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ORIGINAL ARTICLE

Histological tissue healing following high-power laser treatment in a model of suspensory ligament branch injury

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Summary

Background: High-power laser therapy gained popularity recently as a regenerative treatment for tendinitis and desmitis in the horse. However, studies evaluating the effects of laser therapy on tissue repair at the histological level in large mammals are lacking.

Objectives: To evaluate the effects of high-power laser therapy on suspensory desmitis healing, using a model of suspensory ligament branch injury.

Study design: In vivo experiments.

Methods: Standardised lesions were surgically induced in all four lateral suspensory branches of 12 healthy Warmblood horses. Laser therapy (class 4, 15W) was applied daily on two of four induced lesions for four consecutive weeks. Horses were randomly assigned to either short-term study (horses were sacrificed after 4 weeks) or long-term study (6 months). Suspensory ligament samples were scored after staining with haematoxylin-eosin and immunostaining for collagen 1- collagen 3- and factor VIII.

Results: In the short-term study, significantly better (lower) scores for variation in density (17% above cut-off score in treated lesions vs. 31% above cut-off score in controls, $P = .03$), shape of nuclei (54% vs 92%, $P = .02$), fibre alignment (32% vs 75%, $P = .003$) and fibre structure (38% vs 71%, $P = .02$) were found in laser-treated lesions when compared to controls. Collagen 3 expression was significantly higher (32% vs 19%, $P = .006$) in control lesions. In both short- and long-term studies combined, parameters lesion size (44% vs 56%, $P = .02$) and shape of nuclei (53% vs 84%, $P = .05$) scored significantly better in treated lesions. Long-term, significantly better (lower) scores were found in the laser-treated group for lesion size (15% vs 45%, $P = .008$) and a higher percentage above cut-off score for density of the nuclei (27% vs 9%, $P = .02$), compared to controls.

Main limitations: The model of suspensory branch injury is not an exact representation of clinical overstrain lesions.

Conclusions: These results suggest that high-power laser therapy enables better lesion healing than conservative treatment.

KEYWORDS
collagen, fibre, histology, horse, ligament, tendon

1 | INTRODUCTION

Tendon and ligament injuries are common in both humans and horses.[1] Because of the high prevalence, prolonged rehabilitation and high re-injury rate, diverse regenerative therapeutic modalities that aim at accelerating and improving tendon and ligament healing are being explored. In this context, high-power laser therapy has been applied to treat human soft-tissue injuries for decades.[2-6] Randomised, double-blinded placebo-controlled trials on the effect of low-level laser therapy in human tendinopathy show overall positive effects in Achilles tendinitis,[7] with specific examples a significant reduction of inflammation[8] and an acceleration of the recovery process.[9] Likewise, in a single-blinded randomised clinical study in human Achilles tendinopathy, high-power laser therapy significantly reduced pain.[4]

In the past few years, high-power laser therapy is increasingly applied in horses. In a recent retrospective clinical study in 150 sports horses with tendinopathy or desmopathy, a significant improvement in lameness score and ultrasonographic lesion appearance was reported, as a response to a 2-week treatment period using multi-frequency high-power laser.[10] The long-term outcome was also promising, with a re-injury rate as low as 18% at 24 months after the initial injury. Further, the average duration of the rehabilitation period was much shorter than described in previously published studies on different treatment modalities for tendinitis and desmitis in horses.[10] Beneficial effects were also reported in a recent study in 26 sports horses with tendinopathy or desmopathy that were randomly assigned to either high-intensity laser therapy or a non-treatment group.[11] In the treatment group, analgesic and anti-inflammatory scores were better, lameness was improved and better lesion filling on ultrasound was found when compared to non-treated horses.[11] A recent case report describes the successful application of a high-power laser to treat injuries of the medial collateral ligament of the carpus in two horses.[12]

With clinical evidence increasing, standardised experimental studies into the therapeutic effect of high-power laser therapy on the tendon and ligament healing remain, however, scarce. We recently reported on an experimental study in which a high-power laser was applied to a standardised lesion in the suspensory branch in 12 horses.[13] In that study, short- (4 weeks post-lesion) and long-term (6 months) outcomes were evaluated using multiple medical imaging modalities. On ultrasound, enlargement of the lesion was significantly reduced with Doppler signal significantly increased in the laser-treated limbs. On magnetic resonance imaging (MRI), lesion size was significantly smaller, and the mean signal significantly lower in the treatment group vs control.[13]

Where imaging techniques like ultrasound and MRI are commonly used techniques to assess tendon and ligament healing in the horse[14,15] and have been related to histopathologic findings in a standardised lesion model in the equine superficial flexor tendon,[14-16] histology remains the gold standard for the assessment of therapeutic effect at the tissue level. In vivo and in vitro studies covering a wide range of research models, using mainly rodents, have reported beneficial effects of laser treatment on the histology of affected tendon or ligament tissue, including the increased proliferation of fibroblasts, stimulation of collagen synthesis, an increase of collagen fibre alignment, increased tendon tensile strength, increased angiogenesis and micro-vascularisation and reduction of COX-2 and pro-inflammatory mediator expression.[17-22] However, studies in large animals are entirely lacking. This study uses the tissues from the above-mentioned experimental study[13] to investigate the effects at the tissue level of high-power laser treatment in a model of suspensory ligament branch injury. It is hypothesised that the positive effects seen on imaging parameters will be corroborated by histological analysis.

2 | MATERIALS AND METHODS

This study uses material collected in an earlier published study.[13] Full details on animals, experimental design, surgical protocol, laser treatment protocol and exercise protocol are given in Data S1. The following sections provide a brief synopsis of these aspects.

2.1 | Horses, experimental design and follow-up

Twelve healthy adult Warmblood horses (age 4-12 years), sound on standard orthopaedic examination, and without any ultrasonographically visible changes in the flexor tendons or the suspensory ligament were randomly assigned to either the short-term evaluation group (horses were sacrificed after 4 weeks) or the long-term evaluation group (sacrifice after 6 months) by simple randomisation.

After the intervention, horses were subjected to an exercise programme that started on day 1 post-operatively and consisted of lungeing at walk and trot for the first week, followed by hand-walking on a hard surface. The long-term group was subsequently trotted on a soft surface from 3 months onwards, with a weekly increase in exercise duration.

During the entire study period, general appearance, respiratory rate, heart rate, body temperature, appetite, soundness at the walk, and local clinical signs at the level of the suspensory branches such as heat, swelling and pain were recorded daily. Horses were checked for lameness by a clinician and with a gait analysis system (Equinosis Q®) with Lameness Locator® software (Equinosis LLC) on a weekly basis in the first 4 weeks and monthly thereafter.

2.2 | Surgical procedure

A core lesion was created mechanically in all four lateral suspensory branches under general anaesthesia following a modification of the method described by Schramme et al.[15] Briefly, a 4-mm wide shaver (Torpedo®, Arthrex GmbH) was inserted through the skin and advanced proximally into the core of the lateral suspensory

branch to create a 4 cm-long columnar-shaped lesion. Surgical aftercare followed standard protocols and analgesia was secured for 5 days using 2.2 mg/kg PO q12h phenylbutazone (Butagran Equi®; Dopharma).

2.3 | Laser protocol

High-power laser therapy (Prototype, Touch Life Rehab®) was applied daily on two of the four induced lesions for four consecutive weeks, starting on day 1 after surgery. This class 4 laser (maximal output 15 W) emits laser light with four different wavelengths simultaneously: 635, 660, 810, and 980 nm. The output of the device is controlled by a feedback loop regulated by an internal encrypted algorithm that is based on the diffusion of laser light through different coloured tissues measured by the sensor in the handpiece that registers local temperature and micro-impedance at the irradiated area.[3] The approximate amount of power delivered per treated cm^3 tissue was 250 J in each treated limb. All horses were treated by the same veterinarian every day, treatment was applied over a clipped (blade size 0.5 mm) area over the full length of the lesion. The skin was scrubbed and degreased with alcohol and the laser handpiece was held perpendicular to the skin surface at a distance of 0.5 cm after which fast movements were made, in a linear pattern upon the lesion area. Treatment was randomly allocated to left and right for both front- and hindlimbs (simple randomisation) whilst securing an even distribution.

2.4 | Sample preparation

In the short-term study, all lateral suspensory branches were sampled for histopathologic evaluation. All distal limbs were removed within 30 minutes after euthanasia. Ligament sampling was performed 12 hours after limb removal, after MRI evaluation of the cadaveric limbs. A transverse sample was taken at, respectively, 2, 4 and 6 cm proximal to the distal attachment of the suspensory branch onto the sesamoid bone. A longitudinal sample was taken between 4 and 6 cm height. In the long-term group, three horses were randomly selected for histopathologic sampling. The suspensory branch tissue of the remaining three horses was stored at −20°C for future biomechanical testing. Each sample for histological evaluation was processed by prior submersion in a 4% formaldehyde solution for 48 hours, followed by submersion in buffered Phosphate-Buffered Saline (PBS) for 3 days and subsequent dehydration with an ethanol solution in increasing concentrations. Finally, samples were paraffin-embedded for two consecutive days. Samples were cut in 5-μm slices using a Microm (Thermo Fisher) microtome. All samples were stained with haematoxyline-eosine (HE), and for von Willebrand factor (factor VIII), collagen 1 (Col1, Abcam AB138492) and collagen 3 (Col 3, Novusbio-NPB1-05119) by immunostaining. Preparation for immunostaining was done as follows: After deparaffinisation and rehydration, all sections were blocked with peroxidase block using Dako Dual Endogenous Enzyme Block (Dako S2003) and 5% bovine serum albumin in PBS (Col 1 and Col 3, Sigma A3059) to prevent nonspecific binding. For Col 1, antigen retrieval was performed by incubation with 1 mg/mL pronase (11459643001; Roche) and 10 mg/mL hyaluronidase (H3506; Sigma) for 20 minutes at 37°C. For Col 3, antigen retrieval was performed by incubation with Proteinase K (DAKO S302080-2) for 15 minutes at room temperature. For COX-2, no antigen retrieval was required. Thereafter, sections were incubated with the primary antibodies for Col 1 (1:400 dilution, monoclonal rabbit) and Col 3 (1:200, monoclonal mouse). Col 1 and Col 3 were incubated for 1 hour at RT and COX2 was incubated overnight at 4°C. Subsequently, Col 3 sections were incubated with BrightVision Poly HRP anti-Mouse IgG (Immunologic VWRKDPVM110HRP) and Col 1 sections were incubated with BrightVision Poly HRP anti Rabbit (Immunologic VWRDPVR110HRP) as the second antibody for half an hour at RT. All sections were detected by Bright-DAB (Immunologic VWRKBS04-110) for 7 minutes. Nuclei were counterstained with 50% Mayer's haematoxylin. Isotype control stainings were carried out with a normal mouse (IgG1, Dako or IgG1 kappa light chain; Abcam) or normal rabbit (IgG, Dako) antibody at concentrations matching those used for the primary antibodies. For factor VIII, antigen retrieval was performed by incubation with proteinase K (Dako S3004) for 15 minutes at room temperature. Thereafter, sections were incubated with the primary antibodies for VWF (1:800 dilution, polyclonal rabbit, Dako A0082) for 30 minutes at RT. Subsequently, VWF sections were incubated with Envision+System-HRP Labelled Polymer anti-Rabbit (Dako K4003) as a second antibody for half an hour at RT. All sections were detected by DAB+ (Dako K3468) for 5 minutes. Nuclei were counterstained with Gill haematoxylin.

2.5 | Scoring

Evaluation of the histologic samples was performed by two independent blinded observers (MP and AH), one of which is a board-certified veterinary pathologist (AH), and one veterinary surgeon (MP), receiving specific training by a board-certified veterinary pathologist (AG) prior to scoring. Scores were considered separately for further statistical analysis. Longitudinal HE-stained sections were scored for degree of vascularisation (0 = sparse [normal], 1 = slightly increased, 2 = moderately increased, 3 = severely increased), shape of nuclei (0 = spindle shaped [normal], 1 = slightly oval, 2 = moderately round, 3 = predominantly round), density of nuclei (0 = sparse [normal], 1 = slightly increased, 2 = moderately increased, 3 = severely increased), regional variation of density of the nuclei (variation of density) (0 = uniform, 1 = mild regional variation, 2 = moderate regional variation, 3 = high regional variation), fibre alignment (0 = regularly ordered, 1 = mild wave, 2 = moderate wave, 3 = no pattern identified), fibre structure (0 = linear, no interruption, 1 = mild shortening, 2 = moderate shortening, 3 = short with early truncation) and % of normal fibres (regularly ordered, linear, no interruption) (0 = uniform appearance of normal

fibres [100%], 1 = less than 100%, but >50%normal, 2 = 20%-50% normal, 3 = <20%normal). The transverse sections were scored for vascularisation, density of nuclei, variation of density and lesion size (0 = no lesion, 1 = lesion ≤15%, 2 = lesion 15%-20%, 3 = lesion >20%). The presence of factor VIII, both in the lesion and in the surrounding ligament, was scored using a semi-quantitative scale (0 = sparse (normal) presence, 1 = slightly increased, 2 = moderately increased, 3 = severely increased). The stain uptake of collagen 1 and collagen 3 immunostains was quantified using a 0-3 scale. For collagen 1 and 3 stain uptake, the stain uptake was compared to a negative and positive control for each batch. (0 = no staining, 1 = mild staining, 2 = moderate staining, 3 = profound staining). The ratio between collagen 1 and collagen 3 scores were calculated by dividing the collagen 1 score by the collagen 3 score for each sample.

2.6 | Data analyses

To render the outcome variables binary, cut-off values were applied (scores >1 or >2). A cut-off value was determined for each parameter individually beforehand, based on the variation present in the samples. A score > 1 was used for the parameters shape of nuclei, density, vascularisation, fibre alignment, fibre structure, percentage of normal cells, lesion size, ratio collagen 1: collagen 3 and factor VIII. A score of >2 was used for the parameters: variation in density, collagen 1 and collagen 3. For each of the outcome variables, first, a univariable logistic regression was performed to evaluate the effect of treatment and study (SPSS® 25.0; IBM). In a second step, the effect of treatment on each of the outcome variables was evaluated by mixed-effect logistic regression taking into account the effect of the covariables study, observer, height, limb and horse, where the horse was considered as a random effect. These models were built in a stepwise forward fashion. A *P*-value ≤ .05 was considered significant.

3 | RESULTS

In all harvested samples, the lesion area could be clearly identified on all HE stains and showed disrupted fibres, irregular structure and arrangement, focal variations in density of cellularity, rounded nuclei and increased vascularisation (Figure 1). Differences in stainability for factor VIII, collagen 1 and collagen 3 are shown in Figure 2. Descriptive results, including sample size for each parameter, are available in Table S1. Parameters with the significant outcome ($P < .055$) in the mixed-effect logistic regression are listed in Table 1. The significant effects of treatment in the short-term study are summarized in Figure 3. Lower scores for the shape of nuclei ($P = .02$), fibre alignment ($P = .003$), fibre structure ($P = .02$) and variation in density of nuclei ($P = .03$) were found in the treatment group when compared to controls in the short-term study. In the short-term study, also collagen 3 expression was significantly increased in the control group ($P = .006$) when compared to the laser-treated group. For both short- and long-term study combined (Figure 4), the following monitored parameters scored significantly lower in the treatment group: the shape of the nuclei ($P = .05$) and size of the lesion ($P = .02$). In the long-term study (Figure 5), significantly higher scores were found for density of the nuclei ($P = .02$) and the lesion size was significantly smaller ($P = .008$) in the laser-treated group compared to controls. A significant effect of observer 1 vs observer 2 was found for the outcome variable collagen 3. For all other outcome variables, there was no significant difference between observers.

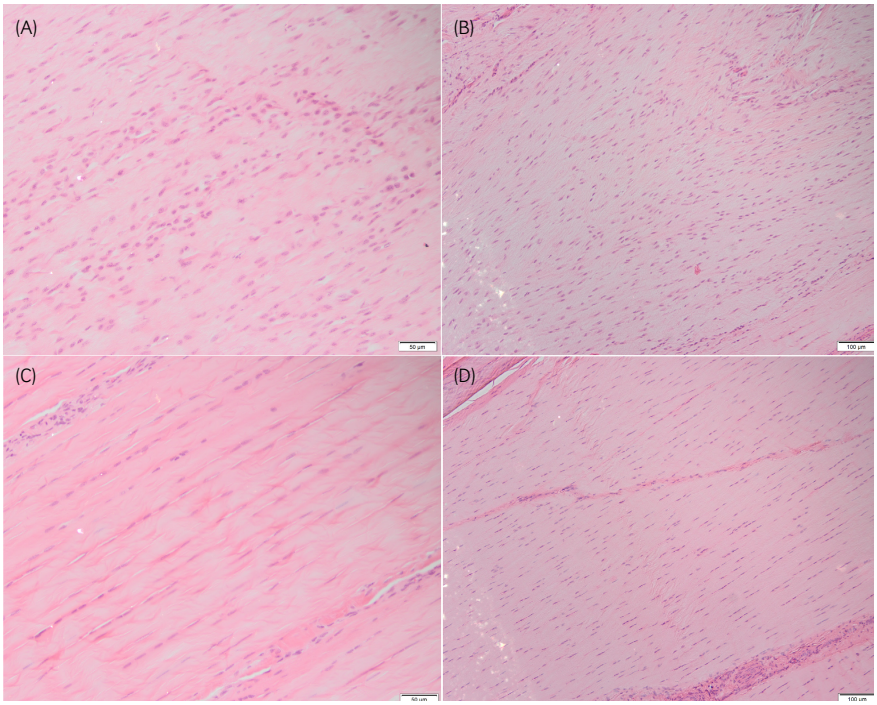

FIGURE 1 Examples of HE-stained sections: (A) control lesion from the short-term study showing clustered round nuclei (a high regional variation of density); (C) laser-treated lesion from the short-term study with regularly dispersed spindle-shaped nuclei (low regional variation of density); (B) control lesion from the long-term study featuring short and irregularly distributed fibres across the ligament; (D) laser-treated lesion from the long-term study with linearly and regularly arranged fibres

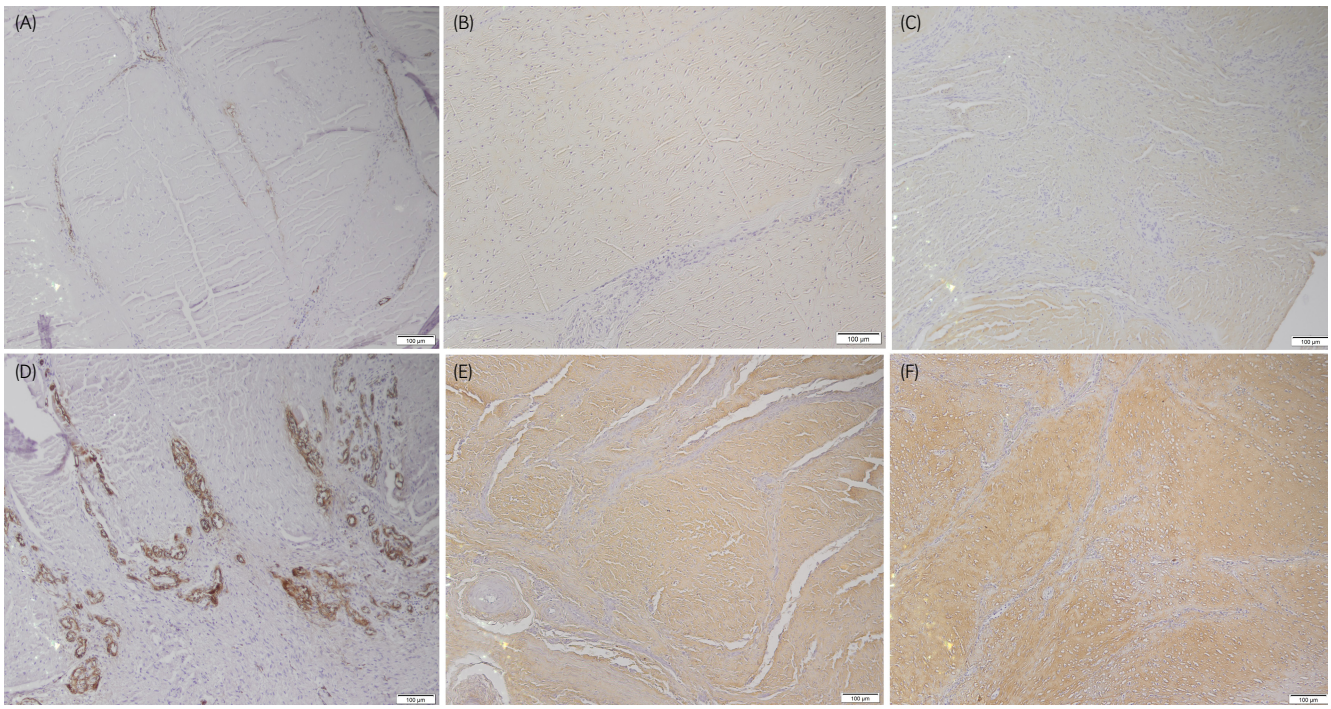

FIGURE 2 Examples of factor VIII immuno-stained sections with score 1 (A) vs score 3 (D); collagen 1 immuno-stained sections showing scores 1 (B) and 3 (E), and collagen 3 immuno-stained sections with scores 1 (C) and 3 (F)

TABLE 1 Significant outcome variables for samples of lesions treated with laser therapy vs samples of control lesions

	Number of sections analysed	*b*-coefficient	Standard error	*p*	Odds ratio	95% Confidence for odds ratio
Effect of treatment short term						
Nuclei > 1[a]	35	−2.061	0.860	0.02	0.127	0.022-0.721
Nuclei ≤ 1[a] (ref)	11					
Fibre alignment > 1[a]	25	−2.541	0.821	0.003	0.079	0.015-0.412
Fibre alignment ≤ 1 (ref)[a]	21					
Fibre structure > 1[a]	26	−2.286	0.907	0.02	0.102	0.016-0.633
Fibre structure ≤ 1 (ref)[a]	20					
Variation density > 2[c]	45	−0.885	0.392	0.03	0.413	0.190-0.895
Variation density ≤ 2 (ref)[c]	141					
Collagen 3 > 2[c]	43	−1.244	0.442	0.006	0.288	0.120-0.690
Collagen 3 ≤ 2 (ref)[c]	115					
Effect of treatment short term+long term						
Size > 1[b]	100	−0.984	0.420	0.02	0.374	0.163-0.856
Size ≤ 1[b]	98					
Nuclei > 1[a]	45	−1.093	0.558	0.05	0.335	0.110-1.022
Nuclei ≤ 1[a] (ref)	25					
Effect of treatment long term						
Size > 1[b]	19	−2.021	0.733	0.008	0.132	0.031-0.574
Size ≤ 1[b] (ref)	45					
Density > 1[c]	16	1.666	0.727	0.02	5.293	1.247-22.469
Density ≤ 1[c]	71					

Note: Data were analysed using a mixed-effect logistic regression with significance accepted at $P < .05$.

[a]Parameters scored on only longitudinal samples.

[b]Parameters scored on only transverse samples.

[c]Parameters scored on all samples are marked.

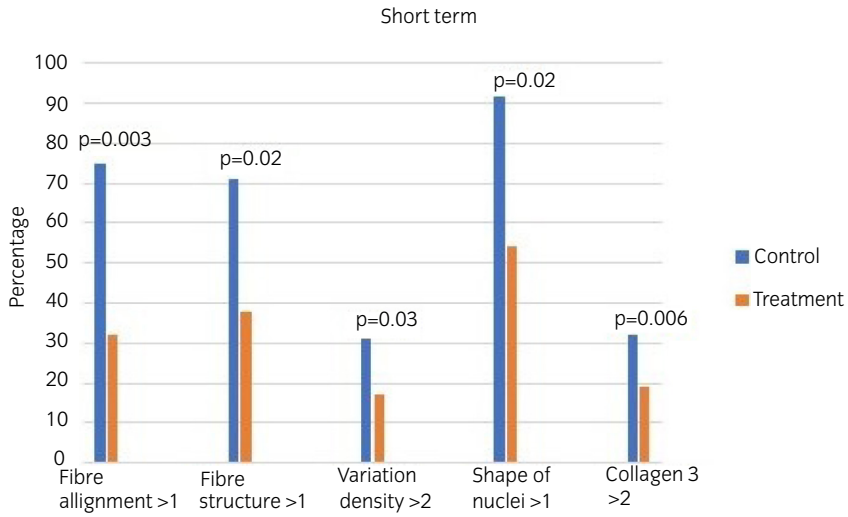

FIGURE 3 Percentage of scores above cut-off values for each outcome variable. Control (blue) vs treatment (orange) in the short-term study. Significantly lower (better) scores for fibre alignment, fibre structure, variation in density, the shape of nuclei and collagen 3 were found in the treatment group

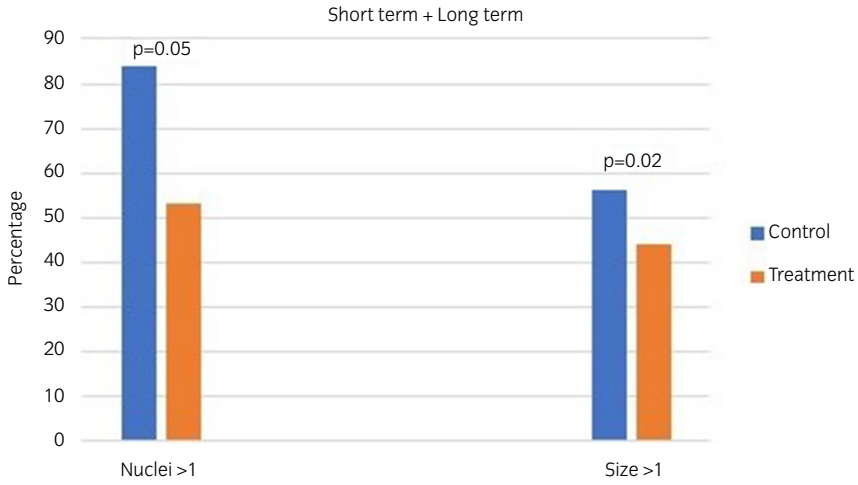

FIGURE 4 Percentage of scores above cut-off values for each outcome variable. Control (blue) vs treatment (orange) in the short-term study and long-term study combined. Significantly lower (better) scores for the shape of nuclei and lesion size were found in the treatment group

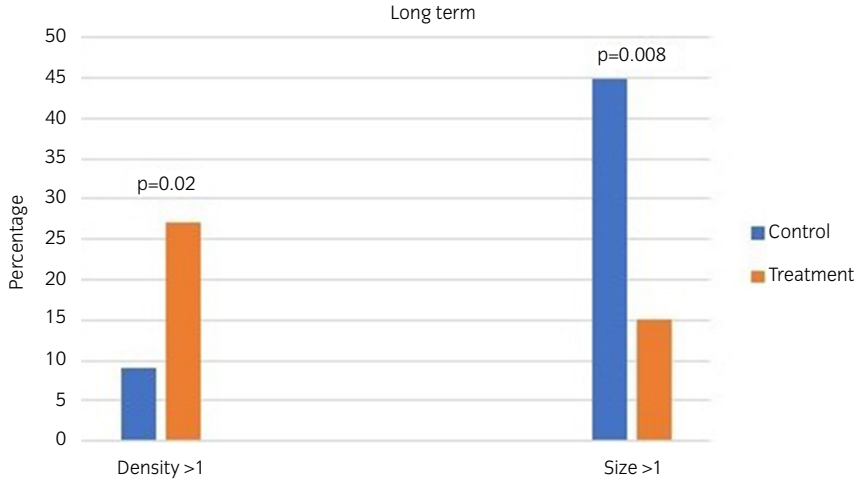

FIGURE 5 Percentage of scores above cut-off values for each outcome variable. Control (blue) vs treatment (orange) in the long-term study. Significantly lower (better) scores for lesion size and significantly higher (better) scores for density were found in the treatment group

4 | DISCUSSION

Significantly better scores for variation in density of nuclei, the shape of the nuclei, fibre alignment and fibre structure were found in the laser-treated lesions compared to controls in the short-term study. In both short- and long-term studies combined, the parameters size of the lesion and shape of nuclei scored significantly better in the treatment group. In the long-term study, significantly better scores were found in the laser-treated group for the lesion size and density of the nuclei compared to controls. In a previous study, a high-power laser significantly improved healing of suspensory branch ligament lesions shown by follow-up using diagnostic imaging.[13] It was hypothesised

that the positive effects of high-power laser therapy seen with imaging parameters would be corroborated by histological analysis and scoring. Results of the current study show significant positive effects on lesion healing at the histological level.

Diagnostic imaging modalities obviously do not provide a detailed view of tendon- and ligament healing at the tissue level. Combined histopathologic staining allows for characterisation of the lesions by assessing cell morphology, fibre alignment, collagen type distribution and neovascularisation, all of which provide information about the degree and quality of healing.

A normal HE-stained tendon or ligament typically shows spindle-shaped nuclei, which are sparse and regularly divided throughout the tendon or ligament. Vascularisation is sparse, and fibres are aligned in a linear and ordered pattern. In humans, Achilles tendinopathy is characterised at the microscopical level by abnormal fibre structure and arrangement, focal variations in cellularity, presence of rounded nuclei, decreased collagen stainability and increased vascularisation.[23] Similar changes are reported in equine tendinitis studies.[24-26] To grade tendinopathy and healing, Movin et al[27] developed a semi-quantitative scale (0-3) to score human Achilles tendinopathy on paraffin-embedded tendon tissue, stained with HE.

In the current study, regional variation in the density of the nuclei was lower in the short-term treatment group ($P = .03$), which indicates a more regular division of nuclei across the injured ligament. In a properly healing ligament, tenocytes and collagen fibres become aligned in the direction of stress.[28]

Both fibre alignment and fibre structure scores were lower (better) in the laser treatment group in the short-term study. In various studies on tendinopathy in the horse, an improvement of fibre structure and alignment is used as an indicator for better tendon healing.[24,29] In a standardised study using a collagenase-induced lesion model in the SDFT in the horse, a significant better tendon organisation was found after treatment with adipose-derived nucleated cell fractions.[29] Multiple studies have described better fibre structure and alignment after laser therapy of tendons in mice,[30] rabbits[31] and rats.[18]

Stainability of collagen 3 in the suspensory branch samples was higher in the control group vs the treatment group ($P = .007$). Williams et al[32] showed that the scar tissue collagen in the equine tendon was more readily extractable and contained a different pattern of collagen types.

Lesion area on transverse HE-stained samples was significantly smaller in the treatment group at both long-term ($P = .0.008$) and short- and long-term combined ($P = .02$), correlating well with a reduced lesion size seen on ultrasound and MRI.[13] In a clinical study with ultrasonographic evaluation after high-power laser therapy, a significant reduction in lesion size was also observed.[10,11]

At the cellular and matrix level of the healing ligament tissue, the shape of the nuclei was significantly more often scored above 1 (2, moderately round or 3, predominantly round) in the control group compared to the treatment group. Rounded nuclei are typically present in immature or activated tenocytes seen in tendinitis.[27,33] The shape of the nuclei was, therefore, significantly better (less round, more mature) in the laser-treated group, both short-term and the short- and long-term groups combined.

The density of the nuclei was higher in the laser-treated lesions and this difference was significant in the long-term study ($P = .02$). Increased cell proliferation is seen after radiation with laser in vitro[34] as well as in tendon lesions of rats.[35] Cells are sparse in a normal tendon or ligament, but in the proliferative healing stage, a tendon or ligament is characterised by increased cellularity.[28] The therapeutic effect of laser on cell proliferation can be explained by the absorption of red or near-red radiation by cytochrome-c-oxidase (Cco) and the stimulation of the production of adenosine triphosphate by the mitochondria, which results in higher cell proliferation.[36] Higher density of tendon cells, besides higher collagen and glycosaminoglycans content, was also seen after a single injection with PRP in a standardised lesion in the superficial digital flexor tendon (SDFT) of the horse and correlated with better organisation of the collagen network and a greater tensile strength.[24]

There was also a clear difference seen between the treatment and control groups in the extracellular matrix. A normal equine suspensory branch is mainly composed out of collagen 1 fibres and a much smaller amount of collagen 3 fibres. In naturally occurring equine flexor tendon injuries, the concentration of collagen is reduced when compared to healthy tendon tissue due to the effect of inflammatory mediators.[32]

A clinically ruptured equine SDFT has substantial quantities (20%-30%) of type 3 collagen in addition to type 1.[32] In an experimental study using a collagenase-induced tendinitis model in the equine superficial flexor tendon, untreated lesions showed low collagen 1 expression and high collagen 3 expression.[37] Treatment of these experimental lesions with either autologous-cultured bone marrow mesenchymal stem cells or bone marrow mononucleated cells resulted in a higher collagen 1:3 ratio compared to controls.[37] Whilst in our study lesions were significantly larger in the control group ($P = .02$), stainability of inferior collagen 3 is likely to be increased in the tissue samples of the control group, whilst the lesions are larger in this group. Although there were significant differences between observer 1 and observer 2 in this study in the scoring of the collagen 3 immunostaining samples, still a statistically significant difference between the treatment and control group was found, after correcting for the observer effect. A possible explanation might be the more subjective scoring due to mild contrast in positive and negative uptake in these stains (Figure 2) and using a semi-quantitative scale instead of real quantitative parameters.

This model of suspensory ligament branch injury is not entirely comparable with the typical overstrain injuries encountered in desmopathy. The main difference is that naturally occurring desmitis has an inflammatory component, whereas this model of suspensory ligament branch injury initially resembles a traumatic insult. Therefore, horses were subjected to 1 week of trotting exercise post-surgery, at which point mild clinical signs of local inflammation and mild lameness were seen. However, a mechanically induced tendon- or ligament lesion model is a more controlled approach to study tendon and ligament healing compared to an enzymatic

model using substances like collagenase because the mechanically induced lesions are more standardised.[13] In this lesion model, all four limbs of each horse were lesioned to reduce the number of experimental animals needed. Therefore, an evaluation of lameness was difficult.

In a retrospective clinical study on the effect of high-power laser therapy on tendon and ligament lesions, follow-up after 6, 12, and 24 months was used for long-term outcome. In that study, rehabilitation protocols used enabled the horses to reach their previous performance level before 6 months.[10] In this study, a similar standardised rehabilitation protocol was used[13] and horses were returned to full exercise including free turnout after 5 months. However, more lengthy rehabilitation protocols are described for naturally healing suspensory lesions.[38] Therefore, a 6-month follow-up for long-term evaluation of the scar tissue might have been insufficient for the natural healing process in the control lesions. The age of the horses was not taken into account as a variable in this study, as only horses in the "adult age range" were selected. The mean- and average age of the studies horses was 7, which matches with the age range reported in the previously published clinical study on high-power laser therapy.[10]

This is the first standardised study on high-power laser therapy in the horse evaluating ligament healing at the histological level. Significant benefits of high-power laser therapy were found, and it is expected that these positive effects are seen in naturally occurring lesions too.

5 | CONCLUSION

In this standardised study focusing on the effect of high-power laser therapy on healing of induced lesions in the suspensory branch of the horse, a significantly better outcome for lesion size, density, variation in density, the shape of the nuclei, fibre structure and fibre alignment was found in the laser-treated lesions. This suggests that high-power laser therapy improves healing in a model of suspensory ligament branch injury.

CONFLICT OF INTERESTS

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

Authors Mathilde Pluim, Berit Boshuizen, Andrea Gröne, Katrien Vanderperren, Ann Martens, Marc Koene, Antonio Luciani and Cathérine Delesalle contributed to the study design. Mathilde Pluim, Annabelle Heier, Saskia Plomp, Berit Boshuizen, Katrien Vanderperren, Ann Martens, Maarten Oosterlinck, Leen Van Brantegem and Cathérine Delesalle to study execution. Mathilde Pluim, Annabelle Heier, Andrea Gröne, René van Weerren, Jeroen Dewulf, Ilias Chantziaras and Cathérine Delesalle to data analysis and interpretation. All authors contributed to the preparation of the manuscript and final approval of the manuscript. Mathilde Pluim and Cathérine Delesalle have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

INFORMED CONSENT

Not applicable.

ETHICAL ANIMAL RESEARCH

This animal study was revised and approved by the Ethical Commission of Ghent University (LA1400077).

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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