

The Biomechanical and Histological Effects of Diabetes on Tendon Healing: Experimental Study in Rats

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Abstract The aim of this study was to investigate the biomechanical and histological perspectives of healing of Achilles tendon in diabetic rats and compare the results with non-diabetic subjects. Fifty four adult Wistar Albino rats weighing 300–350 g were used throughout the study. Six animals were excluded from the study and replaced. Rats were randomly assigned to either the experimental or the control group comprised of 24 rats in each. Diabetes was induced in experimental group with streptozotocin. 3 days after the induction of diabetes, both Achilles tendons were transected 5 mm proximal to their insertions to the calcaneal bone and repaired by using 6/0 polypropylene sutures with modified Kessler method. At weeks 2, 4 and 6, eight rats from each group were euthanized. Left Achilles tendons including the repair site were prepared for histological evaluation and right legs were prepared for mechanical testing. When compared to control group, diabetic animals displayed a lower peak force for failure in each of the second, fourth and sixth week. The differences between the groups in each week were found to be significant in statistical assessments ($p < 0.05$). Histologic assessment revealed that

the diabetic animals had significantly less amount of fibroblast proliferation and lymphocyte infiltration compared to the control group. There is significant delay in tendon strength at the end of week 2, 4 and 6 postoperatively in the diabetic rats. Therefore diabetic individuals require specific postoperative follow up and rehabilitation procedures.

Keywords Tendon · Tendon healing · Diabetes · Wound healing · Rat · Streptozotocin · Biomechanical

Diabetes mellitus (DM) is defined as “a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism resulting from defects in insulin secretion, insulin action or both” [1]. It affects more than 170 million people worldwide and by 2030 these numbers are projected to double [2]. As incidences increase globally, the related complications become more prevalent. Wound healing abnormality is one of the leading causes of hospital admission for people with diabetes [3].

Normal wound healing is a complex and highly integrated cascade of events, requiring the interactions of many cell types, including inflammatory cells, fibroblasts, keratinocytes, and endothelial cells, as well as the involvement of growth factors and enzymes [4]. All stages of this complex process (inflammation, proliferation, and remodeling), which also are associated with tendon healing are impaired in diabetics [5]. It has been shown that abnormalities in the production of cytokines by inflammatory cells, impaired neovascularization, reduced growth factor levels and collagen synthesis interfere with the healing of damaged tissues in diabetic patients [6].

Moreover, DM has been recognized to cause a wide range of musculoskeletal disorders, including tenosynovitis, tendon contracture, Achilles tendon rupture, and rotator cuff tear [7–10]. In addition to impairments in the wound healing cascade, changes in the three-dimensional structure of

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collagen by disrupting the cross-linking due to non-enzymatic glycosylation, is directly related with the tendinous disorders [11, 12].

Although tendinous injuries in diabetic patients are repaired in a similar manner as in non-diabetic patients, it is clear that there should be an individualized protocol of rehabilitation and longer duration of immobilization, due to impairment and delay in healing. The aim of this study was to investigate the biomechanical and histological perspectives of healing of Achilles tendons in diabetic rats and compare the results with non-diabetic subjects.

Materials and Methods

This study was approved by the Istanbul Bagcilar Research and Education Hospital Local Committee on Animal Research Ethics. Fifty-four adult Wistar Albino rats, weighing 300–350 g, were used. Animals were randomly assigned to either the experimental or the control group, comprised of 24 rats each. Each main group was divided into three subgroups containing 8 rats. Three rats that were lost during the induction of diabetes and three rats that failed to develop diabetes with an aimed fasting glucose level were replaced.

Induction of Diabetes

Diabetes was induced by administering a single intraperitoneal injection of 60 mg/kg of streptozotocin (STZ) (Sigma, St Louis, MO, USA) dissolved in citrate buffer (pH 4.5). The control group received only the buffer. Glucose measurements were carried out using Gluco-check strips (Accu-Check Active, Roche Diagnostics, Germany). Animals with blood glucose levels >350 mg/dl on day 3 after injection were considered diabetic.

Surgical Procedure

Three weeks after the induction of diabetes, all animals in the control and experimental groups were anesthetized by an intraperitoneal injection of 60 mg/kg of ketamine (Ketalar, Eczacıbasi, Turkey) and 5 mg/kg of xylazine (Rompun, Bayer, Germany). Both the left and right hind legs were shaved and prepared for surgery. A 2 cm-long posterior midline incision was carried out to expose the Achilles tendon and the Achilles tendon and the plantaris tendon were stripped from the surrounding fascia. The tendon was transected 5 mm proximal to its insertion to the calcaneal bone with a no. 11 scalpel blade perpendicular to the collagen fibers (Fig. 1). The plantaris tendon also was cut to prevent an internal splinting effect. The Achilles tendon was then repaired by using 6/0 polypropylene sutures (Prolene, Ethicon, Cornelia, GA, USA) with a modified Kessler method. The wound was closed with



Fig. 1 The tendon was transected 5 mm proximal to its insertion to the calcaneal bone

5/0 silk sutures (Ipek, Dogsan, Turkey) in a continuous fashion. No wound dressing or casting was applied. The animals were kept in separate cages after the surgery and were fed standard laboratory food and tap water.

At weeks 2, 4, and 6 eight rats from the study and control groups were euthanized. Both hind legs were disarticulated from the hip joints. Strips, 1 cm in length of the left Achilles tendons, including the repair site, were harvested and embedded in 10 % formalin solution for 48 h for fixation. The right legs were prepared for mechanical testing. The whole leg was placed into the upper clamp and the whole foot was placed into the lower clamp of the testing device to prevent slipping of the tendon from the clamp site (Fig. 2).

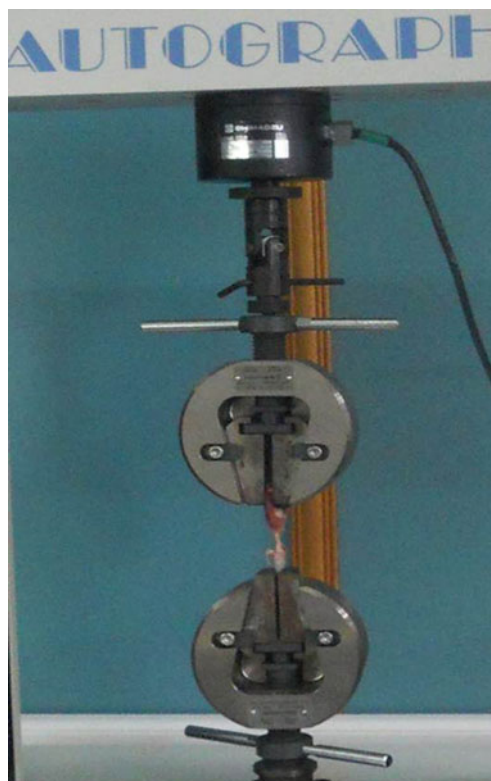


Fig. 2 Right Achilles tendon-calcaneus complexes were stretched using a mechanical uniaxial testing device

Table 1 Peak forces for tendon rupture of control and experimental group ($p<0.05$)

	2nd week (Newton)	4th week (Newton)	6th week (Newton)
Diabetic (Mean \pm SD)	11.53 \pm 1.29	19.72 \pm 1.45	28.30 \pm 1.24
Non-Diabetic (Mean \pm SD)	18.20 \pm 1.22	30.94 \pm 1.60	40.94 \pm 1.43

The formalin fixed tendons were embedded in paraffin, and 5 μ -thick longitudinal sections were obtained. The sections were stained with Masson's trichrome and microscopic evaluation was carried out under 100X and 200X magnification. Collagen deposition, lymphocyte infiltration, and fibroblast proliferation were evaluated subjectively. Alterations to tenocyte morphology and vascular proliferation were noted as well.

The right Achilles tendon-calcaneus complexes were stretched until failure, at a constant speed of 10 mm/min, using a mechanical uniaxial testing device (Autograph, Shimadzu, Japan) (Fig. 2). The amount of peak force (N) that led to tendon rupture was recorded. Peak forces were statistically compared with *t* test using SPSS 10.0 for Windows; $p>0.05$ was defined as significant.

Results

Three rats were lost during the 3 day period between the induction of diabetes and blood glucose level measurement and the three rats with blood glucose levels below 350 mg/dl on day three were excluded from the study. Induction of diabetes was effective in all other animals in the experimental group.

All repaired tendons were intact at the time the animals were euthanized. However, atrophic skin changes were

obvious at the suture site in the diabetic animals, and the wound ends were easily detached with a slight nudge after suture removal. At first glance, tissues of the diabetic animals displayed a yellowish discoloration and sticky structure at the tendon repair site, compared to the reddish and fresh-looking tissue of the control group.

Biomechanical Results

All tendons ruptured at the repair site. The peak forces that led to tendon rupture and standard deviations at weeks 2, 4 and 6 are shown in Table 1. The mean peak loading forces for control group were 18.20 \pm 1.22 N, 30.94 \pm 1.60 N and 40.94 \pm 1.43 N at weeks 2, 4 and 6 respectively. When compared to the control group, the diabetic animals demonstrated a lower peak force for failure in each of the weeks 2 (11.53 \pm 1.29 N), 4 (19.72 \pm 1.45 N) and 6 (28.30 \pm 1.24 N) (Table 1). The differences between the groups in each week were found to be significant in the statistical assessments ($p<0.05$). Figure 3 shows that, the strength of the diabetic tendons in the fourth week was almost equal to the strength of the non-diabetic tendon in the second week. Similar correlation is demonstrated between the strength of the sixth week diabetic and fourth week non-diabetic tendons; there is a delay of almost 2 weeks from the aspect of tendon strength.

Histological Results

The histologic assessment revealed that the diabetic animals had a significantly smaller amount of fibroblast proliferation and lymphocyte infiltration compared to the control group at 2, 4, and 6 weeks (Fig. 4). Interestingly, there was a similar amount of collagen deposition in the diabetic and the non-diabetic animals. Furthermore, osteochondroid metaplasia

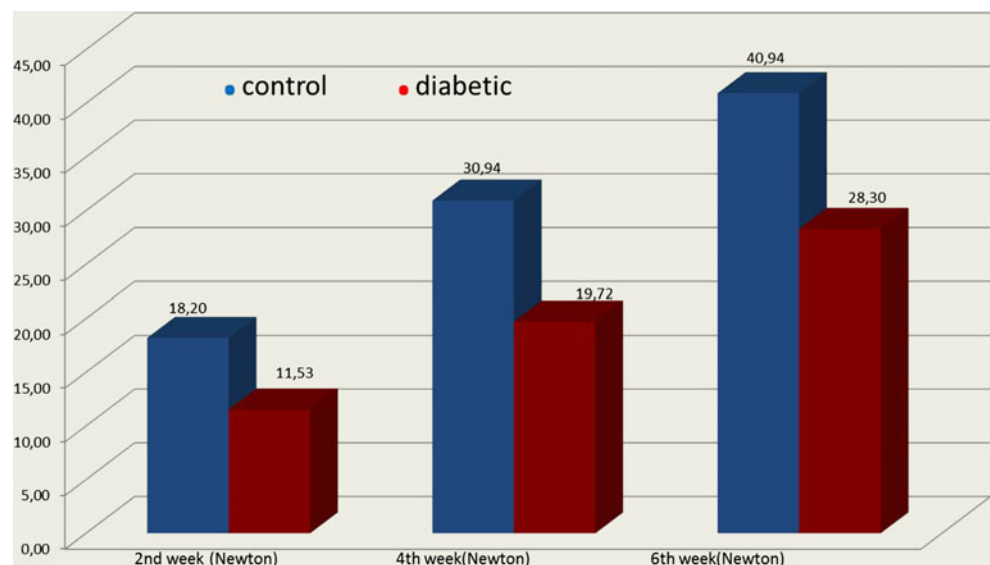
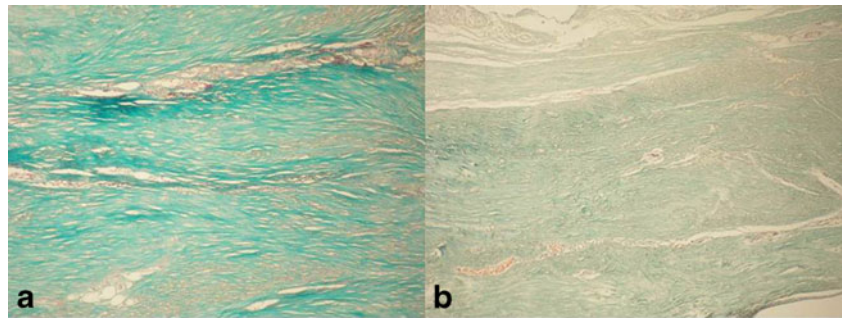
Fig. 3 There is almost two weeks of delay from the aspect of tendon strength

Fig. 4 Masson's trichrome staining of diabetic (a) and non-diabetic (b) tendons at 4th week (X100 magnification)



of some tenocytes was noticed in the diabetic group especially at the sixth week (Fig. 5). Proliferation of the vessels was found to be similar in both groups.

Discussion

Tendons are load-bearing tissues composed of collagen fibers organized in a three-dimensional hierarchy. Increased collagen synthesis and deposition are critical for wound healing and tensile strength [13]. Tendon healing occurs in three overlapping stages: inflammation (in the first 48 h); proliferation which lasts for approximately 6 weeks), and remodeling. The process starts with an inflammation response to tendon injury, predominated by neutrophils and macrophages, including removal of the devitalized tissues and release of chemo-attractive substances to invoke the cells essential for repair. The process develops with proliferation of the fibroblasts, collagen production and extracellular matrix formation. Fibroblasts produce and secrete procollagen, which is hydroxylated and glycosylated to form a triple helix configuration. The triple helix bundles cross-link to form the three-dimensional structure and the synthesized collagen fibers are organized and gain a mature structure in the remodeling phase [14].

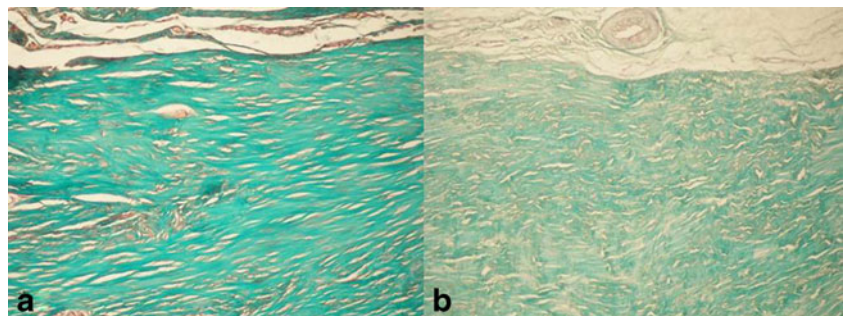
Non-enzymatic glycation contributes to the accelerated aging of proteins in DM [15]. This process involves the non-enzymatic binding of individual sugar residues to proteins via free amino groups [4]. Collagen is one of the major targets for non-enzymatic glycation. Tendons are directly affected by non-enzymatic glycosylation and formation of advanced glycation end products (AGE), due to their rich

collagen content [16]. Non-enzymatic glycation prevents the collagen bundles from forming precise supramolecular aggregates, alters its charge profile and interferes with fibril formation [11, 17]. Longo et al. showed that a high plasma glucose level, even within the normoglycemic range, might be a risk factor for rotator cuff tear [18]. They stated that sustained hyperglycemia is associated with poor healing at the tendon-to-bone insertion site as well as decreased maximum strength. In addition, they showed a significant accumulation of AGEs at the operation site in diabetic animals while they are absent in the control group.

In their recent study, Maffuli et al. noted that Achilles tendon ruptures are prevalent and complication rates are higher in diabetic individuals [19]. The authors do not advocate traditional open techniques or wide dissections, to avoid complications in these patients [19]. Recent studies intending to improve the healing of transected or detached Achilles tendon and achieve tendon repair without surgical intervention may lower the complications due to surgical trauma [20–22]. Reddy demonstrated a significant decrease in the proportion of soluble collagen in glycosylated tendons compared to non-glycosylated tendons. Although this was an *in vitro* study, the authors showed that the degree of collagen cross-linking by non-enzymatic glycation is significantly correlated to the decreased biomechanical attributes of the tendon [23]. In their Achilles tendinitis model, Chbinou and Frenette showed significant impairment of the inflammatory, angiogenic, and proliferative phases, which negatively affect healing [24].

Our study provides information regarding the biomechanical and histological effects of diabetes on tendon healing. In the rat Achilles tendon repair model, the peak forces for failure of the diabetic tendons were significantly lower

Fig. 5 Masson's trichrome staining of diabetic (a) and non-diabetic (b) tendons at 6th week (X200 magnification)



compared to the non-diabetic tendons. The impairment in healing resulted in almost two weeks of delay from the aspect of tendon strength after the second week following repair. Furthermore, the tendons in the diabetic animals with sustained hyperglycemia demonstrated inferior histological healing characteristics. Although the collagen contents were similar in the control and experimental groups, fibroblast proliferation and lymphocyte infiltration were found to be lower in the diabetic animals. These results show that diabetic individuals may need a longer period of immobilization and a more conservative and attentive physical therapy process after tendon repair. Patients may need a longer time for full weight loading and to return to daily life. Future studies are needed to determine the effects of prolonged immobilization after tendon repair in diabetic animals from the aspect of tendon adhesion and onset of physical therapy.

There are several limitations to the present study. First, the STZ-induced DM is the most widely used experimental model. STZ selectively destroys pancreatic β cells and creates type I diabetes. However, type II diabetes, which is more prevalent, involves peripheral insulin resistance. Moreover, this model examines a “worst-case scenario” which is uncontrolled hyperglycemia with acute onset. Longer observations may provide additional information regarding the effects of chronic hyperglycemia on the tendon and its healing. Furthermore, the results of the histological assessments revealed that subjective assessment of collagen content might not be a predictor of impaired healing in such a study. Objective evaluation of collagen organization and three-dimensional architecture of the tendon should be planned.

Conclusion

This study provides objective data especially on the mechanical aspect of tendon healing in diabetic rats showing that there is significant delay in tendon strength at the end of the second, fourth and sixth postoperatively. These results clearly demonstrate that diabetic individuals require specific postoperative follow up and rehabilitation procedures. Further studies should be performed to investigate the appropriate duration of immobilization and method of mobilization in diabetic individuals after tendon repair.

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