Enhancing Achilles tendon healing by using autologous bone marrow in rabbits

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(Received 11 September 2013, Accepted 18 November 2013)

Abstract

The model used in this study was (30) apparently healthy local breed male rabbits, weighing (1.5-2) kg divided into control and treated groups. All animals were anaesthetized generally by intramuscular injection mixture of Ketamine and Xylazine, then operation site was prepared surgically, in control group (left leg) about 3cm skin incision was made caudal to tibia and Achilles tendon splitting was made by used scalpel blade, treated group was subjected to the same procedure (right leg) then (1-2 ml) bone marrow aspirated from iliac crest of the same animal then applied to the injured tendon, skin closed routinely. Biopsies of injured tendon were harvested at 3rd, 7th, 15, 30, and 60 days post operation to show histopathological changes in tendon tissue in each group. Results indicated initiation of healing process in treated group prior to control group, in control group at 3rd day showed persist inflammatory cells associated with congestive blood vessels also at 7th day showed fibroblast proliferation with less new capillary blood vessels whereas at 15 day showed start irregular fibrous connective tissue formation, and at 30 day showed mature fibrous connective tissue formation with dense collagen fiber, while in treated group showed proliferation of fibroblast that producing collagen fibers which give rise for immature granulation tissue formation at 3rd day post operation, also large amount of granulation tissue which formed with deposition of collagen fiber at 7th day whereas at 15 day showed regular fibrous connective tissue formation with contracted collagen fiber that continue to increase collagen formation at 30 day, also at 60 day showed deposition of mature fibrous connective tissue, While in control group at 60 day showed short collagen fibers attached to tendon fibers and still presence same area contain collagen fiber irregularly. According to the results of present study, aspiration of autologous bone marrow from iliac crest and it application directly on injured Achilles tendon may have a better effect in promoting healing in rabbits.

Keywords: Tendon, healing, autologous, bone marrow, rabbit.

تشريع التنام وتر أكيلس باستخدام نقي العظام الذاتي في الأرانب

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الخلاصة

أجريت الدراسة على (30) أرنب من ذكور الأرانب المحلية البالغة السليمة ضعورًا من الأمراض والتي تتراوح أوزانها (1.5-2) كجم قسمت بشكل متساوي إلى مجموعتين مكونتين، تم تخدير الحيوانات تخدير عام باستخدام مزيج الكيتامين والزليانزين حقنًا بالعسلة ، بعدها حضر مكان العملية انحناءاً ظاهراً. بالنسبة لحيوانات مجموعة السيطرة (القائمة البيضى) تم جل من الجلد بطول 3سم خلفياً لعرض القصبة وبعد إظهار وتر أكيلس تم عمل تشريح باستخدام المشرط الجراحى أما بالنسبة لمجموعة المعلجة (الرجل اليمنى) فقد أجريت لها نفس العملية السابقة عدا إضافة (1.5مل) من نخاع العظام الذاتي المنسحب من عظم الحروقة لنفس الحيوان وبعدها تم غلق الجرح بشكل روتيني. جمعت العينات لكلا المجموعتين عند اليوم 1,5,10,15,30 بعد العملية وأخذت لتفتيض ولتلقيح النسيج المرضى للملاحظة للتحريات النسجية المرضية للعظام وعظام الأرانب. أظهرت نتائج التحليل النسيجي المرضى بدل عملية الالتهاب بديل مجموعة المعلجة قبل مجموعة السيطرة عند اليوم الثالث في مجموعة السيطرة شهد استمرار مرحلة الالتهاب، والتي تميزت بوجود الخلايا الالتهابية واحتقان الأدمة الدمية تكون شعارات دموية جديدة بينما شهد تكاثر خلايا الأورام الليفية وقفة الشعارات الدموية عند اليوم السابع بعد العملية. بينما نظرًا لوجود نسيج ليفي غير منتظم عند اليوم 15 ، أما عند اليوم 30 بعد العملية شهد بدأ نضج النسيج اللفيي وخلايا الأورام. أما مجموعة المعلجة شهد عند اليوم الثالث تكون أوعية شعاعية جديدة وتكاثر خلايا الأورام.
Introduction

Tendon tissue is formed by an intricate network of macromolecules that constitute the extracellular matrix and interact with the cellular components composed of tenocytes (1). It is a unit of musculoskeletal tissue that transmits force from muscle to bones, normal tendon consists of soft and fibrous connective tissue that is composed of densely packed collagen fiber bundles aligned parallel to the longitudinal tendon axis and surrounded by a tendon sheath also consisting of extracellular matrix components, tenoblasts are immature tendon cells they are spindle-shaped and have numerous cytoplasmic organelles, reflecting their high metabolic activity, the remaining cellular elements of tendons consists of chondrocytes at the bone attachment and insertion sites (2). Epitenon, a fine, loose connective-tissue sheath containing the vascular, lymphatic, and nerve supply to the tendon, covers the whole tendon, Endotenon is a thin reticular network of connective tissue investing each tendon fiber(3). Tendons receive their blood supply from three main sources the intrinsic systems at the myotendinous junction and periosteal insertion, and intrinsic system through the paratenon or the synovial sheath while Innervations is provided by nerves from the surrounding muscles and from cutaneous nerves, muscular, and peritendinous nerve trunk (2,4). Tendon injuries can be acute or chronic and are caused by intrinsic and extrinsic factors, either alone or in combination, when tendon injured it never restores the complete biological and mechanical property after healing, surgical repair frequently do not fully restore function due to fibrous adhesion or failure arising from the mechanical demand placed on imperfect integrative healing at tendon–tendon or tendon–bone interfaces (2). Many therapeutic approaches have been used to improve tendon healing, including physical therapy, the use of steroidal and non steroidal anti-inflammatory drugs, exercises, injection therapies, shock wave treatments and surgical tendon debridement (5,6). Tendon healing is fraught with complications such as ruptures and adhesion formation due to the formation of scar tissue at the injury site (7). Recently several growth factors have been identified as playing roles in accelerate tendon healing including vascular endothelial growth factor, insulin-like growth factor, platelet-derived growth factor, basic fibroblast growth factor, and transforming growth factor (8). Bone marrow stroma cells are undifferentiated cells from the bone marrow, also Mesenchymal stem cells (MSCs) can be isolated from bone marrow, adipose tissue, cord blood and various fetal tissues, they have the capacity to differentiate into several tissues, including bone, cartilage, tendon, muscle and adipose, and produce growth factors and cytokines that promote hematopoietic cell expansion and differentiation (9). Recently (10) reported considerable success in the use of bone marrow aspirated from the sternum and injected directly into damaged tendon or ligament, experimentally the beneficial use of bone marrow in tendon repair has been demonstrated in rabbits (11). The aim of the present study was to illustrate the effect of autologus bone marrow tissue aspiration from iliac crest on healing stages of Achilles tendon in male of rabbit histopathologically

Materials and methods

The model used on this study was (30) apparently healthy, local breed male rabbits, weighing (1.5-2)kg, they were kept in
(Fig. 1) Show site of operation 3 cm skin incision was made caudally to the tibia over the Achilles tendon, subcutaneous tissue dissected bluntly.

(Fig. 2) Show bone marrow aspiration from the iliac crest by syringe with 18 gage needle.

separate cages and allowed to move freely, animals were divided into two equal groups, control and treated group. Animals were initially weighted and later anaesthetized by intramuscular injection mixture of Xylazine-ketamine hydrochloride (Xylazine: 5 mg/kg B.W and Ketamine: 35 mg/kg B.W). Both hind limbs from stifle joint to tarsal joint prepared surgically in addition to skin around iliac crest by clipping and shaving then coat with cotton saturated by alcohol 70%. In control group animals lie in sternum recumbence, 3 cm skin incision was made in the left hind limb caudally to the tibia over the Achilles tendon, subcutaneous tissue dissected bluntly (fig. 1), then tendon splitting was made in the middle third of Achilles tendon between calcaneus insertion and muscular–tendinous junction using a number (11) scalpel blade, bleeding was carefully arrested after that skin closed by simple interrupted suture 2.0 silk suture. In treated group animals subject to a same operation in the right Achilles tendon then autologous bone marrow (1.5-2 ml) aspirated from iliac crest (9) by syringe 18 gage needle (fig. 2), then applied directly on the site of tendon splitting, skin sutured by simple interrupted suture 2.0 silk suture. Antibiotic therapy was given for 5 days by using penicillin (10000IU/kg BW) and streptomycin (10mg/kg BW) intramuscularly. Animal movement was restricted, Stitches removed after 7 days in all animals. Tendon biopsies were collected under general anesthesia at 3rd, 7th, 15, 30 and 60 day post operation for two groups, then were fixed in 10% neutral buffered formalin for 72 hrs. After which the section was prepared routinely. The slides were stained with Hematoxyline - Eosin stain and Van-Geison stain to demonstrate the connective tissue examination to show tendon healing microscopically in each group (12).

Results

According to clinical follow up of the animals in this experiment, swelling of the limbs were not observed after procedure but lameness persist for 2 days, two animals suffering from tendinitis, that include typical signs of local inflammation, swelling, heat accompanied by well-defined painful area and sever adhesion between tendon and surrounding tissue. Histopathological results in control group at 3 day post operation showed presence of congested blood capillary with slight hemorrhage as well as inflammatory cells infiltration (mainly neutrophils), in the same section showed presence of fibrin meshwork at peritendineous area (fig. 3), at 7 days showed proliferation of fibroblast associated with less new blood vessels formation, also some section refer presence of immature fibroblast in different structure with few fibrin deposition in peritendineous area (fig. 4). At 15 day showed presence of irregular fibrous connective tissue formation infiltrated with mononuclear cells as well as other sections
Fig.(3) Tendon section of control group 3 days (PO) show blood vessels congested (→) with few inflammatory cells infiltration with presence of fibrin mesh work (→) at peritendinous area (H&E X40).

Fig.(4) Tendon section of control group 7 days (PO) show proliferation of fibroblast (→) associated with less new blood vessels formation (→), also some section refer present of immature fibroblast in different structure with few fibrin deposition in peritendineous area (←) (H&E X40).

Fig.(5) Tendon section of control group 15 days (PO) show presence of irregular fibrous connective tissue formation (→) infiltrated with mononuclear cells (H&EX40).

Fig.(6) Tendon section of treated group 3 days (PO) show proliferating of fibroblast (→) that producing collagen fibers give rise for immature granulation tissue formation mixed with fibrin net mesh Deposition (←) in peritendineos area, also new capillary blood vessels formation (→) infiltrated in their lumen (H&EX40).

Fig.(7) Tendon section of treated group 7 days (PO) show proliferation of young fibroblast cell with deposition of collagen fiber (→), also showed large amount of granulation tissue in the peritendineous area accompanied with mononuclear cells infiltration (H&EX40).

Fig.(8) Tendon section of treated group 7 days (PO) show formation of fibrous connective tissue regular in shape, in other section show presence of contracted collagen fiber granulation tissue (→) infiltrated with mononuclear cells (H&EX40).
Fig.(9) Tendon section of control group 30 days (PO) show proliferation of mature fibrous connective tissue (→) consisting of few spindle shape fibroblast cell with dense collagen fiber (H&E X40).

Fig.(10) Tendon section of treated group 30 days (PO) show decrease of fibroblast with increase of collagen fibers (→→) adjacent to peritendineos area with few inflammatory cells (H&E X40).

Fig.(11) Tendon section of treated group 30 days (PO) show regular collagen fiber stained with red color with few blood vessels (V.G X40).

Fig.(12) Tendon section of control group 60 days (PO) show fibrous connective tissue formation characterized by short of collagen fibers (→) attached to tendon fibers (H&E X40).

Fig.(13) Tendon section of control group 60 days (PO) show still presence same area contain collagen fiber irregular in direction stained with red color with numerous congested blood vessels yellow in color (V.G X40).

Fig.(14) Tendon section of treated group 30 days (PO) revealed deposition of mature fibrous connective tissue with few blood capillary (V.G X40).

Refer presence of hemorrhagic area between collagen fibers with newly formed blood vessels (fig. 5), whereas in treated group at 3 days histopathological sections revealed proliferation of fibroblasts that produce collagen fibers give rise for immature
granulation tissue formation mixed with fibrin meshwork deposition in peritendineous area, also showed new capillary blood vessels formation and proliferation of immature fibroblast took different shape (stellar, spindle, oval, rounded with production of few fibrils (fig. 6), at 7 day showed proliferation of fibroblast with deposition of collagen fiber, also showed large amount of granulation tissue in the peritendineous area accompanied with mononuclear cells infiltration consisting mainly of macrophage and plasma cells (fig. 7), at 15 day showed fibrous connective tissue formation regular in shape, in other section show presence of contracted collagen fiber infiltrated with mononuclear cells (fig. 8). In control group at 30 days revealed proliferation of mature fibrous connective tissue consisting of few spindle shape fibroblast cell with dense collagen fiber (fig. 9). Histopathological section in treated group at 30 days refer decrease of fibroblast with increase of collagen fibers formation adjacent to peritendineos area with few inflammatory cells (fig. 10), as well as showed regular collagen fiber stained with red color with few blood vessels stained with yellow color in Van-Geisons stain (fig.11), in control group at 60 day revealed fibrous connective tissue formation characterized by short collagen fibers attached to tendon fibers (fig. 12), as well as in the other section stained with Van-Geisons stain showed still presence same area contain collagen fiber irregular in direction stained with red color (fig. 13). While in treated group at 60 day post operation showed deposition of mature fibrous connective tissue with few blood capillary (fig. 14).

**Discussion**

Tendon healing is a slow process as compared with the healing of most other connective tissue such as bone and skin ,and various treatments have been attempted to improve tendon healing, , collagenous scar tissue formation is the final result of tendon healing rather than restoration of normal tendon tissue (5,13). Male rabbits were used in this study according to many studies were performed on male rat tendon refer that menstrual cycle may has possibly implication or interfere with the process of tissue repair (14). The results of the clinical follow up In each group indicate persistence of lameness for two days without any increase in thickness of surgical site, this condition was reduced gradually till animal gait return to normal, this may be due to pain that originated from a combinations of mechanical factor (break down of collagen and chemical irritant in addition to neurotransmitter that may generate pain (15). Occasionally in this present study animals movement was restricted post operatively due to many investigators (16) reports that immobilization of animal had increased collagen organization ,and increase ratio of type I collagen (indicative of decreased scar). In this present study two animals suffered from tendinitis, the clinical signs may be due to tendon injury accompanied by local infection that cause inflammation (2) . Bone marrow was used in this experiment because several studies (17) indicated that the bone marrow is one of the best source of mesenchymal stem cells which enhance local repair or regeneration of tendon, bone and cartilage when it used locally by either injection or implantation. About (1.5-2ml) of bone marrow was aspiried from the iliac crest and applied on injured tendon due to (18) referred that Injection of large volumes of bone marrow can be deleterious to tendon healing. The results of the clinical follow up in treated group not record any rejected case this agree with (19) who referred to the simplicity of this procedure, inexpensive ,on the other hand there is no record of adverse reaction or rejection due to implantation or injection of autologous bone marrow-derived mesenchymal stem cell in the clinical case of equine tendonitis either locally or systemically. Histopathological results in this study showed at 3rd day in control group persist first inflammation phase, (20) reports that Inflammatiory cells, including neutrophils and macrophage are accumulated in the early stages of the healing process ,suggesting that these cell type are likely to regulate the early event of the healing.
process, while at this period in treated group record early advance in healing process than in control group that showed fibroblast proliferation and granulation tissue formation with new capillary formation this agreement with (21) who referred that bone marrow with its contain have ability to secrete many of bioactive molecule like growth factors also have ability to inhibit scarring, inhibit apoptosis, stimulate angiogenesis, and stimulate the mitosis of tissue intrinsic stem or progenitor cells. In control group at 7th days post operation showed fibroblast proliferation with less of capillary formation continue to form irregular (f.c.t) at 15 day, this agree with (16) who referred that inflammatory phase is followed by a period of new cell proliferation and increased vascularity while concurrently, collagen synthesis begins when the fibers enter a phase of maturation. In treated group showed promotion in healing process when compared with control group, at 7th day post operation continuous proliferation of fibroblast in large area with collagen deposition this progress to show at 15 day post operation regular (f.c.t) formation with shrinkage of collagen fiber this result agree with (9) who refer that in case of tendinitis treated with bone marrow revealed more mature tissue formation with more regular patterns of cell distribution, that’s mean collagen fibril diameter and relative area covered by collagen fibrils were significantly higher at 10 and 20 days postoperatively. Also it has the same opinion with the study of (22,23) whom demonstrated that the stem cells in bone marrow are believed to encourage tendon tissue repair through a complex interaction of bone marrow and its growth factor, which direct cells signaling event and tissue repair through revascularization, collagen deposition, matrix remodeling and reduction in any inflammatory process. Furthermore (24) who report that the end of proliferation stage of tendon healing character by start decrease in cellularity, reduced matrix synthesis, decrease in type III collagen (short collagen) and an increase in type I collagen (long collagen) synthesis, type I collagen fibers are organized longitudinally along the tendon axis and are responsible for the mechanical strength of the regenerate tissue. Consequently dense irregular collagen fiber formation showed at 30 days in control group, whereas it more progress in treated group at the same period that showed decrease in fibroblast cell with presence of regular contracted collagen fiber. While in control group at 60 day showed short, irregular collagen fiber while in treated at the same period showed in same section deposition of mature (f.c.t). this results be in agreement with (25) report that type III collagen synthesis increases during the early phase of repair and remodeling and decreases as type I collagen production increases and becomes highly organized into fiber structures of the extracellular cellular matrix. Whereas (26,2) they report after injury, the healing process in tendons results fibrotic scar formation, the structural, organizational, and mechanical properties of this healed tissue are inferior to that of normal tendon, although these properties improve over time, they do not return to normal levels even after long periods. In conclusion the results that obtained from this present study indicate that autologous bone marrow that is applied directly on injured tendon is readily available treatment for tendon rupture and promote healing as compared with the control group which needs a long time for tendon healing.

References


