BMSCs colonies grew to the confluence at second-passage **(C)** Culture mixture of large, flat, spindle shape cells, round cells and polygonal shape cells on the third passage **(D)** BMSCs are fibroblast-like cells at passage four. Scale bar = 100 µm

3.2. Biomechanical Properties

The average load of the biomechanical evaluation in operated tendons suggested an elevation in the tensile strength throughout the study duration in the experimental group, with a significant increase ($P \leq 0.05$) in the 4th week. During week 8, the mean values of the tensile strength were 98.00±1 N and 33.00±1.69 N in the experimental and control groups, respectively. The results demonstrated the mean values of 165.00±2.5 N and 140.75±2.7 N for the tensile strength in the experimental and control groups, respectively. A significant increase ($P<0.05$) was observed in the tensile strength (232.37±1.5N) of the experimental group, compared to the control group (Table 1).

3.3. Histopathological Evaluation

Cellularity, aggregation of mononuclear cells, uneven collagens, patterns of fibroblasts, and mild neovascularization at the anastomotic site were all observed in the control group in the fourth post-operative week (Figure 3). However, tendon fibers with strong multiplication of tenocytes and mild involvement of collagen fibers and mononuclear cells were observed reaching the area of damage in the experimental group (Figure 4).

During the 8th post-operative week, noticeable mononuclear cells and fibrous connective tissues were infiltrating the tendon fibers at the damage site in the control group (Figures 5 and 6). However, the experimental groups showed mature granulation tissue with less congested blood vessels and regular collagen fibers with few cells attached to tendon fibers (Figure 7). The reference group in the 12th week showed collagen fiber attachment to the newly established tendon tissue with undamaged tissue, swollen blood vessels, expanded endothelial cells, and very few fibroblasts of irregular shape (Figure 7). However, the sections from the experimental group revealed novel tendon fibers with substantial cellular similarity to regular tendon fibers joining the anastomotic extremities and strong cellularity in the healing region (Figure 8).

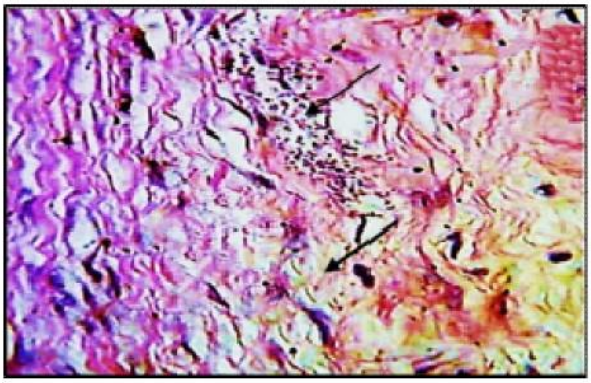

Figure 3. Photomicrograph of defect tendon in control group at 4th week PO, showing hemorrhage (thin arrow) and Irregular highly cellular F.C.T (thick arrows) (H&E, × 200)

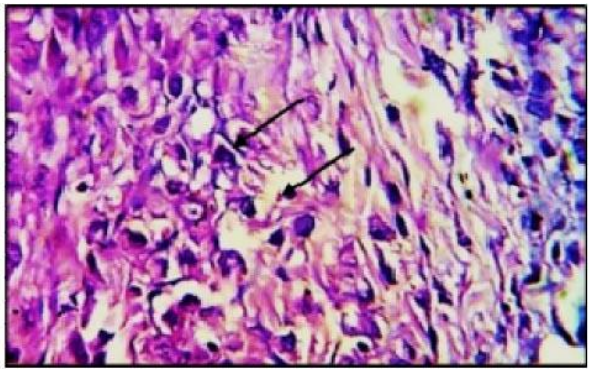

Figure 4. Photomicrograph of defect tendon in combination group at 4th week PO, showing highly cellular F.C.T with intense PMNCs aggregation (arrow) (H&E, × 400)

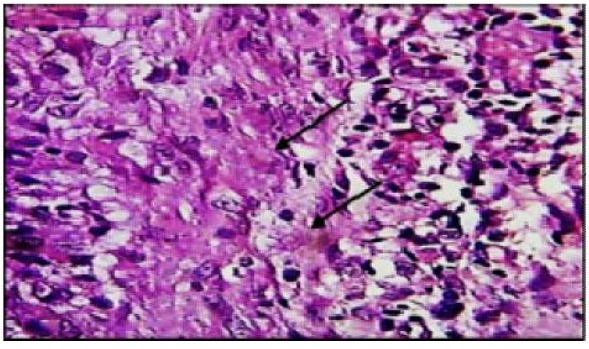

Figure 5. Photomicrograph of defect tendon in control group at 8th week PO, showing immature F.C.T. attached healing (arrows) (H&E, × 400)

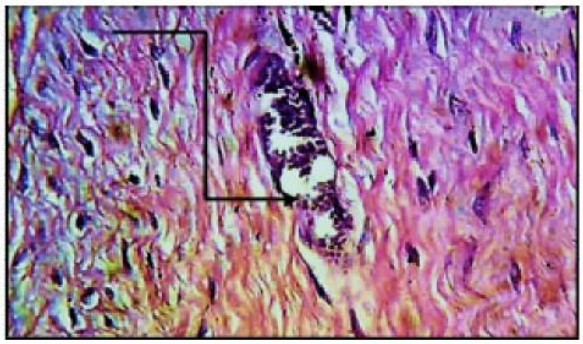

Figure 6. Photomicrograph of defect tendon in combination group at 8th week PO, showing mature granulation tissue consisting with congested B.Vs (arrows) (H&E, × 400)

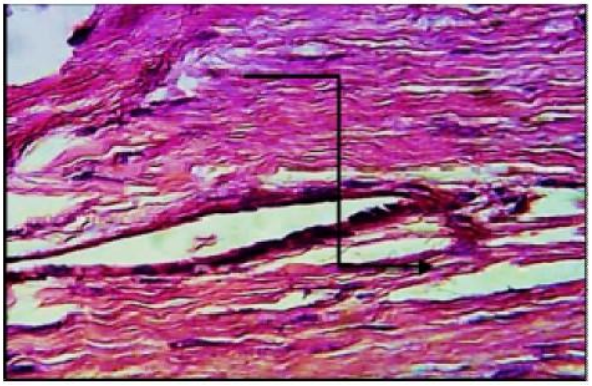

Figure 7. Photomicrograph of defect tendon in the control group at 12th week PO, showing proliferation of F. C. T and remain of scaffold (arrows) (H&E, × 200)

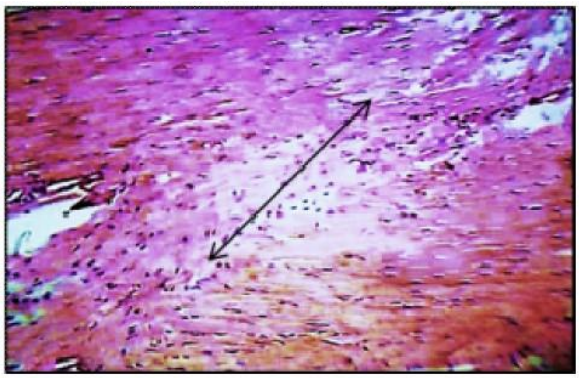

Figure 8. Photomicrograph of defect tendon in combination group at 12th week PO, showing new tendon fibers with highly cellular similar to standard tendon fibers connecting the anastomotic (arrows) (H&E, × 200)

Histopathological exploration scores are illustrated in figure 9. It shows that the experimental group, in which the granulation material with congested BVs was significantly elevated ($P \leq 0.05$) was graded 3, compared to the control group, which was ranked 2-2.5. It is due to the fact that the vascularization at this time frame was essentially reduced ($P \leq 0.05$) in the experimental group which was graded 0.5 and continued to the 12th week. After only a 4-week post-surgery process, the experimental group was scored 3 regarding the inflammatory cell infiltration with a significant increase ($P \leq 0.05$), compared to the control group hich was scored 1.

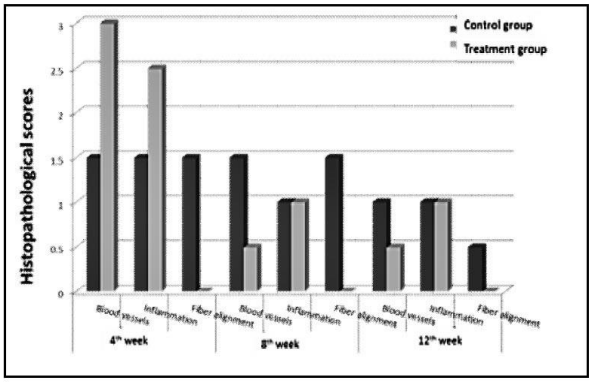

Figure 9. Show the mean values of the histopathological analysis scores

A reduction followed this enhanced inflammatory cell infiltration over time in the experimental group; accordingly, it was scored 2.5, while the score of the control group was 1.5 in this regard. The deposition and direction of the collagen fibers were altogether expanded in the experimental group in the 4th week, which was scored 2.5, while in the control group, it was scored 1.5-2. Moreover, they oriented till at 12th-week post-surgery, when it was scored 1.5, in the experimental group.

4. Discussion

There were no rejections, no infections, and all tendon damage was recovered without any consequences in the present study. This concept was similar to those proposed by Gadupudi, Klaren (16), who had explored it using the dermal patch to enhance

the Achilles tendon in a sheep model. In the present research, at the end of the trial period, there was also no evidence of infections, inflammation, or edema at the operative site (24th weeks). According to Ferrucci and Fabbri, inflammatory cytokines and VEGF function in the capacity of scaffolds to govern the immune system and regulate inflammation; therefore, they can prevent infections at the damaged siteFerrucci and Fabbri (17).

In this study, the increased biomechanical properties of tendon operation in the experimental group may be attributed to potential MSC seeded with scaffold which enhanced the speed of obtaining normal function, Moreover, the results indicated improvements that were associated with the introduction of MSCs into the repair graft. The significantly larger cross-area section of the cells assisted in repair and increased the rate of structural properties. In addition, the histology of this study appears to support the biomechanical findings.

This result agreed with those of a study conducted by Sarrafian, Wang (18), which indicated that TGF-beta1 released from fibroblasts differentiated from cells MSCs could persist mechanical force in the healing process of allo-grafted tendons by promoting the production of collagen I and III, the manufacturing of cross-links, and remodeling of matrices. In the aforementioned research, the biomechanical and immunohistological results revealed favorable outcomes of the MSCs on the affected regions by better tendon remodeling in rat Achilles tendon defect. In that study, after 12 weeks, stem cells had a significant effect on biomechanical assessment.

However, in the present research, scaffolding recovered the biomechanical qualities of tendons that helped to heal and made them sufficiently robust to endure the pressure of motion without breaking or gaping. Present findings were also in line with those of a study performed by M Dohan Ehrenfest, Bielecki (19), who found that the usage of collagen and a polydioxanone implanted sheathing on the healing of an Achilles tendon injury in rabbits increased the biomechanical properties of the wounded location on day 60 after placement. According to them, this was due to the fact that the collagen implant improved the anatomical and physiological qualities of the rebuilt tendon.

Moreover, in the present research, it was found that tendon function and regenerative changes were significantly improved in all the histopathological sections in the experimental group. However, the control group revealed severe irregular collagen fibers, some with vacuolation and necrosis of collagen fibers as well as moderate to severe inflammatory cell infiltration at the injured site. This may be attributed to persistent inflammation and disruption of the pre-existing collagen fibers that were noticed.

This observation was in agreement with a study conducted by Walden, Liao (20) who explained that the tendon healing process is characterized by migration and proliferation of fibroblasts, production of disrupted and randomly arranged collagen fibers, production of extracellular matrix (ECM) during the first three months, tenocytes metabolism and tendon vascularity declined with the gradual development of repair tissue from cellular to fibrous, and then the fibrous tissue change to scar-like tissue which may contain cartilaginous or calcified regions according to the degree of severity of the injury. In the above-mentioned study, it was indicated that the histological changes in the tendons were similar to what occurred during the tendon healing process, which started immediately after tendon injury and lasted for approximately 6-12 months (21).

Nevertheless, the results of the experimental group were close to those of a study performed by Gadupudi, Klaren (16) who explored the sheep model enhancement of the Achilles tendon utilizing the dermal patch as a scaffold. During the trial, they found no signs of infection, inflammation, or edema in the surgical area (24 weeks). The required cytokines and VEGF, present in the scaffold, may play a role in preventing infections at the surgical location due to their capacity to regulate the immune system and

modulate inflammation, as detailed by Meimandi-Parizi, Oryan (22) in a study published in the Journal of the American Medical Association. It has been presumed that implanted ECM demonstrates tissue healing through promoted progenitor cell infiltration, adhesion, and generation association with angiogenesis at the injury site, as well as promotion of granulation tissue formation and deposition of host-derived neo matrix collagen content that outcome in tissue remodeling with diminished scar tissue formation (23).

In addition, the findings of the current study showed the high capacity of the scaffold seeded with BM-MSCs for the induction of a regeneration process in the affected tendon tissues. This reflects the success of implanted stem cells in incorporating into the host tendon tissue (Engraftment) and acting as a critical function in the acceleration of the tendon healing process. It contributes decisively to the reduction of the recovery period and the formation of tendon-like tissue (anabolic effect), compared to the untreated tendons in the control groups, through a number of cytokines and growth factors produced by the MSCs and damaged tissue.

Results of a study conducted by Moshiri, Oryan (24) support these explanation. Moreover, Qasim, Mahdi Mohammed Alakkam (8), demonstrated that MSCs are believed to encourage tendon tissue repair through a complex interaction of MSCs with TNF-α, EGF, IGF-1, and VEGF (trophic effect), in which direct cells are signaling events and tissue repair through neovascularization, collagen deposition, matrix remodeling and a reduction in any inflammatory process. Hafsan, Bokov (10) found that adult MSCs could influence the regeneration process of the damaged tissues via two distinct mechanisms. These mechanisms include direct contribution (differentiation, trans-differentiation, and production of tissue ECM), or indirect contribution (production of bioactive proteins) to tissue healing. The MSCs provide micro-environments for a range of mature tissues that prevent harm and promote a self-regulated reaction to regenerative activity. The activity of a stem cell is thought to be determined by its micro-environment, which includes interaction with neighboring cells, the ECM, the localized milieu, and development and differentiation agents. These mechanisms probably contributed to the reduction in tissue inflammation and improvements in tendon fiber architecture detected in the adult stem cells treated tendons (25, 26).

If xenografts cannot be used to heal a complete tendon lesion, this tissue-engineering strategy using decellularized SIS-ECM packed with BMSCs may be an option worth exploring in place of them. Tissue engineering and regenerative medical uses may benefit from using a natural 3D scaffold implanted with MSCs to aid side-to-side tendon healing by establishing new collagen fibers.

Authors' Contribution

Study concept and design: A. S. H.
Acquisition of data: Q. A. T.
Analysis and interpretation of data: R. H. F.
Drafting of the manuscript: A. S. H.
Critical revision of the manuscript for important intellectual content: A. S. H.
Statistical analysis: R. H. F.
Administrative, technical, and material support: Q. A. T.

Ethics

The present research consents to the Animals' Use and Care Committee, College of Veterinary Medicine, University of Al-Qadisiyah, Al Diwaniyah, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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