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### Original Article

## Collagenase Induced Tendinitis in Forelimb Superficial Digital Flexor Tendon in Donkey (*Equus asinus*): Long Term Ultrasonographic and Biomechanical Assessments

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#### ABSTRACT

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Injuries to tendons are among the most common injuries in competition horses and represent a significant cause of morbidity and mortality. The present study aimed at an eight -week period ultrasonographic and biomechanical assessments of collagenase induced tendinitis in forelimb superficial digital flexor tendon (SDFT) in donkeys. Four healthy male donkeys were used. 1000 U collagenase type I from *Clostridium histolyticum* was injected in the center of the SDFT guided by ultrasonography. Ultrasonographical images of the SDFT were recorded prior to injury and 8 weeks after confirmation of tendinitis on a weekly basis. Images were digitized and lesion area to cross-sectional tendon area ratio (LA/TA ratios) was measured. At the end of the study period, the animals underwent tenectomy and tendon samples were evaluated biomechanically. Collagenase induced tendinitis was confirmed ultrasonographically 10 days post-injection. The animals showed swelling and response to palpation, however, no lameness was found in animals within the study period. There were significant differences between the first and the fifth weeks in operated limbs ultrasonographically ( $p < 0.05$ ). The eight-week period is sufficient to assess the effect of various modalities in tendon healing in the collagenase-induced model in the donkey. The development and expansion of collagenase induced tendinitis until week six after confirmation of tendinitis may disturb findings of the healing effect of various modalities in the tendon, in which tendinitis is still expanding and may mask the healing effect of the modalities used.

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## Introduction

Injuries to tendon are among the most common injuries in competition horses and represent a significant cause of morbidity and mortality. A study of injuries occurring on British racecourses found that 46% of all limb injuries in racehorses were to tendons and ligaments.<sup>1</sup> The incidence rate is also high for horses in training, at 1.9% horse months, recorded in National Hunt training yards in the UK within two racing seasons.<sup>2</sup> Moreover, recurrence of tendinitis after return to competition can be as high as 43%.<sup>3</sup> These issues have been recognized for many years, yet few major advances in treatment have improved the proportion of horses that resume sustained racing.<sup>4</sup> The central core temperature of the superficial digital flexor tendon (SDFT) was reported to increase for up to 45 °C after galloping for 5 min. This high temperature may lead to tenocytes metabolism impairment and eventually cell apoptosis. Raising the central core temperature of SDFT can be attained to 45 °C during exercise is believed to be a possible cause for cell damage and an alteration of matrix synthesis as the histopathologic and biochemical analysis revealed a significant high collagen type III expression and high glycosaminoglycan content.<sup>5</sup> One of the reasons of heat accumulation in tendon is the lack of vascularization and this subsequently leads to longer time of high temperature after exercise. Moreover, the regeneration of damaged SDFT is found to be less vascular than the originally health one which would increase the susceptibility to re-injury.<sup>6</sup>

Treatment of tendon injuries has stimulated much attention in both medical and veterinary literatures, focusing on various materials used to reconstruct damaged or ruptured tendons. Various techniques have been described that provide forceful tendon anastomosis with minimal gap formation, limited adhesion and preservation of the tendon's intrinsic blood supply.<sup>7-9</sup> The ability to accurately induce uniform tendons injuries are very important in studies designed to evaluate and compare new treatments for injured tendons. Various methods were studied for finding an experimental model to standardize tendon injuries.<sup>10,11</sup> The use of a bacteria-derived collagenase was explored to induce tendon damage enzymatically, resulting in lesions similar particulars to those occurring naturally.<sup>12,13</sup> Lesions occur most frequently in the core of the mid-metacarpal region, however, can involve any site from the musculotendinous junction to

the branches of insertion.<sup>10</sup> Tendon healing is a slow process following injury, and tendons rarely regain their original strength and elastic qualities. Suboptimal healing, prolonged rehabilitation time, and a high incidence of recurrence make degenerative tendon injuries difficult to treat successfully.<sup>14</sup>

Ultrasonographic evaluations have been used for the diagnosis of soft tissue injuries in horses. Basically, ultrasonography has been introduced as an appropriate method for the assessment of morphological changes in the tendon and ligament structure and it is known as one of the most accurate and non-invasive tools to evaluate the tendon structure after an injury.<sup>13</sup> The echogenicity and fiber alignment of the core lesions, were considered the major ultrasonographic diagnostic criteria of the severity of the injured tendon.<sup>15</sup>

Tendons are extremely complex in terms of their structural, functional and biomechanical characteristics.<sup>16</sup> Mechanical factors are important in the etiology of tendon and ligament lesion. The distribution of loads among several tendons in the equine limb has been extensively studied.<sup>7, 17-23</sup>

Recent studies have been able to identify the widespread nature of tendon injuries in the horse. Almost all of the data relates to superficial digital flexor tendinitis in the racehorse, as this is the most common strain-induced tendon injury. The incidence of the condition in horses used for other disciplines is, however, poorly documented. Although it is common in all forms of equine athleticism, it is rare in donkeys that represent an experimental model for assessment of tendon healing in research trials.<sup>7,24</sup>

In previous studies long-term assessments in collagenase induced tendinitis in donkeys was not taken into consideration. The present study was aimed at evaluation of ultrasonographic and biomechanical outcomes of collagenase induced tendinitis of SDFT in donkeys in a period of eight weeks. An ultrasonographic tendon reference in donkey was also developed.

## Materials and Methods

### *Donkeys*

Four adult male donkeys approximately 120–140 kg, 4-7 years of age, were included in the present study. The animals were purchased from various localities of Urmia suburbs. They were examined physically and ultrasonographically to exclude any tendon abnormalities and/or tendinous lesions. Animals were

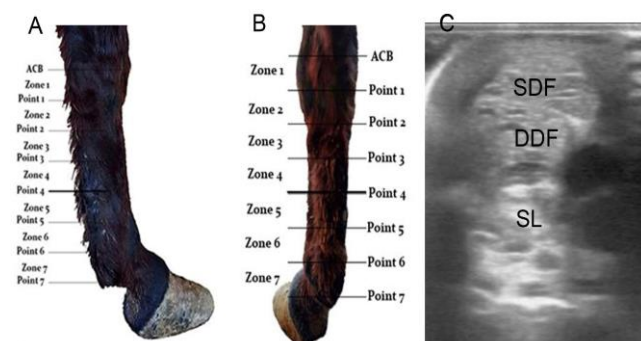
kept in the animal house of Veterinary Medical Teaching Hospital at Urmia University and fed on maintenance balanced hay *ad libitum*. Two weeks before the start of the experiment, all animals were dewormed. During the entire experimental period all animals were kept under similar husbandry and feeding management.

### Ethical Considerations

The present study involved the use of non-experimental animals and procedures that differed from established internationally recognised high standards ('best practice') of veterinary clinical care for the individual patient. This work was approved by Deputy Research, Faculty of Veterinary Medicine, Urmia University, under ID#3/PD/542-12/04/1398.

### Study Design, Tendon Reference Point System and Induction of Tendinitis

In the present experimental study four male clinically healthy donkeys were included. 1000 U/0.05 mL collagenase type I from *Clostridium histolyticum* (BIO-IDEA, Tehran, Iran) was injected in the center of the SDFT of both forelimbs of each donkey guided by ultrasonography.<sup>10</sup> The forelimbs were clipped and prepped using routine aseptic method. The authors developed a seven-point and a seven-zone pattern to provide reference points and zones for ultrasonographical studies of the anatomical structures of the tendons and ligament of the metacarpus in donkeys. The accessory carpal bone (ACB) was selected as a land mark. Seven zones were described starting from ACB and terminating at ergot. Consequently seven



**Figure 1.** Tendon reference point system. **A**, Lateral view of fore limb, **B**, caudal view of fore limb, **C**, Ultrasonographic image (cross sectional view) of the point 4 where the SDF tendon is in the most palmar aspect of the limb. ACB: accessory carpal bone; SDF: superficial digital flexor tendon; DDF: deep digital flexor tendon; SL: suspensory ligament.

points were also described (Figure 1A-B). The animals were premedicated with 2% xylazine hydrochloride (1.5 mg/kg) (Alfasan, Woerden, The Netherlands) and 1.5% acepromazine hydrochloride (0.06 mg/kg) (Alfasan, Woerden, The Netherlands) intravenously followed by lateral recumbency anesthesia using ketamine hydrochloride (2 mg/kg) intravenously. A 24-gauge needle was inserted into each tendon via its palmar surface with about 45° angle, while the tip of the needle was toward the fetlock joint, by ultrasonographic guidance to confirm that the tip of the needle was completely in the middle of the tendon (Figure 2).

### Clinical Observation

Clinical examinations were performed to evaluate heat, response to palpation, presence of swelling, and lameness grade. Following collagenase injection, from the first day of injection to the end of study period the donkeys were submitted to physical examination, palpation and lameness, of the limbs and to progression-controlled walking for 15 minutes.

### Ultrasonographic Assessments

Ultrasonographical images of the SDFT were recorded prior to injury and every day following injection of collagenase. At the end of each week following confirmation of tendinitis, the tendons were assessed ultrasonographically using Chison Q9vet ultrasonographic machine with a 5-10 MHz linear D7L40L probe. Both sagittal and transverse planes were obtained in weight bearing animals from reference point. Ultrasonographical images of the SDFT were recorded prior to injury and for 8 weeks after day zero on a weekly basis. Images were digitized, and cross sectional tendon area (TA), lesion area (LA) and LA/TA ratio were measured.

### Tendon Sample Harvesting and Biomechanical Evaluations

To evaluate the biomechanical indices of the tendons, including ultimate load, stress, strain, yield point, Young modulus, and maximum stored energy the animals were anesthetized at the end of week eight. SDFT specimens were harvested by transecting each tendon 3 cm above and below the injured area. The skin was closed routinely and the animals were recovered after 4 weeks with postoperation care. Postoperation the animals received anti-inflammatory and antibiotics to reduce the likelihood of infection of surgical site. The

samples were frozen in  $-20\text{ }^{\circ}\text{C}$  until assessment. The frozen samples were placed in a normal saline bath at room temperature and were then fixed between fixtures in a mechanical apparatus after thawing. The Santam STM-5 Universal Testing Machine was used for the assessment (Santam Co., Tehran, Iran). The clamps were coated from inside by a piece of sand paper to avoid slipping of the tendon specimens. The initial length was set to 10 mm. Each sample was stretched at a constant rate of 1 mm/min. The load and displacement were sampled 5 times per second. Each sample was stretched to complete tensile failure. Samples were kept wet moist during testing using a drop of normal saline solution to the nerve segments (Figure 3).

### Statistical Analysis

In the present study operated limbs were compared to un-operated limbs biomechanically. Also operated limbs were compared within an 8-week period ultrasonographically. Data were statistically analyzed by repeated measures and One-Way ANOVA with multiple comparison tests using statistical software program (Release 14, SPSS Inc., Chicago, IL, USA). Differences were considered significant at  $p < 0.05$ .

## Results

### Tendon Reference Point System

Ultrasonography of the points showed that in the point 4 the SDFT was in the most palmar aspect and was easily accessible (Figure 1C). Therefore, for induction of tendinitis in the present study point 4 of the mentioned patterns was selected. Hence, in each animal collagenase was injected in the same point compared to the others. This system helped the authors to be able to locate the correspondent identical point in each animal easily.

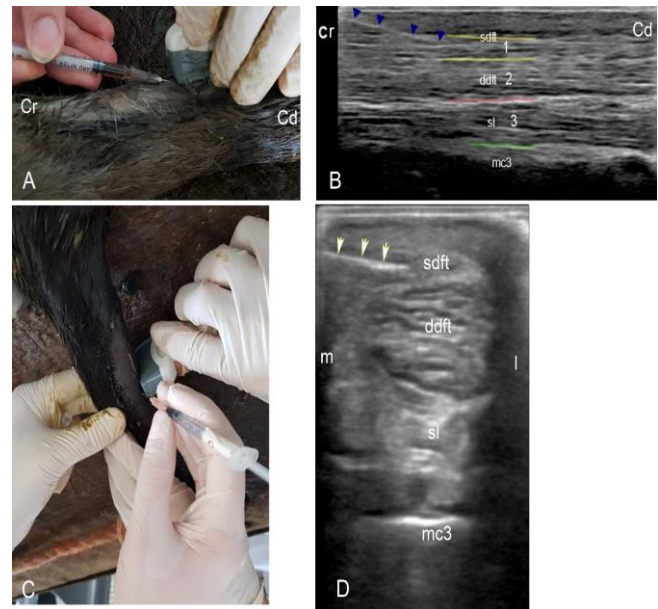
### Clinical Observation

Swelling of the injected site of the tendons was observed on day 7 and reached the maximum amount after on day 10 and gradually reduced over time. The degree of swelling varied among the individuals, however, no lameness was observed in animals.

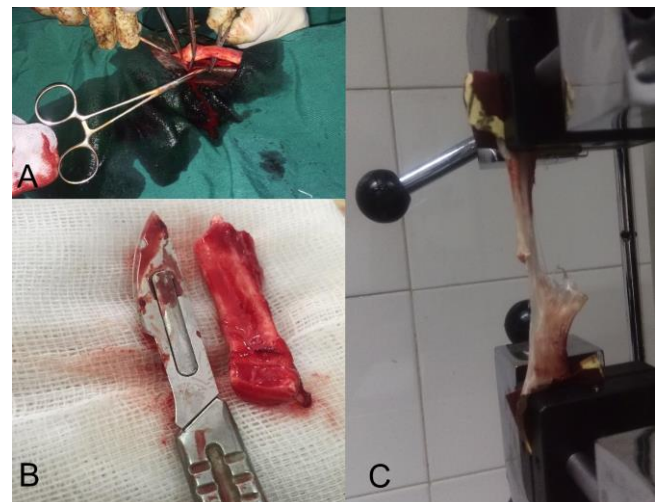
### Ultrasonographical Findings and Confirmation of Tendinitis

On day 10 tendinitis was confirmed and was considered as the day of 0 of the study. The evaluations

were initiated on the day zero and lasted for eight weeks. Table 1 shows LA/TA ratios in which there were no significant differences between first ( $0.27 \pm 0.05$ ) and last weeks ( $0.39 \pm 0.05$ ) post tendinitis confirmation ( $p > 0.05$ ). From the first week to fifth week the ratio was increased to  $0.53 \pm 0.03$  with significant difference between the first and fifth week. Then it was decreased to week eight (Figure 4).



**Figure 2.** A, Ultrasonographic guided injection of collagenase into the superficial digital flexor tendon in sagittal plane. B, Ultrasonographic image of tip of the needle (arrowhead) in the center of the SDFT before injection (B). C, Ultrasonographic guided injection of collagenase into the SDF tendon in transverse plane. D, Ultrasound image of tip of the needle (arrowhead) in the center of the SDFT before injection.



**Figure 3.** A, Intraoperative image of superficial digital flexor tenectomy of left forelimb in anesthetized donkey at the end of the study period. B, The harvested SDFT prepared for biomechanical testing. C, Failure of forelimb SDFT. Arrow indicates the point of SDFT failure from the lesion area.

**Table 1.** Ultrasonographic findings of lesion area to cross-sectional tendon area ratio (LA/TA) ratios in animals within study period. Data are expressed as mean  $\pm$  SD.

Weeks	One	Two	Three	Four	Five	Six	Seven	Eight
LA/TA ratios	0.27 $\pm$ 0.05 <sup>a</sup>	0.33 $\pm$ 0.02 <sup>a</sup>	0.42 $\pm$ 0.05 <sup>a</sup>	0.44 $\pm$ 0.03 <sup>b</sup>	0.53 $\pm$ 0.03 <sup>b</sup>	0.40 $\pm$ 0.02 <sup>b</sup>	0.35 $\pm$ 0.02 <sup>a</sup>	0.39 $\pm$ 0.02 <sup>a</sup>

Values with different superscripts are significantly different ( $p < 0.05$ ).

Difference of LA/TA ratio in week one was significant compared to those of weeks three to six ( $p < 0.05$ ). However, no significant difference was observed in LA/TA ratio in week three compared to weeks four and six ( $p > 0.05$ ). In contrary, LA/TA ratio in week six was significantly different compared to those of weeks seven and eight ( $p < 0.05$ ). However, no significant difference was observed in LA/TA ratio in week seven compared to weeks one, two and eight ( $p > 0.05$ ) (Figure 5).

### Biomechanical Findings

The findings of the present study showed the values of ultimate load, yield point, Young's modulus, maximum stored energy, strain, and stress at tendon failure of donkeys were 437.7  $\pm$  27.3 N, 428.5  $\pm$  13.7 N, 41.9  $\pm$  13.9 MPa, 1673.9  $\pm$  203.5 J, 11.5.7  $\pm$  5.7, 57.6  $\pm$  15.5 %, respectively. Statistical analysis concerning the aforementioned biomechanical indices in operated limbs of the animals, showed there were no significant differences among the harvested samples in operated limbs ( $p > 0.05$ ) (Figure 6).

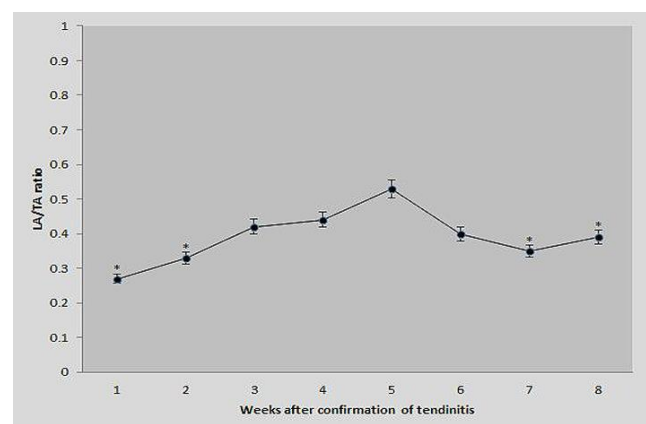
### Discussion

Animal models offer an attractive framework to investigate the etiology of tendinopathy. Unlike horse tissue, which only can be examined during end-stage chronic pathology, animal models provide the opportunity to obtain tissue during all stages of tendinopathy. Additionally, animal models provide the ability to reproduce consistent and repeatable injuries that can be treated in a controlled and quantifiable manner and also allow the evaluation of invasive treatments and assessments that would be unethical with horses.<sup>11</sup> The present study evaluated an animal model that was developed to understand the etiology and pathology of tendinopathy as well as some of its translational implications in horses.

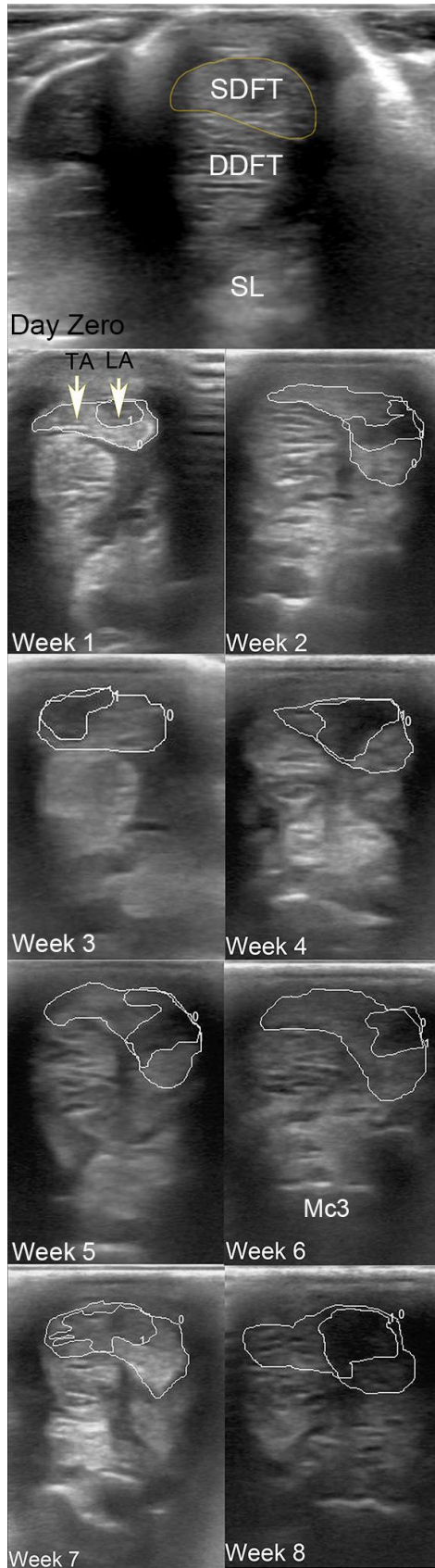
Finding out an optimal technique for tendinitis induction has attracted a lot of researchers to investigate different treatment protocols efficiently. To accurate induction of uniform tendons injuries similar to what happens naturally and to standardize tendon

injuries, various methods were studied and the use of a bacteria-derived collagenase was demonstrated to resulting in lesions similar particulars to those occurring naturally.<sup>25-27</sup> To repair the superficial digital flexor tendonitis in equine, in the new researches, various modalities are being used such as platelet rich plasma, growth factor, hyaluronic acid and physical therapy-laser, electrical shock wave and magnetic field therapy.<sup>28-30</sup> In recent decade, donkeys are mostly used as tendon repair models to study new modalities of treatments for translation to horses.

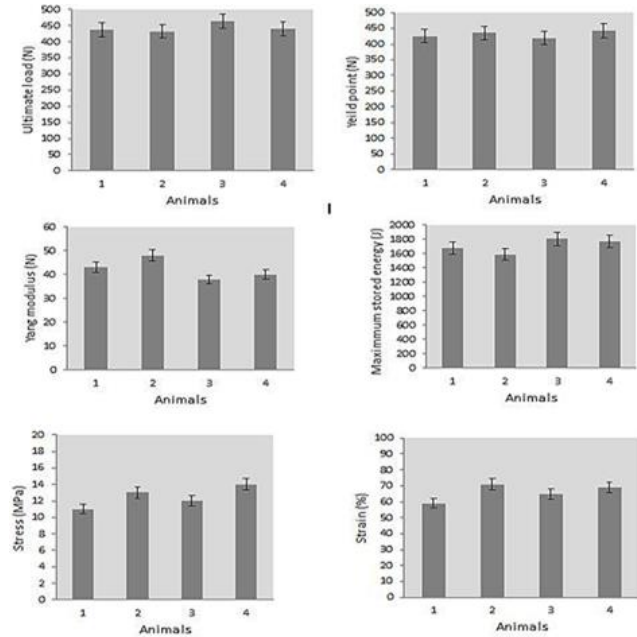
The first step for the diagnosis of tendonitis is the clinical evaluations. For this reason, clinicians need to assess the tendonitis by palpation of the injured area and detect local heat, pain response, local swelling and appearance of the tendon. The degree of lameness is the important criteria to evaluate the severity of the tendonitis too. According to the present study, increased local heat, pain reactions, swelling of the metacarpal region were observed, however, no lameness was observed in the animals. Review of literature revealed that in all experimental collagenase induced tendinitis, the authors found that there were various degrees of lameness in donkey following collagenase induced tendinitis that was in contrary to the findings of the present study.<sup>30,31</sup> Nonetheless, others with surgical core lesion creation in SDFT of horses did not observe lameness in the study period



**Figure 4.** Lesion area to tendon area (LA/TA) ratios within eight week after confirmation of tendinitis. Data are expressed as Mean  $\pm$  SD. \*  $p < 0.05$  vs. other weeks.



**Figure 5.** Serial representative ultrasonographic images of collagenase induced tendinitis within eight weeks following confirmation of tendinitis.



**Figure 6.** Biomechanical indices of tendons harvested at the end of the study period. There were no significant differences between the animals ( $p > 0.05$ ).

that was in agreement with our findings.<sup>32</sup> On the other hand, in studies of others tendinitis was confirmed in a 5-day range from 2 to day 7 following collagenase injections. However, the ultrasonographic findings of the present study revealed that tendinitis was confirmed 10 days following collagenase injection. This could be because of collagenase that was supplied from different companies. Hence, this should be considered when collagenase is supplied from companies other than those used by other authors.

Ultrasonographic evaluation of the metacarpus and metatarsus has been used to diagnose soft tissue injuries of these regions for the past three decades.<sup>8</sup> Therefore, locating the reference points in metacarpus of donkeys seems to be required as they are used as model animals in research clinical trials. However, the reference points in horses could not be used in donkeys because of shorted length of the tendon in donkeys.<sup>33</sup> Therefore, in the present study a tendon reference point system was customized to donkeys. Ultrasonography of the reference points showed that at the point four the SDFT was in the most palmar aspect and could be easily located. We concluded that the point four was the most appropriate one for injection of collagenase and any other agent that may be used as a modality to improve tendinitis.

The ultrasonographic findings of the present study in a two-month period revealed that the tendinitis was expanded from week one to week six following

confirmation of tendinitis and then regressed to the end of the study period that was similar to the week first. These findings showed that a two-month period could be sufficient to assess effect of various modalities in tendon healing in collagenase induced model in donkey. Furthermore, it should be noted that development and expansion of collagenase induced tendinitis until week six post tendinitis confirmation may disturb findings of healing effect of various modalities in tendon, in which the tendinitis is still expanding and may mask healing effect of the modalities used. This may result in unreal positive or negative effects.

The majority of tendon injuries in racehorses occur to the forelimb tendons with the SDFT being injured in 75–93% of cases.<sup>2,34-36</sup> The high susceptibility of the SDFT to injury is not surprising when considering the function of this tendon. In addition to preventing hyperextension of the metacarpo-phalangeal joint, the SDFT plays a vital role in energy storage and release thereby increasing the efficiency of locomotion.<sup>37</sup> Energy storing tendons are required to stretch and recoil and are therefore subjected to high strains. Maximum *in vivo* strain for the common digital extensor tendon, a positional tendon, has been estimated at 2.5% which is almost 4 times lower than the failure strain of 9.7% recorded *in vitro*.<sup>38,39</sup> In contrast, during gallop, *in vivo* strains of 16% have been recorded in the SDFT, similar to the failure strains of 15–17% recorded *in vitro*.<sup>40-41</sup> Therefore, the SDFT is working with a very low safety margin.<sup>17</sup>

For each tendon the force-elongation curve was plotted and the following mechanical parameters were obtained: ultimate load (N), yield point (N), stiffness (N.mm<sup>-1</sup>), ultimate Stress (N.mm<sup>-2</sup>), ultimate strain (%), and energy absorption (N.mm). The ultimate load was defined as the maximum force measured in the tendon during the failure test. The yield point, defined as the point where the curve first deviated from the linear region. Energy absorption values were measured by calculating the area under the force-elongation curve up to the point of maximum force. Ultimate tensile stress was calculated by dividing maximum force values by initial cross-sectional area. Similarly, ultimate tensile strain was calculated by dividing the elongation at the point of maximum force by initial length and was expressed as a percentage. The Young's modulus was determined as the slope in the linear elastic region of the force-elongation curve.<sup>42</sup> In the present study, the biomechanical indices of SDFT in donkeys were found to be widely different from the corresponding indices

reported by others in horses.<sup>33</sup> Therefore, the values of biomechanical properties in horses are threefold greater than the corresponding values in donkeys. Our finding could be attributed to the biomechanical and/or functional differences between donkeys and horses. Difference between members of Equidae should be considered for clinical evaluation during load bearing of donkeys in comparison with horses.<sup>43</sup> Also, it seems possible that there is a proportional relationship between the load and thickness of the tendon; the larger the thickness the greater the load it can bear. The present findings coincided with the suggestion reported by others,<sup>44,45</sup> that determination of the *in vivo* tendon biomechanics is very important since overload of a tendon is best expressed in terms of overstrain that can be clinically avoided by a good understanding of these parameters. Even though our study showed that the eight-week period was sufficient to assess effect of various modalities in tendon healing in collagenase induced model in donkey, determining the molecular mechanisms leading to core lesions remains needs to be investigated. We have not given the histological and molecular evidence for creation of core lesions. This may be considered as a limitation to our study.

In conclusion, the findings of the present study showed that the eight-week period was sufficient to assess effect of various modalities in tendon healing in collagenase induced model in donkey. The development and expansion of collagenase induced tendinitis until week six post tendinitis confirmation may disturb findings of healing effect of various modalities in tendon, in which the tendinitis is still expanding and may mask healing effect of the modalities used.

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### Conflict of Interest

None.

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