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Tendon Healing with Allogenic Fibroblast and Static Magnetic Field in Rabbit Model

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Abstract

Objectives- Tendons are integral parts of musculoskeletal system and are subjected to injury. Fibroblast is used in tendon healing, however, there is no proved and reported result regarding concurrent use of allogenic fibroblast with static magnetic field in tendon healing. In addition, there are some studies done on the effect of magnetic fields on tendon healing but the results are antithesis. The aim of this study is to evaluate the effect of simultaneous application of fibroblast and magnetic field on tendon healing in rabbit model.
Design- Experimental study.
Animals- Eighteen female rabbits, 15 months old and weighing 3.0±0.5 kg were used in this study.
Procedures- Two legs of eighteen rabbits were divided into 6 groups. After skin incision, superficial flexor tendon was exposed and cut transversely and then sutured. In control group tendon injury were created in right and left legs and sutured in bunnell mayer suturing technique. In culture media substance group after tendon injury in two legs, 0.5 cc culture substance was injected in the injured tendon area in two legs. In fibroblast group, fibroblast cells were injected in the tendon injured area in both legs. Then all injuries legs were dressed up, a piece of magnet was placed in the surrounding bandage of the left leg for 7 days and right legs were left empty. After 3 months, rabbits were euthanized, tendons were extracted and biomechanical tests and histopathological tests were performed.
Results- Ultimate Strength showed a statistically significant difference which in fibroblast-magnet group was better than other groups. Also, in histopathological evaluation fibroblast-magnet group showed better result in comparison with others.
Conclusion and Clinical Relevance- Simultaneous use of fibroblast cells and magnetic field has a positive effect on tendon healing, both histologically and biomechanically in animal model.
Key Words- Tendon healing, Fibroblast, Static Magnet, Biomechanics, Histopathology.

Introduction

Tendon tissue is a type of connective tissue which physically binds muscles to skeletal structures; therefore, tendons are crucial for power transition and joint movements.[1-3] They must be capable of resisting high tensile forces with limited elongation.[4,5] However, as tendons are subjected to repeated motion and degeneration over time, they are prone to both acute and chronic injuries.[6] Blood supply to the tendon is reported to be poor, thereby healing often proceeds slowly.[3,7] The healing process in tendon results in formation of a fibrotic scar. The structural, organizational, and mechanical properties of the repairs are inferior to normal tendon.[8-10] These tissues are susceptible to adhesion due to excess fibrous tissue formation.[8]Consequently, failure, resulted from tendon injuries, might last for months and if handle improperly during this period, the tendon won't regain its natural function.[9]
Although, recently, there are some methods used for tendon healing [11], an applied healing technique resulting in both normal physical and functional features is yet to

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be discovered.[12] Among, the most frequent technique is surgical operation. Although minor tendon ruptures may treat spontaneously, but chronic ruptures require surgical intervention.[13] Nowadays, surgeons connect the proximal and distal stumps directly. However, when there is a complete transverse rupture in the tendon or the injured area is vast, tendon graft is recommended.[14,15] In this regard, surgeons can use autograft, allograft, xenograft, synthetic polymers, or absorbable biomaterials. However, they have their own complications, for example: donor-site morbidity for autografts, graft rejection (in allograft and xenograft), nonfunctionality, adhesion formation probability, vascular and neural damage and severe contraction of muscles around the operation area which can all lead to treatment failure.[10,15] For all aforementioned reasons, finding a developed treatment technique resulting in faster healing with less side effects than that of current therapies is of a great interest.[16]

Recently, the major literature in tendon injury treatment is focused on mesenchymal stem cells (MSCs) because these cells are the primary origin of skeletal tissues naturally. In addition, they have a potential ability in tissue engineering making them more applicable in studies. Tenocytes, which are tendon fibroblasts, play main role in producing procollagen, proelastin, and reticulin The point is that in tendons, the main responsibility for producing procollagen, proelastin, and reticulin in tendon injury.[9,17] Therefore, nova days some studies designed to evaluate the effect of fibroblasts application in tendon repair. These cells can be biopsied from tendon itself or the skin tissue. Skin derived fibroblast (SDFs) have some advantages which are: frequently available, easily cultured and isolated and less invasively biopsied (in comparison with MSCs from bone marrow). Moreover, compared to MSCs and autologous tenocytes, SDFs posess less differentiation potential resulting in less exotic tissue formation and harvesting procedures do not induce serious secondary injury to the donor site, respectively.[3,10,18]

Biomagnetics is an interdisciplinary field in which magnetism, biology and medicine overlap and is a popular but controversial method.[19,20] The use of electromagnetic fields in the healing arts dates back as far as the 15th century.[21] In addition, use of static magnetic field (SMFs) in tendon healing with encouraging results has been reported.[22] It has been proved that SMFs can stimulate bone formation by promoting osteogenesis through mechanisms such as neovascularization, collagen production, proliferation and differentiation of osteogenic cells, and the maintenance of the molecular structure of the extracellular matrix.[19,23] Although there is ample evidence supporting the use of magnetic fields to aid bone healing, its application for soft tissue healing, including skin and tendons, is still ambiguous.

The aim of this study is to evaluate the effect of simultaneous application of fibroblast cells and static magnetic field on injured tendon in rabbit model using biomechanical and histo-pathological methods.

Materials and Methods

Animals

Eighteen female white New Zealand albino rabbits, 15 months old and weighing 3.0±0.5 kg were used in this study. Animals were acclimatized for 15 days before the experiment. The experimental protocol was approved by the Animal Care and Experiment Committee of the Shahrekord University, in accordance with the ethics standards of the "Principles of Laboratory Animal Care".

Isolation and in vitro culture of allogenic dermal fibroblasts

In the present study, one healthy rabbit was sedated with acepromazine (0.02 mg/kg, IM, Alfasan, the Netherlands) and the ear skin was prepared aseptically. Anesthesia was induced using Ketamine (30 mg/kg, IM, Alfasan, The Netherlands) then, a 2×2 cm^2 sample of the full thickness ear skin was biopsied bye surgical blade and transferred to laboratory. Dermal fibroblasts were isolated and cultured using a previously described method [24]. The aforementioned sample was rinsed with phosphate-buffered saline (PBS) 3-4 times and then minced into small 1×1 mm^2 pieces. The tissue fragments were rinsed again with phosphate-buffered saline (PBS) followed by digestion with 1.5 mg/mL type II collagenase in serum free Dulbecco's modified Eagel's medium at 37°C on a rotator. The resulting cell suspension harvested at 6 h post-digestion was filtered through a sterile nylon mesh to remove tissue residues. The filtrate was further centrifuged and cell pellets were washed with PBS twice and then re-suspended in DMEM culture medium containing 10% fetal bovine serum (FBS, Gibco), 100 µg/mL streptomycin, 100 µg /mL penicillin and 100 µg/mL ascorbic acid. The extracted cells were plated on 100 mm culture dishes (1× 10^6 cell/dish) and incubated at 39°C in a humidified atmosphere containing 95% air and 5% carbon dioxide. When cultured cells were grew and reached 80-90% confluence, they were detached with trypsin-EDTA solution (0.5% trypsin, 0.2% EDTA in PBS substance) for 5 minutes and then phosphate-buffered saline (PBS) was added to neutralize trypsin. Pellet content was centrifuged 700 *g* for 5 minutes. Floating was performed using culture substance and finally, 30-40 µl of the final mixture was transferred to new pellets containing 5 ml culture substance. They were incubated again and after 80-90% confluence reached, trypsinization and culture steps repeated till passage 3. Cell content was about 7-8×10^6 cell/ml and allogeneic dermal fibroblast cells were ready to use for injection in the injured tendon site.

Surgical techniques

All rabbits in the present study were sedated using Acepromazine (0.02 mg/kg, IM), caudal parts of both hindlimbs between the stifle and tars were shaved and prepared aseptically with povidone iodine and the limb draped with sterile drapes. Anesthesia was induced using ketamine (30 mg/kg, IM). An incision was made directly over the skin of Achilles tendon, superficial digital flexor tendon was exposed and cut transversely and then sutured with nylon 2/0 in a Bunnel-Mayer stitch pattern. Subcutaneous and skin tissues were aligned by common stitch patterns. 18 rabbits were divided in 6 groups according to table 1. In control group (n=6 rabbits) tendon injury were created in right and left legs and also sutured with nylon 2/0 in a Bunnel-Mayer stitch pattern. In culture media substance group (n=6 rabbits) after tendon injury in two legs, 0.5 ml DMEM culture medium was injected in the injured tendon area in two legs. In fibroblast group (n=6 rabbits), allogenic fibroblast cells ($3.5\text{-}4\times10^6$ cells) were injected in the tendon injured area in both legs. Then all injuries legs were dressed up (in all rabbits), a piece of magnet ($10\times10\times1$ mm^3, 2500 gauss) was placed in the surrounding bandage of the left leg for 7 days and right legs were dressed without magnet. After 3 months, rabbits were euthanized humanly (pentobarbital was injected intravenously 100 mg/kg)[25] and treated tendons were excised .

Biomechanical evaluation

Fresh specimens harvested after 3 months were submitted to tensile strength measurement using a biomechanical analyzer (Instron, Canton, MA). Each tendon was loaded by elongating it at a displacement rate of 10 mm/s until a 50% decrease in load was detected.[26,27] During tensile testing no slippage was noted. Load and crosshead displacement data were recorded at 1500 Hz, and load-deformation and stress-strain curves were generated for each specimen. Biomechanical properties including ultimate strength, yield strength, ultimate strain, yield strain, stiffness and stress were measured.

Histopathological evaluation

Immediately after the biomechanical tests[26-30], samples were fixed using formalin solution (10%) and transferred to pathology laboratory. The formalin solution was changed after 24 hours and then after 10 days, tissue samples were sectioned, stained with H&E method, and observed with light microscopy. Histopathological samples were scored qualitatively and semi-quantitatively based on modified Rosenbaum et al and Oryan et al scoring system[26-30] (table 1).[31]

Statistical analysis

Biomechanical test driving data were analyzed by One-way ANOVA test ($p<0.05$ was considered significant). Histopathological driving data were analyzed by Kruskal-Wallis test ($p<0.05$ was considered significant). When p was less than 0.05, then pair wise group comparisons was performed by Mann-Whitney U test (SPSS version 20 for windows, SPSS Inc, Chicago, USA)**.**

Results

There was no intraoperative and postoperative death during the study. None of the rabbits sustained a tendon rupture in the injured area.

Biomechanical evaluation

Biomechanical data are presented in table 2 as Mean ± Standard Deviation (M±SD). There was no significant difference between biomechanical properties except for ultimate strength which was statistically higher in fibroblast-magnet group than that of other groups ($p<0.05$).

Histopathological evaluation

Histopathological data are presented in table 3 as Median (min-max). Only fibrocyte population and collagen fibers' orientation revealed statistically significant difference ($p<0.05$). There was significant differences between fibroblast-magnet group and empty, magnet, and culture-magnet groups and the latter was between fibroblast-magnet group with all others except fibroblast groups. In both of the above markers, fibroblast-magnet group revealed better performance. Moreover, figure 1 shows histo-pathological sections of different groups with fibrocyte population and collagen fibers' orientation.

Table 1. Histopathological scoring system

Marker	Scores
Inflammation degree	0,1, and 2 (qualitative)
Fibroblast population	0, 1, and 2 (qualitative)
Fibrocyte population	0, 1, and 2 (qualitative)
Collagen fiber orientation	1, 2, 3, and 4 (semi-quantitative)
Neovascularization	1, 2, and 3 (semi-quantitative)

Table 2. Biomechanical findings after 90th postoperative day

Tensile strength test criteria	Mean±SEM						
	Control (n=6)		Culture media (n=6)		Dermal Fibroblast (n=6)		P
	Without magnet	With magnet	Without magnet	With magnet	Without magnet	With magnet	
Stress (N/mm^2)	2.12±1.08	1.95±0.79	0.76±0.38	0.81±0.25	2.55±0.59	4.7±3.39	0.0514
Stiffness (N/mm)	10.67±5.51	10.25±3.29	6.03±3.47	4.87±0.31	10.33±3.36	10.8±5.21	0.0939
Yeild Strength (N)	31.42±22.31	23.26±10.93	12.2±3.79	16.22±3.27	28.85±6.01	46.13±10.29	0.0507
Ultimate Strength (N)	39.28±24.19	36.89±22.38	18±4.83	20.96±5.78	40.91±8.66	61.74±13.28[a]	**0.0457**
Yeild strain (%)	28.67±8.69	34.72±3.05	28.8±1.63	39.6±16.61	23.46±13.97	23.32±12.67	0.0544
Ultimate strain (%)	109.9±61.68	90.09±30.04	94.7±17.33	135.8±70.86	61.26±23.44	108.8±35.45	0.167

Significant P-values are presented in bold face.
[a] fibroblast-magnet group showed significant difference (P<0.05), in comparison with other groups

Table 3. Histopathological evaluation results after 90th postoperative day

Histopathological criteria	Med (Min-Max)						
	Control (n=6)		Culture media (n=6)		Dermal Fibroblast (n=6)		P[a]
	Without magnet	With magnet	Without magnet	With magnet	Without magnet	With magnet	
Neovascularization	2 (2-2)	2 (1-2)	1 (1-2)	2 (1-2)	2 (1-2)	1 (1-2)	0.401
Collagen orientation	3 (2-3)	2 (2-3)	2 (2-3)	2 (2-3)	4 (3-4)	4 (4-4)[b]	**0.025**
Fibrocyte	1 (1-1)	1 (1-1)	1 (1-2)	1 (1-1)	2 (1-2)	2 (2-2)[c]	**0.033**
Fibroblast	2 (1-2)	1 (1-2)	1 (1-1)	1 (0-2)	1 (1-1)	0 (0-1)	0.1
Inflammation	0 (0-1)	1 (0-1)	0 (0-0)	0 (0-0)	0 (0-0)	0 (0-0)	0.236

Significant P-values are presented in bold face
[a] Kruskal-Wallis non-parametric ANOVA
[b] fibroblast-magnet group showed better results than all other groups (p<0.05) except fibroblast group
[c] fibroblast magnet group, showed better results than other (p<0.05)

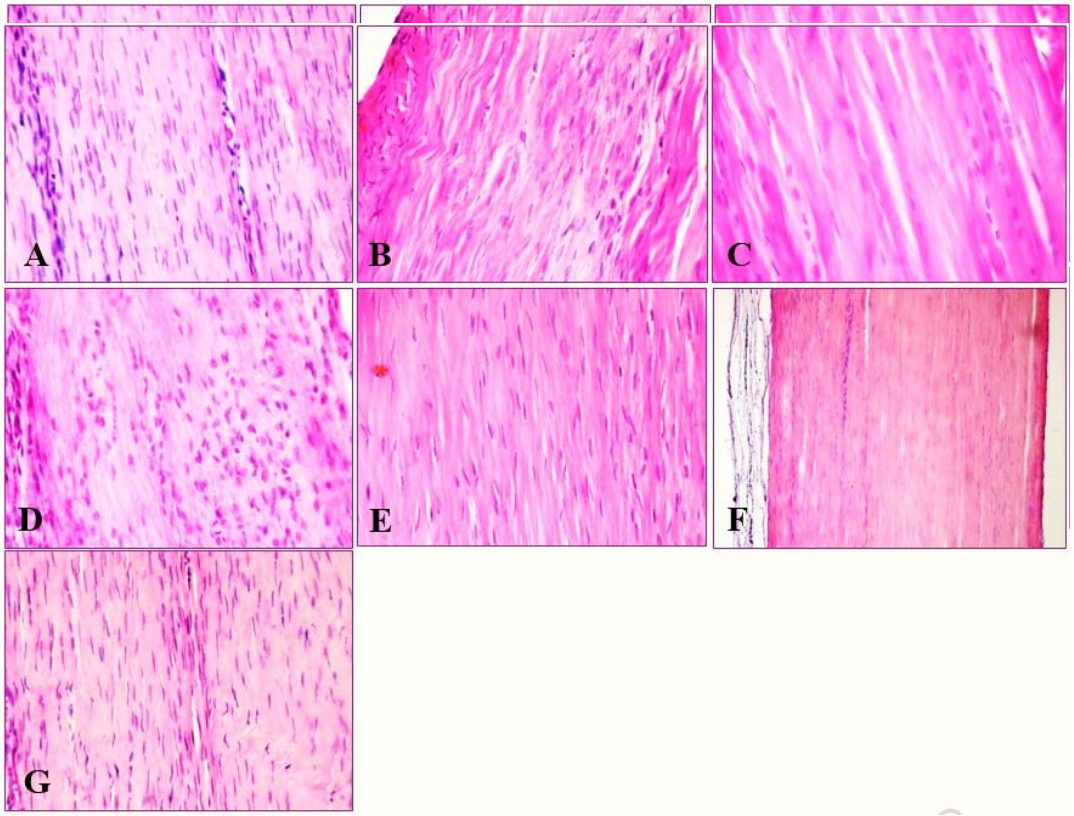

Figure 1- tissue sections of different groups **A)** Normal tendon, thick and dense well oriented collagen fibers and well distributed fibrocytes between the fibers (H&E×10). **B)** Empty group, fairly regular collagen fibers and the dominant population of fibroblasts (H&E×40). **C)** Magnet group, fairly regular collagen fibers and the dominant population of fibroblasts and a few fibrocytes between the fibers (H&E×40). **D)** Culture substance group, irregular collagen fibers' orientation with active fibroblasts (H&E×40). **E)** culture-magnet group, fairly regular collagen fibers with fibroblasts and fibrocytes between the fibers (H&E×40). **F)** Fibroblast group, regular collagen bundles with a great number of fibrocytes and a few fibroblasts (H&E×20). **G)** Fibroblast-magnet group, dense collagen bundles with adult fibrocytes well oriented and distributed between fibers (H&E×40).

Discussion

Tendons are structures which are usually at risk of injury due to severe trauma, over use cause of sports, doing difficult bodily stuffs, and even daily activities, thus, tendon injuries from the major part of orthopedic procedures.[2,13,14,32] Tendon problems are of great importance due to both their role in body functions and complication in healing process.[3] There are three major complications related to tendon healing:

1. Low blood supply: tendon healing period is remarkably higher than other types of connective tissue such as bones.[3,7]
2. Healing doesn't lead to the normal histological structure, i.e. healing occurs forming a scar tissue whose quality is less than that of normal tendon. Therefore, healed tendon doesn't have normal function and may reinjure.[8-10]
3. Tendons are susceptible to adhesion due to excess fibrous tissue formation.[8]

In severe and vast tendon injuries, it's even worse because there's no scaffold to orient cells migration toward the injured area. Therefore, these cells migrate and proliferate in different orientations, in addition, due to fibroblasts movement toward tendon fascia, the healing capacity decreases, muscular fibrous is also probable. These tendon injuries may remain nonunion.[33,34] Other limitation of this type of injury is muscular atrophy.[35] Consequently, tendon healing failure may last for months and inefficient management of injury during this period, this tendon will remain functionless.[9] Although there are some methods helping tendon healing these days, none are applied techniques leading to both physical and biomechanical progress.[12] Therefore, finding a developed treatment technique resulting in fester healing and less side effects than that of current therapies is of a great interest these days.[36]

In this regard, current study was designed and performed. The main aim was to answer this key question: "Does concurrent application of fibroblast

cells and static magnetic field accelerate tendon healing and increase its quality in long term or not?"

Dermal fibroblasts have been used in tendon engineering due to their abundant supply, ease of harvesting, and reprogrammability. They have multi differentiation potential and have been shown to develop into brain, glia, muscle, and adipose lineages. [37] In vitro experiments have shown promise in tendon engineering.[38] In our study histopathological evaluation revealed this phenomenon in the dermal fibroblast injection groups (with or without magnet) in the tendon injured site. Also, Connell et al. showed that dermal fibroblasts could be expanded, stretched, and induced to lay down collagen in a similar fashion to tenocytes.[39]

In a randomized trial of 60 cases of patellar tendinopathy, comparing ultrasound guided intratendinous injection of dermal fibroblasts to plasma controls, a faster response to treatment and significantly greater reduction in pain and improved function was noted in the treatment group.[40] One patient in the treatment group experienced tendon rupture, and subsequent biopsy showed relatively normal tendon tissue with type I collagen and tenocytes with normal morphology, and no ectopic tissue was noted.

In our study, histo-pathological evaluations revealed that fibrocyte population was significantly higher ($p<0.05$) in fibroblast-magnet group than those of no substance, magnet, and culture medium groups. Most probably, it's due to injection of fibroblasts in the defected area. However, simultaneous application of culture substance and static magnetic field might have a positive effect on cell proliferation by disposing nutrients and direct fibroblast stimulation.[41] Collagen fibers' orientation in fibroblast-magnet group was significantly higher ($p<0.05$) than those of empty, magnet, culture medium, and culture substance-magnet groups, which might indicate that fibroblasts play the main role in this case. In fact, magnetic field alone, even if is effective, couldn't show its influence after a long time (3 m) after operation.

In the present study, ultimate strength in fibroblast-magnet group was significantly higher ($p<0.05$) in comparison to culture medium group. This may be due to both fibroblasts (higher collagen I/III) and magnetic field (increasing blood supply and stimulating fibroblasts) effects.[41-44] As Strauch et all in 2006 showed that skin fibroblast application in tendon healing results in a 69% increase in tensile strength of rats Achilles tendon and forms an applicable tendon, at least biomechanically and Lui et al in 2006 claimed that dermal fibroblasts increase the tensile strength of treated tendon to 76% in 26 weeks.[18]

According to our results, in the other criteria of biomechanical evaluation, significant difference was not revealed. We proposed that it's cause of the long after operation period. I.e. all the groups had acceptable progress in healing procedure.

Conclusion

Since in the present small groups to conclude, in response to the main question, we can claim that simultaneous application of dermal fibroblasts and static magnetic field has positive effects on tendon healing; however, in long after operation periods, it's most likely due to presence of fibroblasts.

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چکیده

ترمیم آسیب تاندونی با استفاده از فیبروبلاست آلوژنیک و میدان مغناطیسی ثابت در مدل حیوانی

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هدف- تاندون جزیی از ساختار اسکلتی- عضلانی محسوب می‌شود که دچار آسیب می‌گردد. امروزه از فیبروبلاست در ترمیم زخم پوستی و تاندونی استفاده می‌شود. همچنین از میدان مغناطیسی نیز در درمان آسیب های تاندونی استفاده می‌شود ولی نتایج ضد و نقیضی حاصل شده است. هدف از انجام این مطالعه بررسی استفاده همزمان از فیبروبلاست و میدان مغناطیسی ثابت در ترمیم تاندون آسیب دیده می‌باشد.

نوع مطالعه- تجربی

حیوانات- ۱۸ خرگوش بالغ

روش کار- دو اندام خلفی در ۱۸ خرگوش به ۶ گروه تقسیم شد. در گروه کنترل در دو اندام خلفی ضایعه تاندون خم کننده سطحی ایجاد شد. در گروه محیط کشت بعد از ایجاد آسیب و بخیه پوست در محل آسیب ۰/۵ میلی لیتر محیط کشت در محل تزریق شد. در گروه فیبروبلاست بعد از ایجاد آسیب و بخیه پوست در محل آسیب سلول های فیبروبلاست در محل تزریق شد. در تمامی خرگوش ها اندام‌ها پانسمان شدند و در پای چپ آهن ربا به مدت ۷ روز داخل پانسمان قرار داده شد و پای راست بدون آهن ربا رها گردید. بعد از ۹۰ روز خرگوش ها به روش انسانی معدوم شده و تاندون‌های مورد درمان و کنترل جهت انجام آزمایش بیومکانیک و پاتولوژی خارج گردیدند.

نتیجه‌گیری و کاربرد بالینی- در آزمایش هیستوپاتولوژی و بیومکانیک گروه فیبروبلاست به همراه آهن ربا نسبت به بقیه گروه‌ها نتایج بهتری را نشان دادند.

کلید واژگان- ترمیم تاندون، فیبروبلاست، آهن ربا.