A novel approach for tendinopathy induction in donkeys using artificial heat stimulation strategy

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ABSTRACT

Animal models for tendonitis are essential for studying the disease's mechanism and pathogenesis, and evaluating different therapeutic protocols. The temperature seems to play a significant role in tendinitis initiation. The aim of this study was the generation of a novel, safe and cheap tendinitis model, and validation of its reliability. The effect of microwave diathermy (30 watts for 30 min) on the flexor tendons of donkeys as animal models was investigated after 15, 30 and 60 days. The evaluation was based on geometric analysis, ultrasonography, histomorphometric analysis and scanning electron microscopy (SEM). Microwave diathermy was capable of successfully inducing well-defined lesions in the superficial digital flexor tendon (SDFT) as well as the deep digital flexor tendon (DDFT). The results showed that all the animals exhibited signs of lameness, starting on day 15 and reaching on maximum on day 30. A significant increase in limb circumference was also detected on day 30 ($P<0.05$). Furthermore, the geometrical analysis of the proportion of induced lesion (PIL) in correlation with the tendon diameter, revealed that PIL was at the maximum width on day 30 ($20.6 \pm 1.2\%$ for SDFT and $15.7 \pm 0.7\%$ for DDFT), as detected by ultrasound. Moreover, a high number of rounded tenocytes, bleeding, severe matrix disruption, and an increase in fiber thickness were detected by histomorphometric analysis. Also, the matrix alignment was severely disrupted in both SDFT and DDFT by day 30, as confirmed by SEM. In conclusion, using microwave diathermy for induction of tendonitis in donkey is a reliable, minimally invasive, and cost-effective tendonitis model.

Key words: equine; tendinitis; superficial digital flexor tendon; microwave diathermy; large animal model

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Introduction

Superficial digital flexor tendon (SDFT) injuries ultimately compromise athletic performance and may result in a tragic career-ending injury (DOWLING et al., 2000). The incidence of SDFT injuries in thoroughbred (T.B.) racehorses was estimated as 7 - 43% (ROONEY and GENOVESE 1981; DOWLING et al., 2000) and 24% in national hunt horses (AVELLA et al., 2009). This relatively high risk is believed to be due to high racing speeds or the high speeds associated with jumping.

So far, there is no ideal large animal model for tendinitis that can mimic naturally occurring tendinitis. Experimental tendon injury has been induced in horses by two main methods, collagenase injection (DEHGHAN et al., 2007; WATTS and AJ, 2011) or a surgical approach (SCHRAMME et al.,

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2010). The collagenase-induced model, however, is considered the most common tendinitis model (WILLIAMS et al., 1984). The output lesions are highly variable in size and extent from the site of injection. Moreover, animals under investigation in this method require approximately three weeks to stabilize before starting the experiment. Thus, this may poorly mimic clinical injuries (BOSCH et al., 2010). In the surgical approach, disruption of the paratenon may occur due to the invasive procedure, which is different from natural tendinitis in clinical cases (SCHRAMME et al., 2010). Collectively, most of these tendinitis induction techniques could produce significant and well-defined core lesions, but they are invasive and require anesthesia.

Recently, coblation technology via radiofrequency energy (ZEDLER et al., 2008) and a 980-nm diode laser (VALLANCE et al., 2012) have also been proposed as a safe method to induce lesions in equine SDFTs. However, there is a considerable cost involved in this technique.

Diathermy is electrically induced heat, or high-frequency electromagnetic currents as a form of physical or occupational therapy to deliver moderate heat directly to pathological lesions in the body's deeper tissues.

In our previous study, we used donkeys as a large animal preclinical model to assess the efficacy of the proposed microwave method for tendinitis induction. The output of this study revealed that a microwave setting of 30 watts/30 min was able to increase the temperature of SDFT and DDFT in cadaveric limbs to 47.5±0.6 and 45.5±0.6 C° (ELSAYAD et al., 2020).

In the present study, we hypothesized that microwave diathermy would induce consistently sized lesions in SDFT and DDFT, with hypercellularity and collagen disruption that resembles naturally occurring tendinitis in clinical cases. The evaluation methods used were clinical examination, and the macroscopic and microscopic appearance of microwave-treated tendons compared to untreated control ones.

Material and methods

Animals. Nine, healthy local breed Egyptian male donkeys, weighing from 150 to 200 kg, 3-5 years old, were used in the present study. All the animal procedures were carried out according to on protocol approved by the institutional animal care and use committee (IACUC), Alexandria University, Egypt (AU01320190226115). The donkeys were examined clinically and by ultrasound (US) to exclude pre-existing tendinitis. The tendons were considered reasonable if there were no clinical signs of tendinitis or ultrasonography findings of tendinitis. Also, the tendons examined by US had to have homogeneous echogenicity and normal fiber alignment, and their cross-sectional area did not exceed 1.2 cm2. The SDFT was evaluated by US at two-centimeter intervals, starting 4 to 12 cm distal to the accessory carpal bone (DACB).

Tendonitis induction. For tendinitis induction, microwave therapy equipment (CFT-2100, Jiangsu Rich Life Science Instrument Co., LTD, China) was used. The system is equipped with a 2450 MHz microwave generator, with a maximum output power of 30 watts. The probe (18 cm length and 2 cm diameter) was fixed on the donkey's right metacarpal region by a plastic holder at 10 cm DACB for 30 min daily for 14 days. The radiation power was determined on the basis of our previous investigation as 30 W for 30 minutes (ELSAYAD et al., 2020) without using any nerve block or local anesthetic medication.

Clinical examination. Clinical examinations of the right forelimbs were performed on day 0 (baseline) and weekly until the end of the experiment. The donkeys were evaluated for lameness scores on a scale from 0 to 5, according to the American Association of Equine Practitioners (AAEP) grading system. The score was considered as follows: 0: Lameness not perceptible with flexion test, 1: Lameness is difficult to observe and is not consistently apparent with flexion test, 2: Lameness apparent with flexion test, 3: Lameness is consistently observable at a trot, 4: Lameness is obvious at a walk and 5: Lameness produces minimal weight bearing in motion (Practitioners 1991). Moreover, the limb circumference at the tendinitis induction site was measured using a measuring tape on days 1, 7, 15, 30, 45, and 60. The degree of pain was also evaluated on the basis of the animals' response to palpation of the flexor tendons. A total score was assigned, with a scale of 0 (no reaction) to 4 (severe reaction) (REDDING et al., 1999).

Ultrasonography (US) examination. The tendon structure was evaluated by US (Sonoscape E1EXP Machine, Sonoscape Co., China) with a 5- 16 MHz linear transducer on days 1, 15, 30, and 60. All US examinations were performed under light sedation with Xylazine HCl (Xylaject®, ADWIA Co., Egypt (0.5 mg/kg, IV). Transverse and longitudinal images were obtained at the level of 10 and 14 cm below the DACB. The images were captured digitally, and Sonoscape Scan Software was used to measure the proportion of the induced lesion (PIL) according to the following equation: PIL (%) = lesion cross-section area (LCSA) / tendon cross-section area (TCSA) × 100) at the maximum injury zone (MIZ). Also, the degree of echogenicity of the flexor tendons at the MIZ was recorded in the cross-sectional image. The degree of echogenicity was evaluated as follows: Degree 1, a hypoechoic lesion with more white than black. Degree 2, a hypoechoic injury with equal amounts of white and black. Degree 3, a hypoechoic injury with more black than white. Degree 4, an anechoic lesion with all black and no white (SMITH, 2008). Furthermore, the fiber alignment at the MIZ in the sagittal image was evaluated subjectively on a scale from 0 (76%–100% parallel fibers; standard) to 3 (0–25% of parallel fibers) (SMITH, 2008).

Gross pathology of the tendon. Three donkeys were euthanatized, using thiopental sodium (Thiopental®, EIPICO, Egypt; 2gm/100kg, IV), on days 15, 30, and 60. The SDFT and DDFT of the forelimbs were dissected within two hours of death. Gross photographs were taken for the whole SDFT and DDFT. The length of the lesion was measured, and the degree of hemorrhage was assessed (from a scale of 0 to 3) (MAFFULLI et al., 2008; SMITH, 2008). A longitudinally cut surface of each tendon segment at the lesion level was also created for the macroscopic examination.

Histomorphometric analysis. Paraformaldehyde fixed histological sections were stained with hematoxylin and eosin (H&E), Alcian blue, and Masson's trichrome. The area of the defect in the histological sections (n=2) from each animal (n=9) was analyzed. The evaluation of the histopathological section was according to (Schnabel et al., 2009) (Table 1). All tendon parameters were scored between 1 (normal) and

4 (severe changes) on the basis of the tenocytes' shape and density, bleeding, neovascularization, inflammatory cell infiltration, and collagen fiber linearity and uniformity.

Table 1. Histological scoring system, modified from (SCHNABEL et al., 2009)

Variable	Score	
Tendon cell shape	1	Linear
	2	Oval
	3	Moderately round
	4	Predominantly round
Tendon cell density	1	Sparse
	2	Mild increase
	3	Moderate increase
	4	Severe increase
Hemorrhage	1	None
	2	Mild/multifocal
	3	Moderate/coalescing
	4	Predominant hemorrhage
Neovascularization	1	None
	2	Mild increase
	3	Moderate increase
	4	Marked increase
Inflammatory infiltrates	1	None
	2	Mild
	3	Moderate
	4	Severe
Collagen fiber linearity	1	Linear
	2	>50% Linear
	3	20–50% Linear
	4	No linear areas
Uniformity of collagen fiber diameter	1	All fibers uniform
	2	>50% Uniform
	3	20–50% Uniform
	4	Complete disarray

Scanning electron microscope (SEM) analysis. Specimens from SDFT and DDFT were prepared for scanning electron microscope (SEM). Images were captured using scanning electron microscope (JEOL JSM -5200LV, JEOL, Ltd., Japan) and examined for fiber arrangement.

Statistical analysis. All the data are presented as mean ± standard deviation (SD). The one-way ANOVA was used to evaluate statistical differences in the present study using SPSS software (IBM SPSS version 25, 2017). Quantitative data plots were performed using Excel 2016. Statistical significance was identified as $P<0.05$.
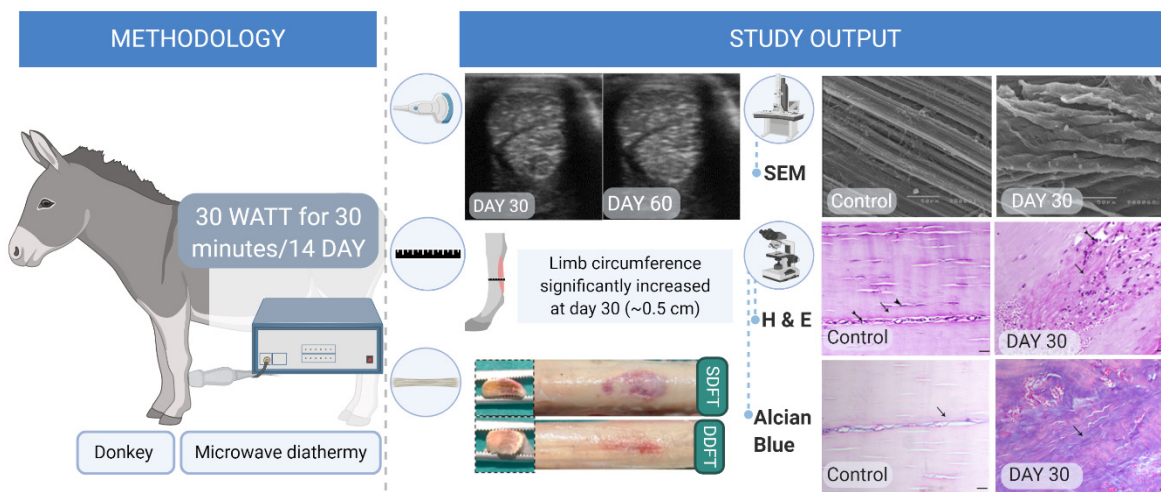
Graphical abstract of artificial heat stimulation strategy for tendinopathy induction

Results

Clinical signs. All the donkeys used developed lameness that started as grade 1 on day seven, then grade 2 on day 15, and reached grade 3 on day 30. It is also worth mentioning that the lameness decreased without any interference to grade 1 on day 60. In addition, all limbs developed mild diffuse limb swelling, heat, pain, and edema in the palmar mid-metacarpal region, that increased gradually until it reached the maximum on day 30, then decreased progressively and disappeared on day 60. The quantitative analysis of limb circumference after exposure to the microwave revealed a LOG phase from day one until day 30, followed by a LAG phase from day 30 to day 60.

Ultrasonography data. SDFT tendinitis induction was confirmed by the US on which centrally located lesions, without disruption of the epitenon, were detected at the site of microwave exposure (Fig. 1). Measuring the TCSA revealed

that the tendon diameter increased significantly after microwave exposure on days 15 and 30 (0.38 ± 0.03, $P<0.05$ and 0.42 ± 0.03, $P<0.01$) in comparison to 0.31 ± 0.02 on day 1. However, on day 60, the TCSA was similar to day 1 (0.33 ± 0.01, $P>0.05$). The LCSA measurements also revealed that lesions were significantly higher in diameter on day 30 than day 15 (0.09 ± 0.01 vs. 0.05 ± 0.01, $P<0.01$, Fig. 2). However, LCSA was reported to decrease again after day 60, to reach similar values to day 15 (0.05 ± 0). Moreover, the PIL (%) of the lesions on day 30 were significantly higher than day 15 (20.6 ± 1.2% vs. 14.5 ± 0.9%, $P<0.01$). However, the PIL (%) of lesions on day 60 was significantly lower than day 30 (15.16 ± 0.5%, $P<0.001$, Fig. 2) but the difference was non-significant until day 15 ($P>0.05$). Similarly, lesions were well-defined and centrally located in the DDFT of all tendons subjected to microwave. The TCSA

of the DDFT after 15 days of microwave treatment was significantly higher than on day 1 (0.48 ± 0.02 vs. 0.41 ± 0.02, $P<0.01$, Fig. 2). On day 30, TCSA was significantly higher than on day 1 (0.53 ± 0.03, $P<0.01$) but not on day 15. However, on day 60, TCSA was significantly lower than the previous three-time points (day 1, day 15, and day 30) (0.33 ± 0.01, $P<0.001$). The LCSA measurement data revealed that after 15 days, there was a detectable lesion about 0.06 ± 0.01, $P<0.001$, in size, followed by a very slight non-significant increase on day 30 (0.08 ± 0.01, $P=0.07$). On day 60 the lesion tended to decease to reach similar values as day 15 (0.06 ± 0.01). Furthermore, the PIL (%) on day 30 was significantly higher than both day 15 ($15.7 \pm 0.7\%$ vs. $12.5 \pm 0.5\%$, $P<0.01$, Fig. 2) and day 60 ($13.1 \pm 1.0\%$, $P<0.05$). Qualitative analysis of the echogenicity at the MIZ of the SDFT revealed that the induced lesions increased gradually from grade 2 on day 15 to grade 3 on day 30. However, the echogenicity of the DDFT injuries was relatively lower and not all the lesions reached grade 3 on day 30, but some were grade 2. The fiber alignment of the SDFT was also evaluated and the score was significantly higher on day 30, between grade 2 (26–50% of standard parallel fibers) and grade 3 (0–25% of standard parallel fibers). However, the fiber alignment score was enhanced by day 60, in

which 76%–100% of the parallel fibers were regular. Similarly, the changes in the fiber alignment of the DDFT were less severe than the SDFT, where the score did not exceed grade 2 on day 30 and almost recovered by day 60, when 76%–100% of the fibers were normally parallel.
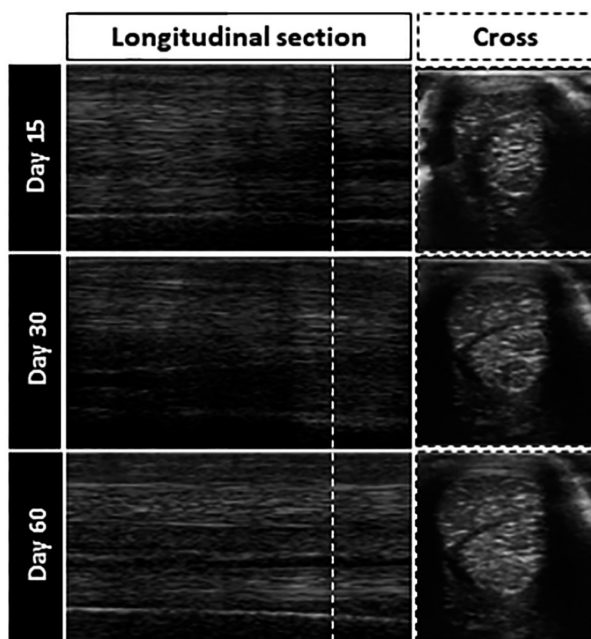

Fig. 1. Representative ultrasonograms (longitudinal and cross-section) of SDFT and DDFT on days 15, 30, and 60.
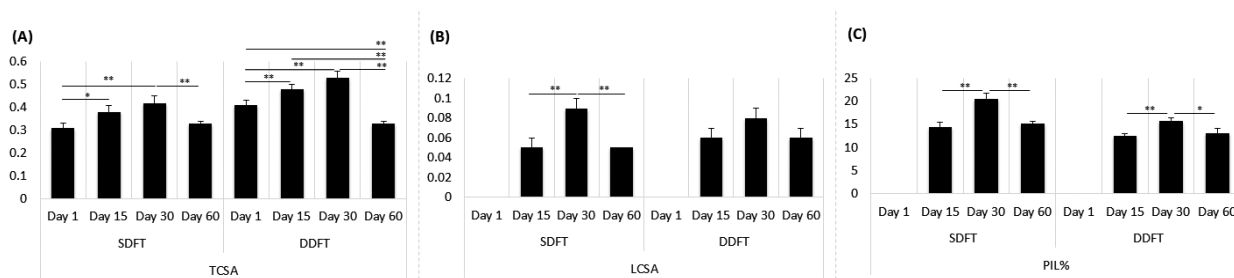

Fig. 2. Assessment of microwave effect on SDFT and DDFT using ultrasonography at four time points: on days 1, 15, 30, and 60. (A) TCSA: Tendon cross-section area, (B) LCSA: Lesion cross-section area, and (C) PIL %: Proportion of the induced lesion (%). The data are expressed as the mean ±SD (* $P<0.05$ and ** $P<0.01$).

Findings of gross morphology. After euthanizing the animals, the SDFT and DDFT tendons were carefully inspected for lesions. The lesions at the SDFT were relatively small and centrally located at the site of microwave exposure on day 15. The lesions' size was noticeably larger on day 30 with detectable hemorrhage spots. On day 60, the injuries were almost undetectable visually with less or no bleeding (Fig. 3).
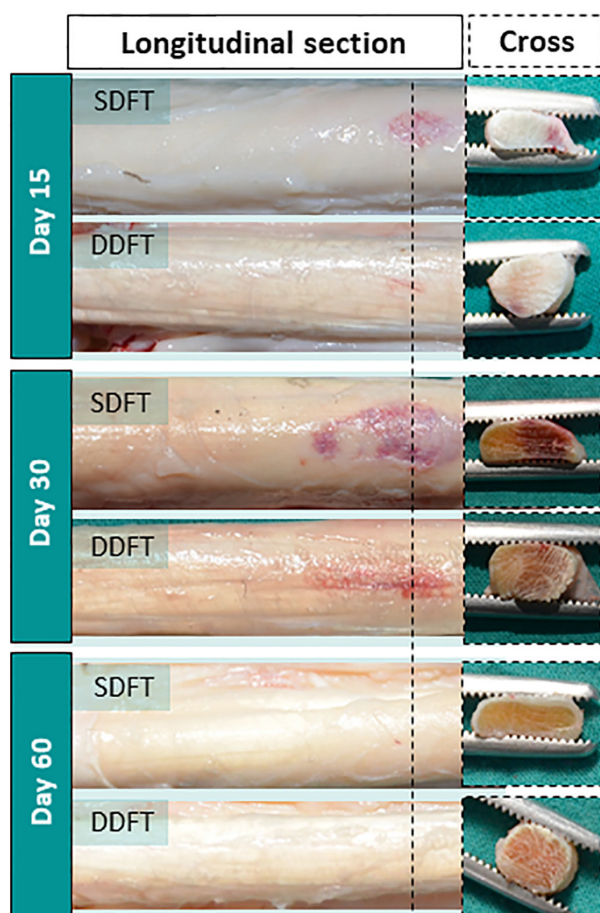

Fig. 3. Gross pathology of superficial digital flexor tendon (SDFT) and deep digital flexor tendon (DDFT) on days 15, 30, and 60.

Histomorphometric findings. Histological sections from SDFT and DDFT at the site of microwave exposure were stained by H&E, Masson trichrome, and Alcian blue (Fig. 4 A & B). Then quantitative analysis was carried out at the 3 time points on days 15, 30, and 60 (Fig. 4 C). Subjective and quantitative analyses of the SDFT on day 15 using H&E stained sections revealed that the defect site exhibited relatively rounded tenocytes with a low hemorrhage rate. Moreover, there was a moderate increase in inflammatory cells and mild matrix disorganization (Fig. 4 A). Furthermore, a slight increase in collagen fiber thickness and glycosaminoglycan (GAG; proteoglycan) content was detected by Masson Trichome and Alcian blue. Comparatively, on day 30, the number of round tenocytes and the amount of hemorrhage were significantly higher. Also, there was a significant increase in inflammatory cells with severe matrix disorganization. Moreover, the thickness of the collagen fiber and proteoglycan content was significantly greater than on days 15 and 60. Collectively, quantitative analysis revealed that the histological score on day 30 was significantly higher than the score on day 15 ($P<0.0001$, Fig. 4 C). However, on day 60, the histological staining exhibited slightly oval cell morphology and the absence of hemorrhage. Also, there was mild inflammatory cell infiltration and slight matrix disorganization (Fig. 4 A).

The histological score of the DDFT showed a similar pattern to SDFT through the three-time points on days 15, 30, and 60, but was significantly lower in severity ($P<0.01$). Interestingly, however, the histological score of both SDFT and DDFT on the end of the experiment day 60 was significantly lower than the score on day 15 ($P<0.001$), and substantially higher than the non treated Control ($P<0.001$ for SDFT and $P<0.01$ for DDFT, Fig. 4 C). Furthermore, SEM was conducted to visualize the linearity of the collagen fibers and matrix alignment. SEM images on day 15 revealed a moderate disruption of collagen fiber and filament damage in both SDFT and DDFT. More severe degree was also detected on day 30. However, on day 60, there was only mild disruption of collagen fiber and filament damage in both SDFT and DDFT (Fig. 5).
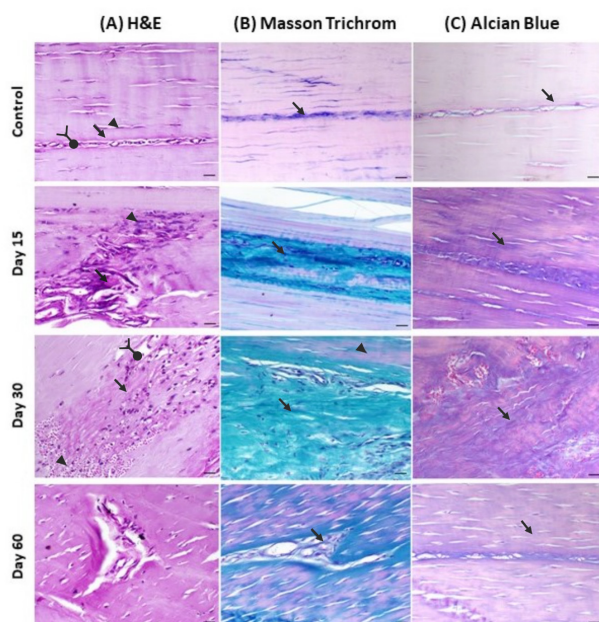
Fig. 4 A. Histological analysis of SDFT in the control limbs and on days 15, 30, and 60 after tendinitis induction. (A) H&E, (B) Masson trichrome, and (C) Alcian blue. On day 0 the histological sections revealed normal parallel collagen fibers (arrow) with peripheral tenocytes (arrowhead) and the presence of a fibrovascular layer around the collagen bundle (tailed-arrow). However, on days 15, 30, and 60, the images revealed collagen fiber degeneration (arrow) with a notable increase in the activity of the fibroblastic cells, as confirmed by Masson trichrome (arrowhead) and high glycogen content, as confirmed by Alcian staining. Scale bar: 40 µm.
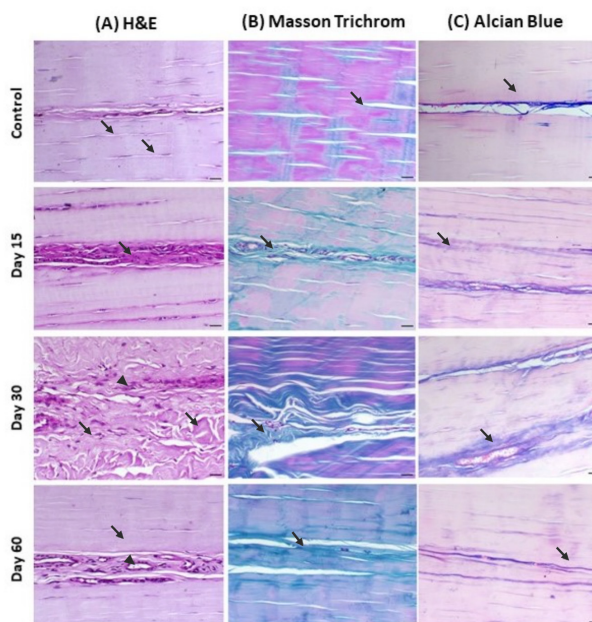
Fig. 4 B. Histological analysis of DDFT in the control limbs and after15, 30, and 60 days of tendinitis induction. (A) H&E, (B) Masson trichrome, and (C) Alcian blue. On day 0 the histological sections revealed normal parallel collagen fibers (arrow) with peripheral tenocytes (arrowhead) and the presence of a fibrovascular layer around the collagen bundle (tailed-arrow). However, on days 15, 30, and 60, the images revealed collagen fiber degeneration (arrow) with a notable increase in the activity of the fibroblastic cells, as confirmed by Masson trichrome (arrowhead) and high glycogen content, as confirmed by Alcian staining. Scale bar: 40 µm.
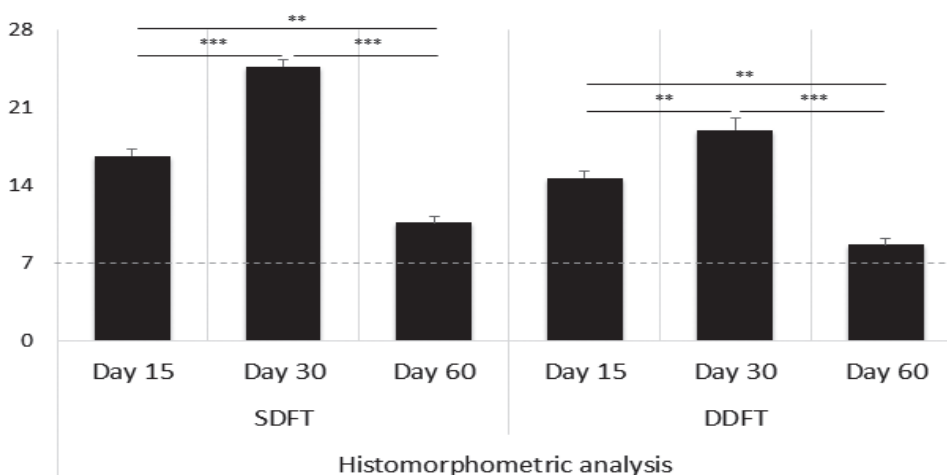

Fig. 4 C. Graph representing the quantitative histomorphometric analysis of SDFT & DDFT on days 15, 30, and 60. The dotted line indicates the control tendons. The data are expressed as the mean ±SD (** $P<0.001$ and *** $P<0.0001$).
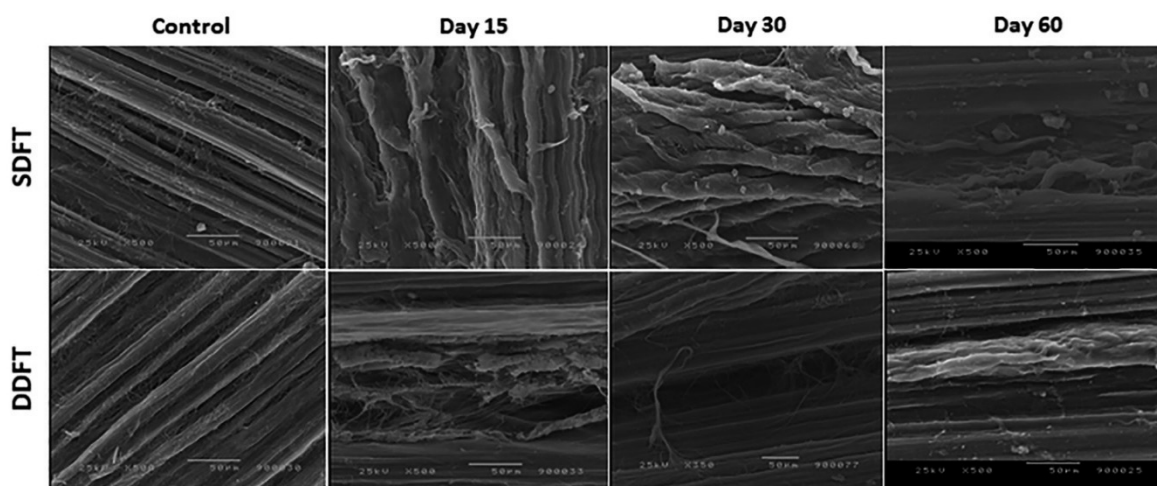
Fig. 5. Scanning electron microscopy of SDFT and DDFT collagen fibers in control limbs and 15, 30, and 60 days after tendinitis induction. Scale bar: 50 µm.

Discussion

The present study data show that microwave diathermy has the potential to induce tendinitis similar to clinical cases. This finding was evident on the macro and micro-overview of the treated tendons. On the macroscopic view, a well-defined lesion was detected by US and gross pathology, and confirmed histologically by H&E, Trichrome, and Alcian blue staining. These findings reflect the notion that tendinitis is initiated by raising the tendon's local temperature due to over-exercise.

Microwave power is known to be absorbed in the tissues by the movement within the electromagnetic field in an aqueous solution, resulting in vibrational energy, later translated as heat (GRANT, 1981). For this reason, microwave radiation was used as a therapeutic modality in which it was able to target the deep tissue, heating without causing any adverse effect on the skin (GOATS, 1990). To our knowledge, there are no previous published data that demonstrate the impact of microwave diathermy on tendon tissue for tendinopathy induction in an animal model.

In our previous cadaveric study, we demonstrated that microwave power of 30 watt for 30 min was able to raise the temperature of SDFT and DDFT to 47 and 45 °C, respectively, similar to that reported after exercise (ELSAYAD et al., 2020). However, the local temperature in the current study was expected to be less than 45 °C due to circulating blood supply and water content within the tissue. Further investigations are needed to report the ultimate core temperature of SDFT and DDFT in live animals.

Here, general clinical signs were prominent in all animals, including lameness, pain, and swelling, which was also confirmed by detection of a well-defined lesion on US imaging. All these signs showed a LOG phase lasting from day seven to day 30 of heat stimulation, followed by a LAG phase from day 30 to 60.

US examination revealed that the induced lesions were hypoechoic from the beginning of heat stimulation due to cell and fluid accumulation, similar to the findings reported by (WATTS et al., 2012; ELSAYAD et al., 2020). These lesions were reported to expand until four weeks, which resembles clinical cases (GENOVESE et al., 1990; MARR et al., 1993). Furthermore, the US examination revealed that the epitenon was intact, mimicking the tendinopathy of natural clinical cases. However, using other methods which require injections usually results in mild to moderate epitenon disruption, depending on the size of the needle used (WATTS and AJ, 2011; AHRBERG et al., 2018).

Interestingly, the lesion size induced by the heat stimulation using microwaves was comparable and

significantly prominent to the lesions produced by diode laser (PIL = 20.6 ± 1.2% to 12.2 ± 1.7%, P<0.01) (VALLANCE et al., 2012). Notably, the lesion size showed high consistency using a heat stimulation technique (PIL range was 19-22%, median 20.6%) in comparison to collagenase gel injection (PIL range was 19–43%, median 25%) or traditional collagenase injection (PIL range was 10–89%, median 45.5%) (WATTS et al., 2012).

In the present model, tendinitis induction requires a shorter period of time than the over-exercise model, which may require from 4.5-18 months (PATTERSON-KANE et al., 1997; SMITH et al., 1999). Further investigations are needed to test the additive/synergistic effect of combining mechanical overload and heat stimulation strategy to overcome the former technique's limitations.

It is well known that tendon healing passes through 3 main steps of acute inflammation, which may last up to 10 days, followed by a proliferation stage that may last from 4 to 45 days, and then the remodeling stage, which may last as long as 45 days (GOLDIN et al., 1980; SILVER et al., 1983). Histologically, we reported that the tendon lesions in our experiment passed through the natural inflammation stage in a similar manner to the steps mentioned above in clinical tendinitis cases. For instance, the specimens harvested on days 15 and 30 were in the proliferative phase. There was a marked influx of fibroblastic cells, small hemorrhagic spots, and occasional macrophage left from the initial inflammatory phase. Meanwhile, the specimens harvested on day 60 confirmed active remodeling, as the collagen fiber linearity improved and normalized. However, the collagen fiber linearity was ordinary. The collagen fiber bundles had not yet matured, and remained small by subjective assessment resembling the signs reported by previous researchers (WATKINS, 1992).

Furthermore, the histological analysis revealed a significantly high GAG deposition on the site of microwave exposure. This finding confirms the efficacy of tendinitis induction as it has been reported previously that chondrocyte-like cells (responsible for GAG production) were detected on the degenerated regions of tendons in tendinopathy patients (FU et al., 2007; PARKINSON et al., 2010).

Conclusion

The microwave diathermy technique is a non-invasive, cheap, safe, and realistic method for induction of tendinitis in the donkey model. It induces lesions similar to clinical cases.

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SAŽETAK

Uporaba životinjskih modela je neophodna za proučavanje mehanizma bolesti, patogeneze i za procjenu protokola liječenje tendinitisa. Čini se da temperatura igra značajnu ulogu pri nastajanju tendinitisa. Cilj istraživanja bio je stvaranje novog, sigurnog i jeftinog modela za proučavanje tendinitisa te potvrda njegove pouzdanosti. Učinak mikrovalne dijatermije (30 vata tijekom 30 min) na tetive fleksora u magaraca kao životinjskih modela istraživan je nakon 15, 30 i 60 dana. Procjena se temeljila na geometrijskoj analizi, ultrazvuku, histomorfometrijskoj analizi i skenirajućoj elektronskoj mikroskopiji (SEM). Mikrovalna dijatermija uspješno je inducirala dobro definirane lezije u površinskoj digitalnoj tetivi fleksora (SDFT) kao i dubokoj digitalnoj tetivi fleksora (DDFT). Rezultati su pokazali da su sve životinje pokazivale znakove hromosti, počevši od 15. dana i dostižući maksimum 30. dana. Također, 30. dana je uočeno znakovito (P<0,05) povećanje opsega ekstremiteta. Nadalje, uporabom ultrazvuka i primjenom geometrijske analize udjela inducirane lezije (PIL) u korelaciji s promjerom tetive, ustanovljeno je da je PIL bio maksimalne širine 30. dana (20,6 ± 1,2% za SFT i 15,7 ± 0,7% za DDFT). Osim toga, histomorfometrijskom analizom otkriven je povećan broj zaobljenih tenocita, krvarenje, teži poremećaji u matriksu i povećanje debljine vlakana. Do 30. dana, SEM analiza je pokazala izražene poremećaje u poravnavanju matriksa i u SDFT i u DDFT. Zaključno, primjena mikrovalne dijatermije za indukciju tendinitisa kod magaraca je pouzdan, minimalno invazivan i troškovno učinkovit životinjski model za proučavanje ove bolesti.

Ključne riječi: kopitari; tendinitis; površinska digitalna tetiva fleksora; mikrovalna dijatermija; veliki životinjski model