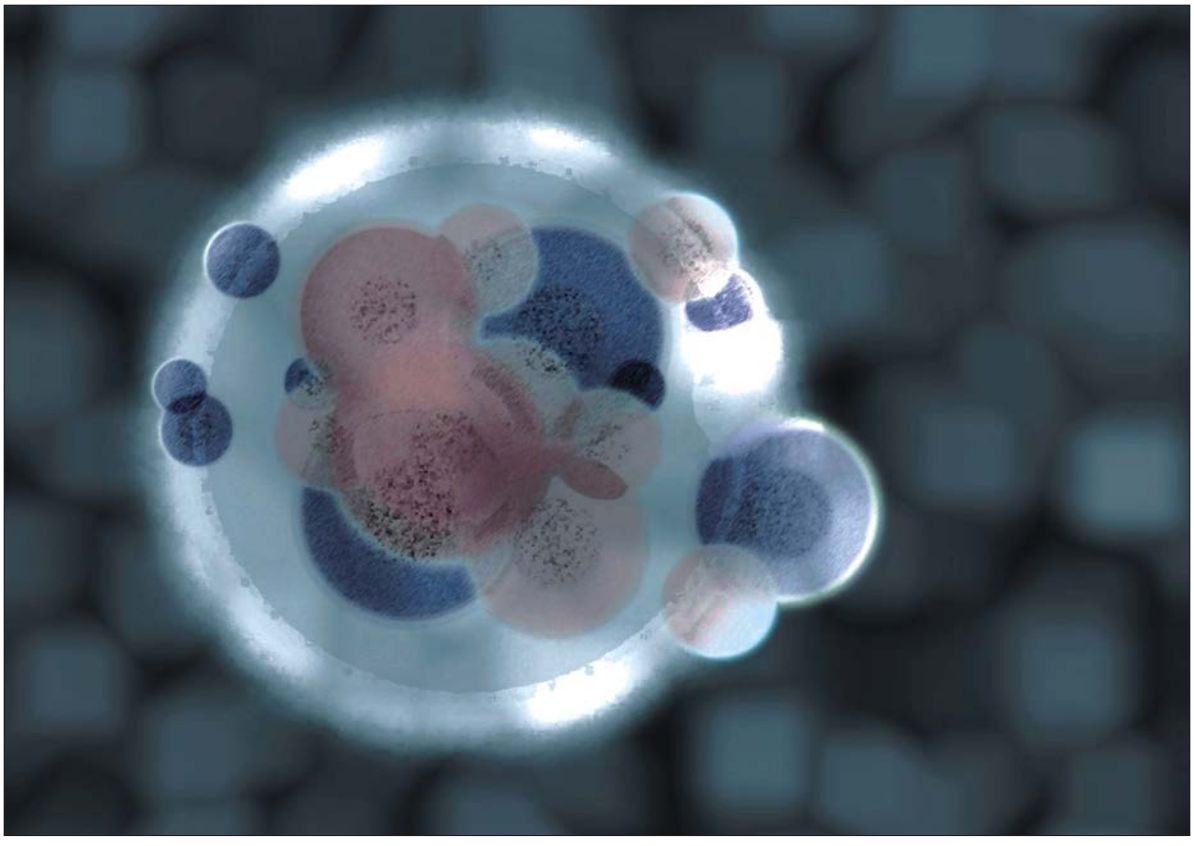

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Experimental Study of the Tendon Healing and Remodeling After Local Injection of Bone Marrow Myeloid Tissue in Rabbit

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Abstract: The aim of this study was to clarify whether of Bone Marrow Myeloid tissue decreases the period of regeneration of tendon and enhances healing. Fifteen New Zealand white rabbits with body weight ranging from 2.5 to 3 kg were used in this study, divided into three groups, five of each. The Achilles tendons were damaged by collagenase injection. Bone Marrow aspirated from the iliac crest and were injected to the injured tendon of the right leg and normal saline into the control leg. The animals were euthenized after 10, 20 and 30 days, respectively and tendon samples were taken for histopathologic examinations. Histopathologic observation demonstrated that Bone Marrow injections resolved edema, swelling and inflammatory cell infiltration in injured tendons. Also different degrees of increased fibroblast hypercellularity and vascularity were seen. In this experiment, the cellularity, vascularity, collagen density and collagen fibril organization were examined within and between the groups 10, 20 and 30 days after collagenase injection ($p>0.05$). The results showed no significant differences within the control and treatment groups and also between the control groups ($p>0.05$). However, significant differences were obvious between the treatment groups of 10, 20 and 30 days after collagenase injection ($p<0.05$). According to the results of the present study, injection of the bone marrow myeloid tissue may have effects on tendon healing in rabbit, as assessed by histopathological examination, compared to control group.

Key words: Bone marrow, tendon regeneration, rabbit, myeloid tissue

INTRODUCTION

Tendon tissue is formed by an intricate network of macromolecules that constitute the extra cellular matrix and interact with the cellular component composed of tenocytes (Dowling and Dart, 2005). The major constituent of the extracellular matrix are collagen type I and noncollageneneous glycoprotein's and proteoglycans. The interaction between these components is important for the function of the tendon, the conduction and withstanding of tensile loads, particularly when subjected to the heavy stress (Cribb and Scott, 1995).

In case of tendon injure, absolute or relative overloading will result in the tearing of tendon fibrils, with ensuing. Many therapeutic approaches have been used to improve hemorrhage and edema (Cribb and Scott, 1995). After the initial inflammatory response, the defect will be filled by granulation tissue consisting of fibroblasts, which will differentiate into new tenocytes that, at a certain stage, will start to produce new components of the extracellular matrix (Cherdchutham *et al*., 1999; Haupt *et al*., 2006).

During tendon injury, as with damage to any tissue, there is a requirement for cell infiltration from the blood system to provide the necessary reparative factors for tissue healing (Fenwick *et al*., 2002).

Severe tendon injuries are difficult to manage. Surgical repairs frequently do not fully restore function due to the fibrous adhesions or failure arising from the mechanical demands placed on imperfect integrative healing at tendon-tendon or tendon-bone interfaces (Chen *et al*., 2004).

Many therapeutic approaches have been used to improve tendon healing, including physical therapy, the use of steroidal and non steroidal anti-inflammatory drugs (NSAIDs), surgical intervention and use of support bandage which have been shown to alleviate the load on the tendon (Willmen *et al*., 1999; Gibson *et al*., 2005; Hosaka *et al*., 2005).

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Intralesionally, polysulphated glycosaminoglycans have been reported to reduce the inflammatory reaction in case of acute tendonitis in horses (Dow *et al*., 1996). Also sodium hyaluronate has been advocated as having an anti-inflammatory effect (Howard and Mcllwraith, 1996). The drug beta aminopropionitril fumarate (BAPN-f) has been reported in animal models to have an effect on the formation of scar tissue when interalesionally applied by reversibly inhibiting the enzyme lysyle oxidase, thus blocking lysine deamination which as an important first step in formation of covalent HP and LP crosslinks (Cohen, 1985; Sardari *et al*., 2007).

A new approach recently advocated is tissue engineering of tendon (Woo *et al*., 1999; Butler and Awad, 1999). As the name implies, tissue engineering is the laboratory based design and development of tissues and organs to replace or support the function of defective or injured body parts. Molecules, macromolecular assemblies and scaffolds are combined with progenitor cells and induced to differentiate into the desired tissue (Young *et al*., 1998; Awad *et al*., 1999; Randell *et al*., 2005).

This is not to say that a stem cell populated biomaterial would not enhance repair, only that, as with cartilage and ligament repair, stem cell technology has not as yet attained acceptable performance (Koob, 2002).

Both *in vivo* and *in vitro* studies have clearly indicated that tendons retain an intrinsic capacity for tissue repair (Lundborg and Rank, 1980; Becker *et al*., 1981). Moreover, tendon repair is accomplished primarily by the resident tendon fibroblasts. However, extrinsic factors may influence the efficacy of the repair response mounted by endogenous tenocytes, as well as the involvement of peripheral tissues. The relative contribution of each depends upon the nature of the trauma, the anatomical position of the injury and subsequent repair, vascularity and most important, the presence or absence of a synovial sheath. Complications derived From extrinsic sources are detailed below in the cell biology section (Koob, 2002).

Bone marrow contains numerous cell populations including adult Mesenchymal Stem Cells (MSCs) that can differentiate, along multiple lineages, to form tendon (Forslund, 2003; Awad *et al*., 2003).

The present study was carried out to verify the effects of bone marrow myeloid tissue in tendon healing.

MATERIALS AND METHODS

Animals: Fifteen skeletally mature male, New Zealand white rabbits (age, 6 months, weigh 2.5-3 kg) were used at the present study.

They were kept in soft bedding cages with free access to food and water. Rabbits were assigned to three groups of experiments, 5 of each. Rabbits of 1st, 2nd and 3rd groups were euthanized 10, 20 and 30 days, respectively.

General anesthetize was performed by ketamine HCl 40 mg kg^{-1} and xylazine HCl 0.01 mg kg^{-1} intra muscullary and followed by sodium thiopental for euthanized.

Experimental set-up: A single dose (0.1 mL; 400 IU) of collagenase (Sigma, St. Louis, MO, USA) was injected into the central region of the Achilles tendon, 3 cm above the calcaneal tuber in left and right limb of each rabbit.

Bone marrow was aspirated from the iliac crest and collected into polypropylene tubes containing 1000 units mL^{-1} preservative-free heparin. The bone marrow and heparin were well mixed and then injected to the left leg of each rabbit. Pure Normal saline was injected to the right leg of each rabbit as the control group.

After euthanized specimens, taken for histopathology, were fixed in 10% phosphate buffered formalin and longitudinal tissue sections were prepared for histology. The tissues were dehydrated, cleared in xylene, embedded in paraffin, sectioned in the following sequences and finally mounted on microscopic slides.

Sections were stained with Pic Indigokarmin, Hematoxylin-eosin (H and E) and Van Gieson for evaluation cellularity, vascularity, collagen density at day 10, 20 and 30 post-injection of bone marrow.

Pic Indigokarmin in polarized light microscope were used to evaluate collagen fibril organization of healing Achilles tendon at day 10, 20 and 30 post-injection of bone marrow.

Statistical analysis: For statistical analysis, ten slides from each sample and ten microscopic field (magnification x400) from each slides were Considered. The mean of these one hundred numbers were used in grading data.

Statistical analyses were performed using the SPSS 9 program for windows (SPSS Inc., Chicago IL, USA). The grading data within and between group were analyzed using Paired sign test and Kruskal wallis, respectively. Differences were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Ten days after Bone Marrow Myeloid Tissue treatment a large number of plum-shaped and spindle-shaped fibroblasts from peritendon recruited into the lesion site and turned into hypertrophied cellular tissue (Fig. 1).

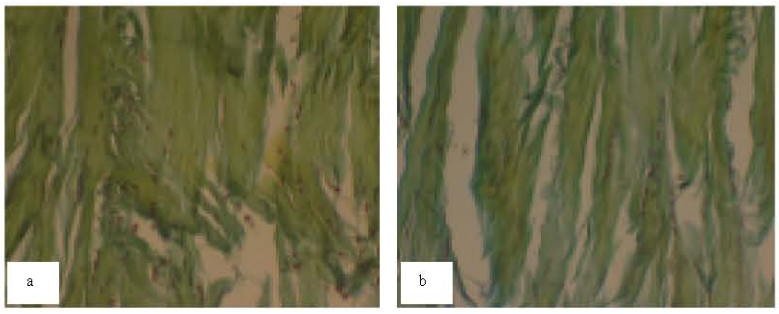

Fig. 1: Pic Indigokarmin (x250) staining of histological changes in Bone Marrow-Derived cells treatment of *Achilles tendonitis* after 10 days of test group (a) and control group (b)

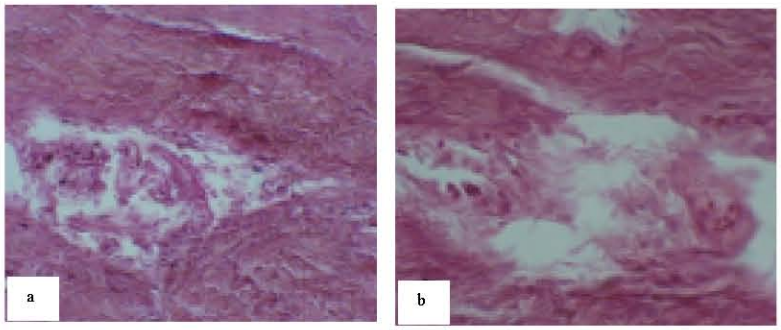

Fig. 2: Hematoxylin-eosin (x250) staining of histological changes in Bone Marrow-Derived cells treatment of *Achilles tendonitis* after 20 days of test group (a) and control group (b)

Twenty days after Bone Marrow Myeloid Tissue treatment, inflammation was gradually resolved and intensive blood capillaries and extracellular matrix production were observed in lesion site and spindle-shaped tenocytes gradually oriented into tendon bundles. Intensive tendon fibril continued to be produced. Fibril increased in size to become histologically visible thin wavy forms (Fig. 2).

Thirty days after Bone Marrow Myeloid Tissue treatment, lesion site appeared to be fused by a fibrous bridge. The granulation tissue and inflammation cell infiltration was completely improved. Well-aligned tendon fiber bundles gradually formed parallel to the long axis of the tendon (Fig. 3).

Under polarized light microscopy, normal tendon showed well-organized collagen bundles. The birefringence of bundles of collagen fibers were longer, wider and in better longitudinal alignment along with the cell axes compared to that of control tendons in 10, 20 and 30 days tendons (Fig. 4).

No significant differences was seen in cellularity, vascularity, collagen density and collagen fibril organization within treatment and control groups in 10, 20 and 30 days after collagenas injection ($p>0.05$; Table 1). No significant differences was seen between control

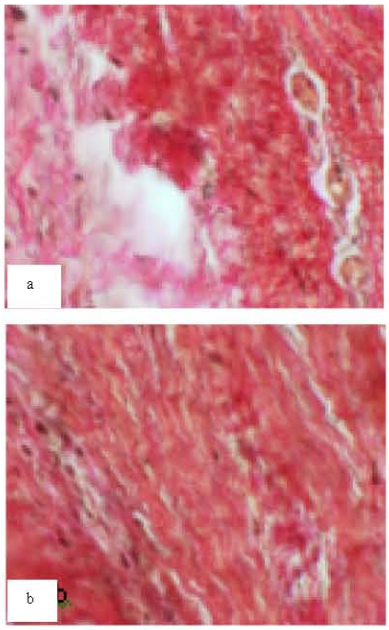

Fig. 3: Van Gieson (x450) staining of histological changes in Bone Marrow-Derived cells treatment of chilles tendonitis after 30 days of test group (a) and control group (b)

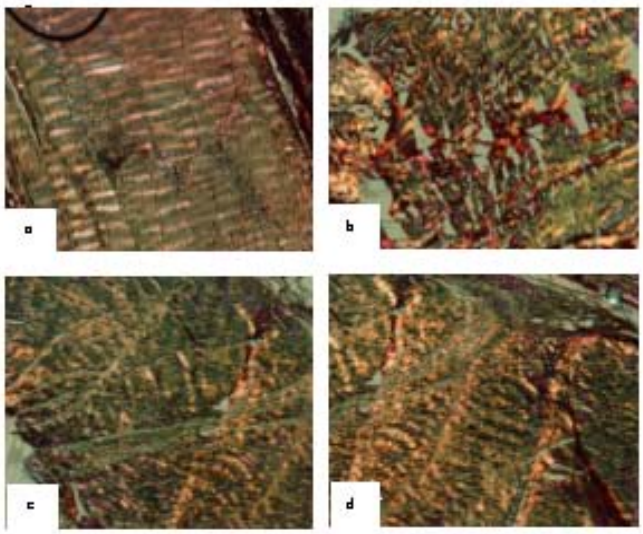

Fig. 4: Pic Indigokarmin (x250) Observation of section of healing Achilles tendons under polarized light microscope, (a) normal Achilles tendon, (b), (c) and (d) the healing Achilles tendon at days 10, 20 and 30 post-operation

Table 1: Percentile of the total cellularity, vascularity, collagen density and collagen fibril organization in fifteen rabbits in various sampling time

		Day 10			Day 20			Day 30				
Percentiles		Test	Control	p-value (within group)	Test	Control	p-value (within group)	Test	Control	p-value (within group)	p-value (between group)	
Cellularity	25	1	0.0	NS	2	1	NS	2.0	1	NS	Control	S
	50	1	0.0		2	1		2.0	1		Test	S
	75	1	0.5		2	1		3.0	1			
Vascularity	25	0	0.0	NS	2	0	NS	2.0	0	NS	Control	NS
	50	1	0.0		2	0		2.0	0		Test	S
	75	1	0.0		2	0		2.0	0			
Collagen	25	0	0.0	NS	1	0	NS	0.0	0	NS	Control	NS
density	50	0	0.0		1	1		0.0	0		Test	S
	75	1	0.0		1	1		0.5	0			
Collagen	25	1	2.0	NS	1	2	NS	0.0	2	NS	Control	NS
fibril	50	2	3.0		2	3		0.0	3		Test	S
organization	75	2	3.0		2	3		1.0	3			

Significant difference between groups ($p<0.05$), NS: No significant difference between groups ($p<0.05$)

groups in vascularity, collagen density and collagen fibril organization ($p>0.05$; Table 1), but significant differences was seen between treatment groups in 10, 20 and 30 days after collagenas injection ($p<0.05$; Table 1).

. The aim of the tendon treatment is to normalize walking and obtain finger movement. It has been reported that using viscose material (Juncosa *et al*., 2003; Atasoyn *et al*., 2001), artificial sheet (Leung *et al*., 2002) and nonsteroid antiinflammatories give successful results in tendon treatment (Wang *et al*., 2003, 2006).

Inflammatory cells, including neutrophils and macrophages are accumulated in the early stages of the healing process, suggesting that these cell types are likely to regulate the early events of the process.

These cells are probably derived from bone marrow, although it is also possible that macrophages are derived from the synovial membrane or synovial fluid (Gameti *et al*., 2005).

Of note, there was no proliferation of intrinsic tendon cells, indicating that the tendon does not directly contribute to the early healing process (Kawamura *et al*., 2005).

The collagenase-induced model is a well-established model for the study of tendinitis (Yamamoto *et al*., 2002).

The acute swelling, matrix destruction and inflammation and matrix destruction in tendons are similar to those seen in naturally occurring tendon injuries (Dahlgren *et al*., 2002).

In the present study, it demonstrated that bone marrow-derived cells treatment promotes repair of collagenease-induced Achilles tendonitis.

The use of collagenase-induced Achilles tendonitis to an excellent model for clarifying the cellular and histological mechanism that biological strategies such as Bone Marrow-derived cells treatment uses to stimulate tendon repair *in vivo* (Ouyang *et al*., 2004).

There are many speculations to understand the mechanism of tendon injury repair. Increasing fibroblast proliferation and biosynthesis of extracellular matrix including collagens are crucial stage for the return of normal tendon strength (Chen *et al*., 2004).

Inflammation is the initial response to tissue injury. The cellular events associated with the inflammatory response have been well studied in numerous physiologic processes, including skin wound healing and tendon injury (Chhabra *et al*., 2003).

The migration of inflammatory cells to and within the damaged tissue is facilitated by chemotactic cytokines that are rapidly secreted locally following tissue damage (Harris *et al*., 2004).

Neutrophils are typically the first inflammatory cells to accumulate in a wound site, followed by sequential accumulation of monocytes, macrophages and lymphocytes (Kawamura *et al*., 2005).

Tendon healing is a slow process, compared with the healing of most other connective tissue (e.g., bone, skin) and can be extrinsic (healing starts from the surrounding soft tissue) or intrinsic (healing starts from the tendon itself (Hefti and Stoll, 1995; Stashak, 1991; Watkins *et al*., 1985). The final result of tendon healing is the formation of collagenous scar tissue, rather than the restoration of normal tendon tissue (Watkins *et al*., 1985; Koike *et al*., 2005).

Many and various treatments have been attempted to improve tendon healing and to minimize the formation of scar tissue including none or minimally invasive techniques such as exercise protocols (Marxen *et al*., 2003) and the use of cell therapy such as bone marrow-derived cells (Kawamura *et al*., 2005).

Macrophages appear to play a central role in tendon healing. Macrophages are responsible for cellular debridement and are known to produce soluble cytokines that can contribute to healing by induction of angiogenesis, fibroblast mitogenesis and extracellular matrix synthesis and degradation (Moyer *et al*., 2003; Ackermann *et al*., 2003; Anaguchi *et al*., 2005).

The histological findings support the concept that tendon fibroblasts are responsible for tendon repair. The tendon fibroblasts synthesizes and organizes the newly deposited collagen within the area of injury. They are responsible for splicing the newly deposited collagen fibers with the residual collagen fibers and organizing them into a functional tendon, a process that appears to have the qualities of regenerative repair (Garner *et al*., 1988; Weiler *et al*., 2002; Moyer *et al*., 2003).

Therefore, it seems that application of bone marrow-derived cells in tendon injuries induces the release of angiogenic growth factors after 10 days (Fig. 1), while cell proliferations and formation of neovessels are affected in approximately 30 days (Fig. 3). The neovascularization may lead to the improvement of blood supply and play a role in tissue regeneration at the tendon (Chbinou and Frenette, 2003).

In this study, no significant differences was seen between control groups in vascularity, collagen density and collagen fibril organization ($p>0.05$; Table 1), but significant differences was seen between treatment groups in 10, 20 and 30 days after collagenas injection ($p<0.05$; Table 1).

In conclusion, it seems that injection of the bone marrow myeloid tissue may have effects on tendon healing in rabbit, as assessed by histopathological examination, compared to control group.

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