EXPERIMENTAL STUDY

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The effects of resveratrol on tendon healing of diabetic rats

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Objective: The aim of this study was to determine the effects of resveratrol on the tendon healing process of streptozotocin-induced diabetic rats.

Methods: The study included 16 male Sprague-Dawley rats. Streptozotocin was administered to induce diabetes and bilateral tenorrhaphy of the Achilles tendons was performed. Intraperitoneal resveratrol was injected in the experimental group (n=8) and saline in the control group (n=8) during the postoperative period. Rats were sacrificed at the 14th day. Right side tendons were evaluated biomechanically and left side tendons histologically.

Results: Difference in mean tendon tensile strength was not statistically significant between groups ($p>0.05$). Histologic evaluations of the repair zones showed greater configuration of the newly synthesized collagen in the experimental group. The ratio of the newly synthesized collagen area to the healing region area was significantly higher in the experimental group then the control group ($p<0.01$).

Conclusion: Resveratrol appears to have a positive impact on the process of tendon healing in diabetic conditions in the first 14 days.

Key words: Diabetic; resveratrol; tendon healing; tensile strength.

Tendon injuries resulting from traumatic injuries of the hand and forearm form a large portion of emergency plastic surgery cases. Tendon repairs have begun to yield successful results due to the development in tendon repair techniques and the increasing importance of postoperative physical therapy protocols.

Patient's age, concomitant diseases and patient compliance are important factors affecting the healing of the tendon. As tendon healing follows the same principles as wound healing, conditions that delay or accelerate wound healing have similar effects in the tendon healing process.

Plastic surgery in diabetic patients should be approached with caution. Wound healing problems may occur due to impaired microcirculation. Tendon repair studies in diabetic rat models have shown that tendon healing is slower than in non-diabetic rats.[1]

Resveratrol is an antioxidant agent that has anti-dia-

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betic, anti-inflammatory, anticarcinogenic and neuroprotective effects.[2-5] Antioxidant substances are known for their positive effects on wound healing. However, only a few studies showing the effects of antioxidant substances on tendon healing have been peformed.[6,7]

The aim of this study was to determine the histological and biomechanical effects of resveratrol on tendon healing. In this respect, results of our study can be useful in clinical practice for diabetic patients.

Materials and methods

The study protocol was approved by the Marmara University Animal Studies Ethical Committee and was consistent with the Declaration of Helsinki principles. The study included 16 male Sprague-Dawley strain albino rats weighing between 250 and 300 grams. Rats were housed individually at ambient room temperature and provided with adequate water and laboratory chow throughout the study.

Blood samples were taken from the tails of all subjects and basal glucose levels were measured (within 1 mg/dL) and recorded. Animals with a blood glucose level between 80 and 300 mg/dL were included in the study.

Rats were intraperitoneally administered 55 mg/kg streptozotocin (STZ; Sigma-Aldrich Corp., St Louis, MO, USA) stored at -20°C and dissolved in a pH 4 citrate buffer.[8]

At the 6th hour after application, 2 ml of 10% glucose solution was administered subcutaneously and fluid resuscitation was performed with a 2-4 cc balanced isotonic solution once per day. Animals were kept in the same feeding and housing conditions and cages were cleaned on a daily basis. At the end of the 2nd week, blood glucose levels were measured. Rats with glucose levels under 300 mg/dL were excluded from the study. The 16 rats with a blood glucose level above 300 mg/dL were divided randomly into experimental and control groups with 8 rats each.

All surgical procedures were performed under sterile conditions by the same investigator. Subjects were anesthetized intraperitoneally with 5 mg/kg xylazine hydrochloride (Rompun; Bayer, Turkey), and 100 mg/kg ketamine hydrochloride (Ketalar; Pfizer, Turkey). A 2-cm incision was made on the posterior part of both legs to dissect the gastrocnemius muscle and Achilles tendon. The tendon was cut as full-thickness with the aid of a

Fig. 1. **(a)** Dissection of the Achilles tendon. **(b)** Full-thickness cut of Achilles tendon at 0.5 cm proximal to the adhesion point with the calcaneus. **(c)** Repair of the cut tendon in accordance with the Kessler method. **(d)** Closure of the skin. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

scalpel at 0.5 cm proximal to the adhesion point with the calcaneus. The cut ends were immediately repaired with 6/0 round polypropylene sutures using the modified Kessler method. Skin incisions were then closed (Fig. 1). Splinting was not performed to protect the repaired tendons due to the technical difficulty in application on rats.

Beginning on the 1st postoperative day, the experimental group received an intraperitoneal daily dose of 10 mg/kg resveratrol (Sigma-Aldrich Corp., St Louis, MO, USA) dissolved in 50 mg/ml ethyl alcohol for 14 days. The same amount of saline was given intraperitoneally to the control group daily.

Rats were sacrificed on the 14th day. Histological examination was performed by taking biopsies of the anastomotic region of the left Achilles tendon. Sections of a thickness of 5 μm were obtained by microtome from the samples placed in paraffin blocks. The samples were stained with both Masson's trichrome and hematoxylin-eosin. Parameters were evaluated semi-quantitatively and quantitatively. Semi-quantitative evaluation was performed by the same histologist with a light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan). Histologic findings that were found in the sections were taken with a digital camera (Olympus DP-2 BSW; Olympus Corp., Tokyo, Japan).

In the semi-quantitative evaluation, parameters such as the presence of inflammatory cells, fibroblast density and the sequence of collagen fibers were measured.[1,9]

Quantitative evaluations were performed by the same pathologist under polarized light with the same microscope (Olympus BX51). The ratio of the newly synthesized collagen area to the healing region area was calculated on aggrandized images of the entire incision of the repaired area using microscope camera software (Olympus DP2-BSW) in μm^2 (Fig. 2). Rates for both groups were compared.

Fig. 2. Ratio of the newly synthesized collagen area to the healing region area was calculated on aggrandized images of the entire incision of the repaired area using microscope camera software. Collagen areas in the central region are highlighted in pink and white under polarized light. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

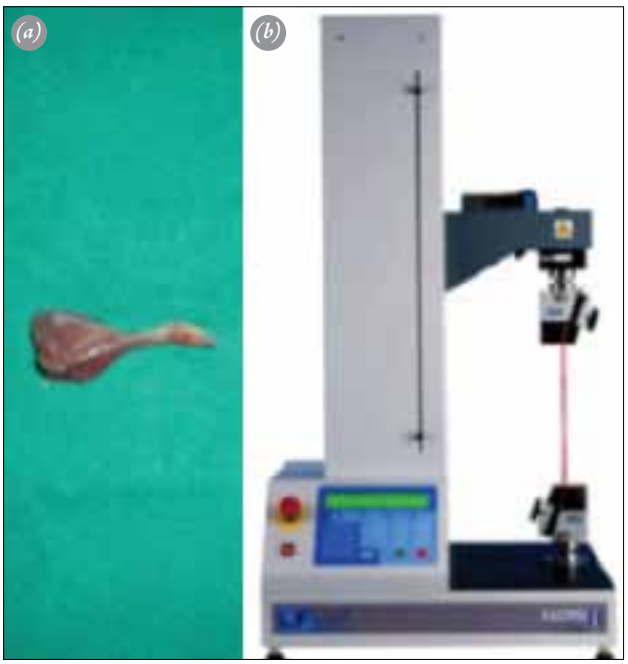

Fig. 3. **(a)** The Achilles tendon that was excised together with the calcaneus and gastrocnemius muscle. **(b)** Tensile testing machine. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

The right Achilles tendons of all rats were excised along with a small amount of the gastrocnemius muscle. Proximal and distal parts of the gastrocnemius muscle and Achilles tendons excised together were super-glued to the flat side of the sandpaper and the rough side was connected to a tensile testing machine (Lloyd Tensile Testing Machine; String Technology UK, Nottingham, England) for biomechanical evaluation (Fig. 3).

The tendon was attached to the device at the proximal and distal ends and uniaxial constant force was applied. Maximum load before rupture was measured in Newtons. Tendon diameters after healing were not measured.

Statistical evaluation was performed using the NCSS (Number Cruncher Statistical System) 2007&PASS (Power Analysis and Sample Size) 2008 statistical software program (NCSS, LLC, Kaysville, UT, USA). The Mann-Whitney U test was used for comparison among groups of parameters without normal distribution. The Fisher's exact test was used for comparison of quantitative data. The significance level was set at $p<0.05$.

Results

Sections from both the experimental and control groups stained with hematoxylin-eosin and Masson's trichrome were examined and grading was performed considering inflammatory cell density, density of fibroblasts and collagen fiber sequence (Table 1).

A large number of polymorphic leukocyte cell were found through the collagen fibrils in the control group in the hematoxylin-eosin-stained sections. Additionally, an increase in fibroblast cells was observed in all studied sections. This increase was more prominent in the experimental group. The parallel structure of solid tendon parts in the anastomotic region was evaluated while analyzing the arrangement of collagen bundles. Most of the collagen bundle alignments in the control group were at different directions in the sections (Fig. 4).

Inflammatory cell density rates between groups were not statistically significant ($p>0.05$). However, inflammatory cell density rate was 'high', in 50% of the control group (n=4). None of the rats in the experimental group had 'high' inflammatory cell density rate. This difference was very close to significant. The fibroblast density was significantly higher in the experimental group than the control group ($p<0.05$).

The areas with newly synthesized collagen in the scar tissue at the repair area and the areas of scar tissue were calculated separately for each sample. Rates of the newly synthesized collagen areas at the sections were then calculated (Fig. 5). Results are shown in Table 2.

The rate of newly synthesized collagen over the scar tissue was statistically significant ($p<0.01$).

The maximum tendon tensile strength was not statistically significant among groups ($p>0.05$) (Table 3).

Discussion

Diabetes is one of the most important systemic diseases affecting wound healing and can cause circulatory problems and wound dehiscence.[10] Initial research emphasized the adverse effects of diabetes in wound healing and microvascular occlusive disease. In the presence of hyperglycemia, the sorbitol that arises as a toxic metabolite of glucose results in renal and vascular damage.[11] In addition, non-enzymatic glycosylation of collagen compromises the wound healing process. Systemic diseases that adversely affect wound healing affect tendon healing at the same rate.

Few studies have been published that illustrate the effects of diabetes on tendon healing. Egemen et al. evaluated tendon healing in rats and found that tendon healing at the 2nd, 4th and 6th week was significantly superior in non-diabetic rats.[1] In a study on tendinopathy in diabetes, Babu et al. concluded that an important role in the pathogenesis of tendinopathy is played by glycosylation of collagen and increases in cross-linkage and that administration of green tea decreased these

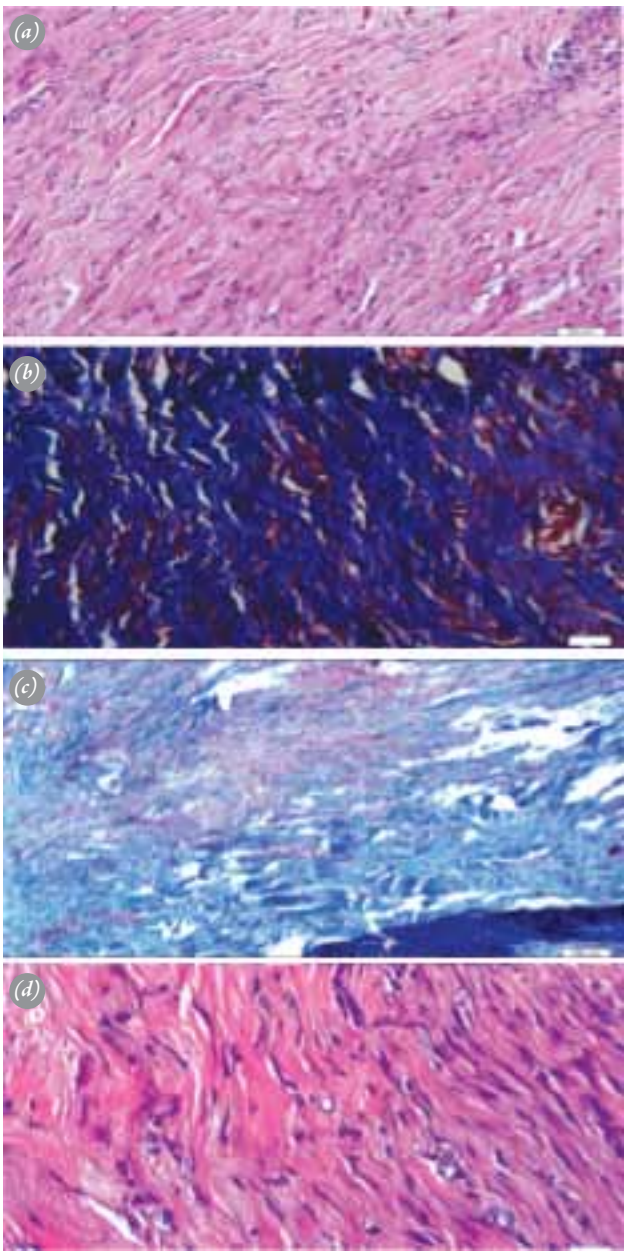

Fig. 4. **(a)** C6: Abundant inflammatory cells scattered among the irregular collagen bundles in cross-sections stained with hematoxylin-eosin (x20). **(b)** E6: Increased fibroblastic cell density in the cross-section stained with Masson's trichrome (x40). **(c)** C3: Irregular arrangement of collagen fibers and some gaps in the cross-section stained with Masson's trichrome (x20). **(d)** E5: Regular alignment of collagen bundles and increased fibroblast density in anastomosis area in the cross-section stained with hematoxylin-eosin. (x40) (C: control group, E: experimental group). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

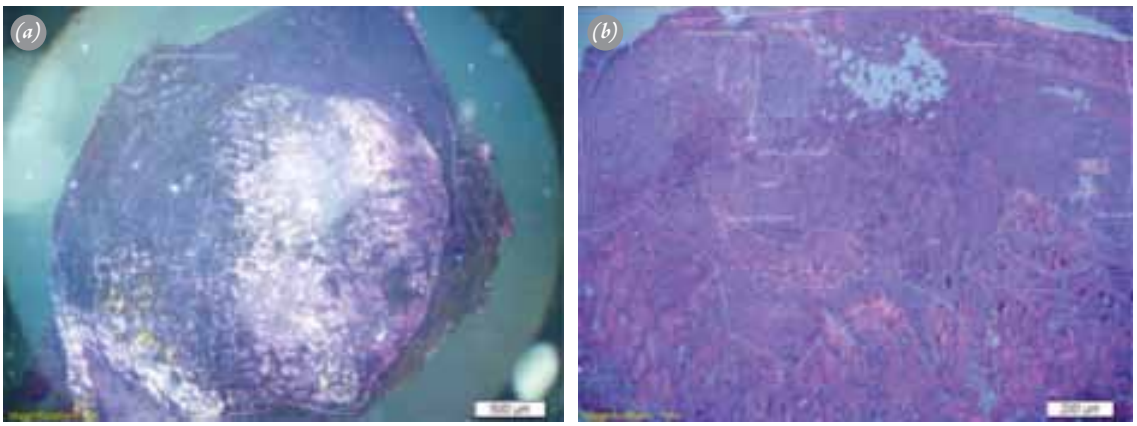

Fig. 5. **(a)** E5: Scar tissue sections stained with Masson's trichrome and newly synthesized collagen areas. **(b)** C5: Scar tissue sections stained with Masson's trichrome and newly synthesized collagen areas. (C: control group, E: experimental group). [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

adverse effects.[12] The effect of diabetes in tendon healing has been studied at the molecular level by Ahmed et al.,[13] who reported that tendon healing was impaired in a diabetic rat model mainly due to altered expression of collagen and matrix metalloproteinases reflecting decreased degradation of matrix proteins and impaired tissue remodeling. Therapeutic modulation of collagens or matrix metalloproteinases might be targets for new regenerative approaches in operated, injured or degenerative tendon diseases in diabetes. A study evaluating the effect of low-level laser therapy on the healing of tenotomized Achilles tendons in STZ-induced diabetic rats revealed that the use of lasers in both the non-diabetic and diabetic experimental groups significantly increased the strength and maximum stress of the repair.[14] De Oliveira et al. studied the biomechanical properties of the Achilles tendons in rats chemically induced to diabetes mellitus and reported that moderate-intensity aerobic training restored the normal mechanical properties of the tendons in diabetic animals.[15]

Few studies on the effect of antioxidants in tendon healing have been reported.[6,7] In the current study, the effects of the powerful antioxidant and effective anti-diabetic resveratrol on tendon healing in diabetic rats have been studied.

Resveratrol has recently gained attention as a supplement prolonging life. Found in several vegetables and mainly in grapevines, phytoalexins are protective substances that are synthesized by plants against ultraviolet radiation, bacterial and fungal infections.[16,17] A broad spectrum of the drug's benefits are due to its strong antioxidant properties. It has shown cardioprotective, neuroprotective, anti-diabetic, antitumor, antiviral and

Table 1. Semi-quantitative assessment of some histological parameters.

	Control Group (n=8)		Experimental Group (n=8)		p*
	n	%	n	%	
Inflammatory cell density					
Low	1	12.5	5	62.5	0.119
Medium	3	37.5	3	37.5	1.000
High	4	50.0	0	0	0.077
Fibroblast density					
Low	3	37.5	0	0	0.200
Medium	5	62.5	3	37.5	0.619
High	0	0	5	62.5	0.026†
Alignment of collagen fibers					
Regular	2	25.0	6	75.0	0.132
Irregular	6	75.0	2	25.0	

*Fisher's exact test. †p<0.05.

Table 2. Collagen synthesis rates in the groups.

	Control Group (n=8)	Experimental Group (n=8)	p*
Scar Tissue (μm^2)			
Min	1383243.0	863905.0	0.834
Max	31812641.0	31812641.0	
Med	4533194.5	4641327.5	
Newly Synthesized Collagen (μm^2)			
Min	75619.0	335937.0	0.172
Max	10088523.0	4480913.0	
Med	240011.0	1792576.0	
Synthesized Collagen / Scar Tissue			
Min	0.022	0.228	0.009†
Max	0.360	0.839	
Med	0.080	0.393	

*Mann-Whitney U-test. †p<0.01.

Table 3. Maximum tensile strength of the tendons.

	Control Group (n=8)	Experimental Group (n=8)	p*
Maximum Load (Newton)			
Min	7.56	5.61	0.834
Max	35.46	37.78	
Med	17.91	23.97	

*Mann-Whitney U-test.

anticancer effects.[2-5]

Resveratrol has been shown to lower glucose in obese and STZ-induced diabetic rats.[18,19] In some studies, resveratrol reduced the levels of HbA1c, an indicator of prolonged glycaemia.[20] Resveratrol's anti-diabetic effect mainly works through three mechanisms; by increasing the number and activity of membrane proteins GLUT[4,21] by protecting pancreatic β cells to oxidative stress, and by breaking insulin resistance by reducing body fat mass.[20]

The tendon repairs of two groups of diabetic rats that did and did not receive resveratrol were evaluated. The resveratrol group showed higher resistance to maximum load before rupture than the control group. However, the difference was not statistically significant. Similarly, histological healing was better in the resveratrol group. In many tendon studies, histological evaluation was performed semi-quantitatively using light or electron microscopes.[22] No quantitative histologic evaluations were performed in the literature. In the current study, measurement of the newly synthesized collagen areas in healing region areas showed a significantly higher rate in the resveratrol group.

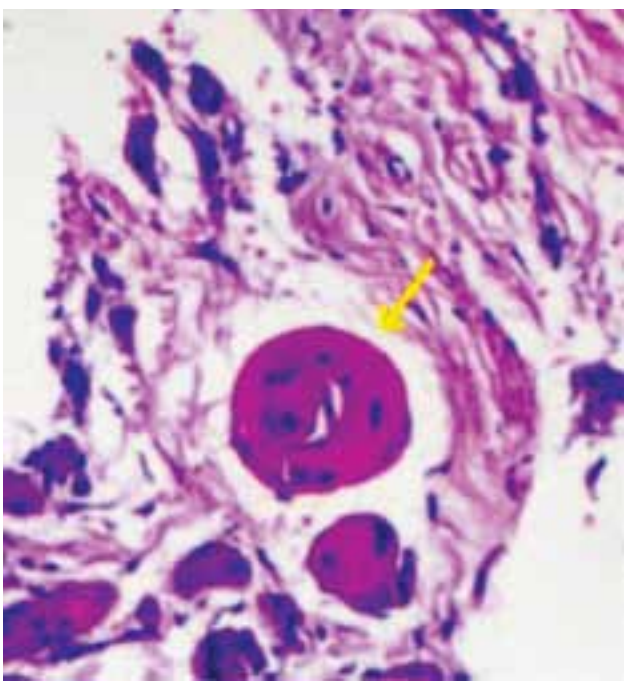

Fig. 6. Skin section stained with hematoxylin-eosin (x10). Hyaline arteriolosclerosis; vascular finding of diabetes is shown by the arrow. [Color figure can be viewed in the online issue, which is available at www.aott.org.tr]

Surgery was performed after 2 weeks of STZ administration. In studies in which rats were accepted as diabetic, blood glucose levels were taken as reference at the 1st, 2nd, 4th and 6th weeks.[1,23,24] In our study, blood glucose levels were measured prior to surgery and those higher than 300 mg/dL were accepted in the study. Although not included in the protocol of our study, hyaline arteriosclerosis, a small vessel disease of diabetes, was observed on some of the randomly sampled hematoxylin-eosin-stained sections (Fig. 6).

Findings from earlier and later periods may provide more comprehensive information or different results of biomechanical analysis. The healing period of rats is shorter than that of humans. However, we performed the examination on Day 14 when an acceptable level of healing could be detected in humans. Additionally, as dramatic weight loss and general poor health was observed after STZ administration, the study was limited to 14 days.

The lack of weight measurement before and after resveratrol treatment can be considered a limitation of our study. Such measurements may provide additional clinical information about the effects of resveratrol.

Studies on the maximum functional recovery after tendon repairs, remedial work related to surgical technique, administering exogenous material to accelerate tendon healing and the effects of additional patient-related diseases on tendon healing have been reported in the literature.[25-29] In contrast, in our study, we believe that a concomitant metabolic disease, such as diabetes, can be controlled during administration of an exogenous substance to accelerate tendon healing. Resveratrol has no known side effects and had an extremely positive impact on tendon healing. Only one detailed study documenting the effects of resveratrol on tendinopathies has been published. Resveratrol decreases the amount of inflammatory mediators such as IL-1β by inducing SIRT-1 (Sirtuin) genes in human tenocites.[30] Due to its substantially high cost, this herbal product cannot be used in clinics on a routine basis. However, it may be recommended to selected patients such as diabetics.

In conclusion, resveratrol, a powerful antioxidant extracted from plants, has a positive impact on the tendon healing process in diabetic subjects, particularly in the first 14 days.

Conflicts of Interest: No conflicts declared.

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