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RESEARCH

Use of adipose-derived stem cells in an experimental rotator cuff fracture animal model☆

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KEYWORDS
Cell therapy;
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Abstract
Aim: Rotator cuff repairs have shown a high level of re-ruptures. We hypothesized that the use of adipose-derived stem cells (ASC) could improve the biomechanical and histological properties of the repair.
Materials and methods: Controlled experimental study conducted on 44 BDIX rats with section and repair of the supraspinatus tendon and randomization to one of three groups: group A, no intervention (control); group B, local applications of a fibrin sealant; and group C, application of the fibrin sealant with 2×10^6 ASC. At 4 and 8 weeks a biomechanical and histological analysis was performed.
Results: There were no differences in load-to-failure at 4 and 8 weeks between groups. The load-to-failure did increase between week 4 and week 8. Histologically the tendon-to bone union showed a disorganized fibrovascular tissue. Group C showed a different inflammatory pattern, with less presence of neutrophils and more presence of plasma cells.
Conclusion: The use of ASC does not improve the biomechanical or histological properties of the repair site. More studies are needed to improve techniques that enhance the healing site of the repair.
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PALABRAS CLAVE
Terapia celular;
Reparación;
Manguito rotador;
Experimental;
Celula troncal derivada de lipoaspirado

Uso de células troncales derivadas de lipoaspirado en un modelo experimental animal de rotura de manguito rotador

Resumen
Objetivo: La reparación del manguito rotador tiene una alta tasa de fracaso. Se investiga si la aplicación de células troncales derivadas de lipoaspirado mejorará la resistencia de la reparación y recreará la entesis original.
Material y métodos: Estudio experimental en 44 ratas BDIX con sección y reparación con sutura del tendón supraespinoso y asignación aleatoria a uno de 3 grupos: grupo A, nada (control); grupo B, aplicación local de vehículo de fibrina; y grupo C, aplicación de 2×10^6 células troncales derivadas de lipoaspirado. Se realiza estudio mecánico en célula de carga y estudio histológico en hematoxilina-eosina.
Resultados: En el estudio mecánico no hubo diferencias entre grupos. La carga hasta el fracaso aumentó de los grupos de 4–8 semanas. En el estudio histológico se observó la unión hueso-tendón mediante un tejido fibrovascular desorganizado. En el grupo C se observó un aumento de células plasmáticas a las 4 y 8 semanas.
Conclusión: La utilización de células troncales derivadas de lipoaspirado no recrea la organización celular de la entesis ni mejoran las propiedades biomecánicas de la misma. Son necesarios más estudios para investigar técnicas que mejoren la cicatrización del tendón.
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Introduction

The existence of re-ruptures after rotator cuff repairs is still a frequent problem, with some studies reporting an incidence of up to 94% in massive tears.[1,2] In addition, the functional results of patients with complete repairs are better than those obtained in cases where the repair failed.[3]

The majority of tears present degenerative changes in the local biology of the tendons which include, among others, an increase in cellular apoptosis and alterations in the levels of matrix metalloproteases and various growth factors. Repair of the rotator cuff does not manage to recreate the native structure of the enthesis or its original biomechanical strength.[4,5]

The 2 factors affecting healing of the enthesis include the mechanical resistance of the repair and the biological environment, which affects scarring. There has been considerable work focused on increasing the mechanical resistance of repairs, however, there is less information regarding modification of the local biological environment through the use of therapies to improve scarring.[6] The use of mesenchymal stem cells has been one of the strategies explored to biologically improve these repairs.[7] Stem cells are fibroblastic cells which are able to differentiate toward various different cellular types, including osteoblasts and chondrocytes. Their 2 main sources are the bone marrow and adipose tissue, which is more accessible and has greater proliferative power.[8]

Our hypothesis is that the local application of adipose-derived stem cells (ASC) in a rotator cuff animal model would improve the mechanical resistance of repairs, as well as the histological structure of the enthesis.

Materials and methods

In order to study tendon-to-bone unions in a rotator cuff model we used a total of 24 syngeneic BDIX rats aged between 6 and 8 weeks in the biomechanical study and 18 animals for the histological study. The work was approved by the Ethics Committee for animal experimentation at our center and we followed all the international guidelines for experimentation with animals (86/609/CEE).

Collection of adipose-derived stem cells (Appendix A)

The method to obtain ASC has been published previously.[9] In short, the cells were extracted from the epiploon and subcutaneous fat of 2 animals and were then submitted to mechanical disaggregation and enzymatic cellular digestion. Next, the cellular phase was selected and the ASC were isolated. We verified the cell lineage through differentiation of the cells into adipocytes, chondrocytes and osteocytes, as well as by flow cytometry studies of membrane markers (positive CD 90, CD 73 and CD 105 and negative CD 34, CD 44). Lastly, we multiplied the cells in an adequate medium and carried out a cell count before application.

Surgical technique

The surgical technique has been described previously.[10] In short, a longitudinal incision was made on the proximal part of the front leg with horizontal section of the deltoid, exposing the rotator cuff of the animal. Next, the tendon of the trochiter supraspinatus was sectioned and the animals were

Table 1 Biomechanical results of the repair of the supraspinatus tendon after 4 weeks.

Group	Load until failure (N)	Maximum deformation (mm)	Rigidity (N/mm^2)	Energy absorbed (J)
Control	12.83 ± 3.79	3.99 ± 2.11	5.27 ± 3.11	0.069 ± 0.035
Fibrin sealant	11.57 ± 2.08	4.21 ± 1.72	3.50 ± 1.68	0.051 ± 0.019
ASC	11.72 ± 3.98	5.13 ± 1.78	2.93 ± 1.07	0.080 ± 0.080
P	.67	.41	.11	.47

ASC: adipose-derived stem cells.

randomly divided into 3 groups using an electronic method (Excel, Microsoft, Redmond, VA, USA). The first group underwent repair of the supraspinatus tendon to the trochiter through an anteroposterior bone tunnel using 5/0 monofilament non-resorbable suture. The second group underwent the same repair with a fibrin sealant applied on the repair area (Tissucol, Baxter, Deerfield, IL, USA). The third group underwent tendon repair and application of 2×10^6 ASC imbibed in fibrin sealant. After assigning each subject to an experimental group, we closed the deltoid with reabsorbable suture and the skin with 3/0 silk suture.

Histological and biomechanical study (Appendix B)

The animals were sacrificed after 4 or 8 weeks from the repair. The specimens were prepared for the biomechanical study by individualizing the scapula, the myotendinous unit of the supraspinatus and the humerus, and introduced in a 2 ml syringe with a polymethylmethacrylate (PMMA) bone cement medium (CMW-1, De-Puy, Johnson & Johnson, Warsaw, IN, USA) in order to include them in a lineal electrohydraulic load cell (ControlTest, Servosis, Madrid, Spain). The specimens were preloaded at 0.1 N and tested until failure at a speed of 14 μm/s. The maximum load until failure and the location of the failure were recorded. The rigidity of the sample was calculated by determining the lineal part of the ascending slope of the load-displacement curve.[7]

For the histological study we individualized the scapula and upper limb up to the elbow and fixed them in a 10% formalin solution with a decalcifying solution (EDTA) and imbibed them in paraffin in order to obtain histological sections. The sections were 5 μm in the coronal plane and stained with hematoxylin and eosin. The histological study was carried out by a pathologist who was unaware of the experimental group to which each sample belonged. The study was conducted in 2 different periods, in order to make it more homogeneous. A third reading of the study was obtained when discrepancies arose. We paid special attention to the trochiter, insertion of the tendon-bone repair and supraspinatus tendon *per se*, using an optical microscope. We carried out a study of the microscopic histological qualities, like inflammatory infiltrate, vascularization and cellular and fibrillary organization of the enthesis.

Results

Two animals died due to anesthetic complications and were replaced so as to maintain the predetermined experimental figure. All the animals had achieved normal mobility by 7 days after the intervention. There were no other postoperative complications.

The biomechanical study showed no significant differences between any of the groups for any of the studied variables. The mean load until failure was 12.83 N for group 1, 11.57 N for group 2 and 11.72 N for group 3 after 4 weeks ($P = .67$) (Table 1). In the animals analyzed after 8 weeks, the mean load until failure was 15.11 N in group 1, 15.94 N in group 2 and 14.35 N in group 3 ($P = .88$) (Table 2).

The necropsy test did not reveal any significant differences between the study groups, although the tissue was found to be more robust in the groups sacrificed after 8 weeks than in those sacrificed after 4 weeks.

The histological study found an immature enthesis in the groups sacrificed after 4 weeks evolution, with a more disorganized and highly cellular structure, and an abundance of inflammatory cells, compared to the groups sacrificed after 8 weeks, which presented a slightly more organized structure, with a better alignment of the collagen fibers and with no differences between groups (Fig. 1). The samples analyzed with mesenchymal cells only presented a lower inflammatory response after 4 weeks, although we did not carry out any additional studies to quantify the difference, which disappeared in the groups sacrificed after 8 weeks (Fig. 2). We observed the presence of cells marked with green fluorescent protein after 4 weeks.

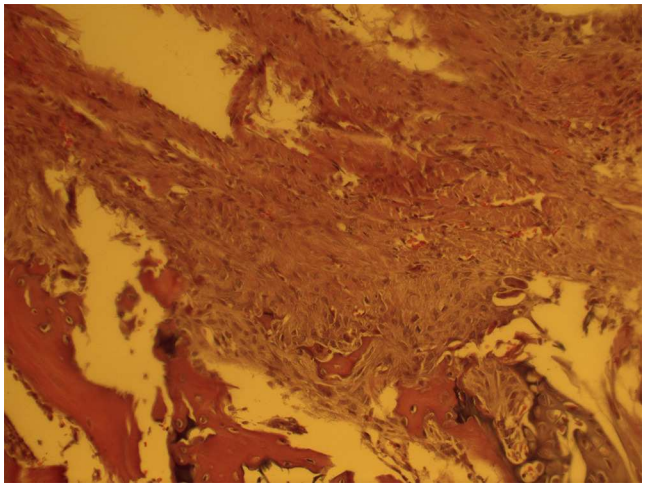

Figure 1 Histological section (hematoxylin–eosin, ×40) of the enthesis after 4 weeks evolution. The enthesis appears highly cellular and notably disorganized.

Table 2 Biomechanical results of the repair of the supraspinatus tendon after 8 weeks.

Group	Load until failure (N)	Maximum deformation (mm)	Rigidity (N/mm^2)	Energy absorbed (J)
Control	15.11 ± 5.75	8.32 ± 4.83	4.10 ± 1.90	0.120 ± 0.051
Fibrin sealant	15.94 ± 5.50	5.09 ± 3.10	4.30 ± 1.94	0.079 ± 0.067
ASC	14.35 ± 4.16	6.46 ± 1.69	3.53 ± 1.61	0.096 ± 0.37
P	.88	.23	.79	.33

ASC: adipose-derived stem cells.

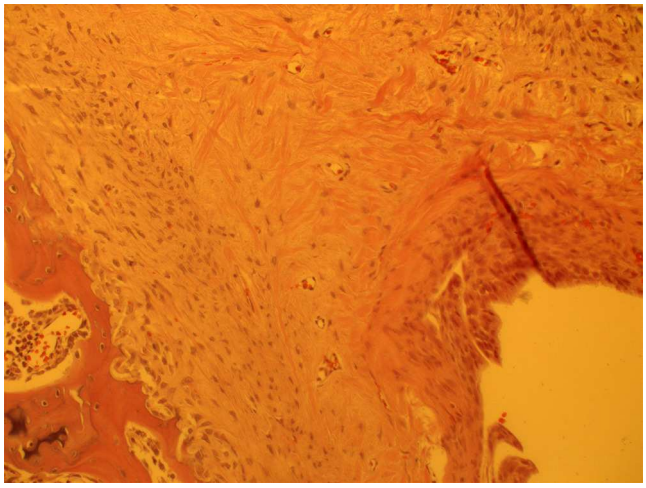

Figure 2 Histological section (hematoxylin–eosin, ×40) of the enthesis after 8 weeks evolution. The fibrillary structure of the enthesis shows a more organized arrangement, although its orientation is still not parallel to the native enthesis.

Discussion

The high rate of failures following surgical repair has raised the existence of an intrinsic biological problem with the tendon. The enthesis is not regenerated by the surgical repair but instead is substituted by scar tissue which has less mechanical resistance than native tissue and may predispose it to suffer new ruptures.[5] The objective of biological therapies is to improve the biological environment around the repair in order to restore the microscopic structure of the native insertion. Growth factors and extracellular matrices have been used with modest results.[11–15] Cellular therapy could play an important role in the treatment of rotator cuff tendon lesions.[7,16,17]

The present work considered the hypothesis that local application of adipose-derived stem cells would improve the biomechanical resistance of the repair as a result of an improved cellular organization. However, the results of the study have not supported this hypothesis.

Mesenchymal stem cells from bone marrow have improved scarring of the tendon to the bone in some previous experimental models of knee cruciate ligament and *flexor hallucis longus* in rabbits.[16,17] The use of isolated stem cells derived from bone marrow did not improve the histology or biomechanical properties of rotator cuff repair in rats. Possible causes considered included the existence of a small contact area between the tendon and the bone, nonexistence of postoperative immobilization and the possibility of insufficient molecular signaling necessary to achieve scarring in such a complex area, which would point to the biological complexity of the repair in these lesions. The use of various biological therapies in combination seems to improve the biomechanical and histological results.[7,18,19]

ASC are easily obtained, genetically stable, with a nearly unlimited proliferative potential and immunoprivileged, as they lack the HLA-DR haplotype. Guilak et al. proved that these cells are able to reduce inflammation, differentiate and induce revascularization.[20] In studies on humans, they have been shown to be safe and effective in the treatment of perianal and tracheoesophageal fistulas.[21,22] We did not register any complications derived from the use of stem cells and confirmed the experience of other authors.[23,24] Assuming equal results as those obtained with stem cells derived from the bone marrow, these advantages would make them attractive for use in rotator cuff repairs.

In our experimental model, ASC did not improve the mechanical resistance or histological appearance of the repair. Despite the fact that some authors suggested a possible influence of mesenchymal cells after 8 weeks of evolution, we were not able to demonstrate any differences.[7] Moreover, we did not find any significant differences after 4 weeks. Perhaps an analysis after 2 weeks would have shown differences that we were not able to detect. The number of cells used was equal to that in other previously used and validated models, but it is possible that this amount was insufficient or that the site of the repair lacked the molecular signals necessary to induce differentiation of the cells we applied.[7,13,16] Other authors have pointed to the difficulty in establishing the correct dose and adequate sealant to achieve true tendon regeneration rather than scarring of the enthesis.[25] It is possible that dispersion of the cells in a fibrin sealant was not capable of containing the cells locally or that the dose used was insufficient or else that the complexity of the necessary molecular signals for the repair without scar cannot be reproduced by the isolated addition of cells.

Further studies focused on combining various inducers of cellular differentiation are required to increase our current knowledge about the specifics of scarring of the rotator cuff.

Level of evidence

Level of evidence I.

Ethical responsibilities

Protection of people and animals. The authors declare that this investigation adhered to the ethical guidelines of the

Committee on Responsible Human Experimentation, as well as the World Medical Association and the Declaration of Helsinki.

Confidentiality of data. The authors declare that this work does not reflect any patient data.

Right to privacy and informed consent. The authors declare that this work does not reflect any patient data.

Financing

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Conflict of interests

The authors have no conflict of interests to declare.

Appendix A. Collection and culture of adipose-derived stem cells

The cells were obtained from the adipose tissue of 2 additional rats according to a protocol described in humans with minimal modifications.[24] In short, the adipose tissue was washed with saline solution buffered with phosphate (PBS; Gibco, Invitrogen, Paisley, UK) and exposed to collagenase (type I; Gibco, Invitrogen) for 30 min at 37 °C. Enzyme activity was neutralized by the addition of 10% fetal bovine serum (FBS; Gibco) and the mixture obtained was centrifuged at 300 G for 10 min. The cellular remnant was treated with 160 mM ammonium chlorhydrate for 10 min to produce the lysis of any remaining erythrocytes. The cells were washed and suspended in Dulbecco modified Eagle medium (DMEM) and 10% fetal bovine serum. We carried out a third centrifugation and the resulting cellular suspension was filtered through a 70 μm nylon mesh. Subsequently, the material obtained was suspended in DMEM with glucose and pyruvate, 10% bovine fetal serum, 1% 2 mM glutamine, 10 μm/ml streptomycin and 1 IU/ml penicillin. The product thus obtained was called vascular stromal fraction (VSF). These cells were cultured in 100 mm trays in concentrations of 10–15 cells/ml at a temperature of 37 °C in a humid atmosphere with 5% carbon dioxide in DMEM with 10% fetal bovine serum and 1% streptomycin/penicillin (Gibco, BRL). The medium was changed to remove non-adhered cells 24 h after incorporation in the tray and subsequently every 4 days. For derived cultures, the cells were disaggregated with 0.05% trypsin (v7 v) in a saline solution buffered with phosphate when a cellular confluence of 70–80% was obtained. We carried out studies of cellular characterization by flow cytometry analyzing the expression of surface markers CD90, CD29, CD45 and CD11b in order to confirm the ASC phenotype of the cultured cells.

We marked the cells with green fluorescent protein (GFP) following the indications of the manufacturer.

Appendix B. Histological analysis

We carried out euthanasia of the animals at the predetermined periods using an intracardiac injection of chlorhydric potassium under general anesthesia and obtained the specimens for the histological study. The specimens consisted of the complete glenohumeral joint, including the deltoid muscle. These were fixed with a 4% formaldehyde solution, decalcified in EDTA for 14–21 days, and imbibed in paraffin. The samples were analyzed using a conventional optical microscope. The samples marked with green fluorescent protein were analyzed with a Leyca DMI 6000 inverted microscope.

B.1. Biomechanical analysis

Sample preparation. Three groups with comparable weights were established preoperatively and at the time of sacrifice. At 4 and 8 weeks, the animals were euthanized with an intracardiac injection of chlorhydric potassium under general anesthesia and the specimens for the biomechanical study were obtained. Each sample was dissected to isolate the scapula, humerus and supraspinatus tendon. The humerus was placed in a 2 mL syringe and fixed with polymethylmethacrylate cement. During polymerization of the cement and to avoid damage caused by the exothermic effects of the reaction, the tendon was protected in a saline solution. The specimens were frozen at a temperature of -80 °C at the time of the biomechanical analysis and unfrozen at room temperature 12 h before the test.

Biomechanical test. The biomechanical test was carried out in a specific electromechanical device consisting of a lineal encoder with a position sensor and 2 load cells of 20 N and 200 N, calibrated to detect small loads. The data were registered in the software program (PCD-2K) designed specifically for Windows 7. We obtained tension-deformation curves and carried out calculations of maximum load until failure (N), rigidity (N/mm^2), absorbed energy (J) and maximum deformation (mm). The scapula was fixed in one of the load cells with a support in order to enable the tendon to be aligned with the appropriate load direction. The specimens were preloaded at 0.10 N and then loaded until failure of the tendon at a rate of 14 μs^{-1}.

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