GENE THERAPY USING PLASMID DNA ENCODING BONE MORPHOGENETIC PROTEIN 2 AND VASCULAR ENDOTHELIAL GROWTH FACTOR 164 GENES FOR THE TREATMENT OF EQUINE PROXIMAL SUSPENSORY DESMITIS: CASE REPORTS

M. Kovac[1], *I.V. Berestov*[1], *A.M. Aimaletdinov*[2], *C.S. Rutland*[3], *A.A. Rizvanov*[2], *E.Yu. Zakirova*[2*]

[1] Equine clinic – New Century, Moscow State Academy of Veterinary Medicine and Biotechnology, 23 Akademika Skryabina St., Moscow, 109472, Russia;
[2] Institute of Fundamental Medicine and Biology, Kazan Federal University, 18 Kremlevskaya St., Kazan, 420008, Russia;
[3] Faculty of Medicine and Health Sciences, School of Veterinary Medicine and Science, University of Nottingham, Nottingham, LE12 5RD, UK.

* Corresponding author: lenahamzina@yandex.ru

Abstract. Injury to the proximal part of the equine suspensory ligament (SL), called proximal suspensory desmitis (PSD), commonly causes lameness in horses. PSD is extremely difficult to manage and treat, with present methods often unable to achieve full recovery, especially in chronic cases. The present study was the first to use gene therapy to restore moderate and severe injuries of the proximal suspensory ligament in horses. Plasmid DNA encoding species specific bone morphogenetic protein 2 (*bmp2*) and vascular endothelial growth factor (*vegf164*) was injected into the site of proximal suspensory ligament injury, followed by box rest and a controlled exercise program. Clinical observations and ultrasound imaging were used to evaluate effectiveness over a period of 12 months. No negative side effects were observed. Clinical improvements were observed, especially in the forelimb affected horses, by day 30. In horses with chronic hindlimb PSD, few clinical improvements were reported. Echogenicity and the fiber alignment scoring improved but no concomitant changes to cross section area, dorsopalmar thickness or lateromedial width of the proximal suspensory ligament were observed. The transfer of *bmp2* and *vegf164* genes into the equine PSL exhibited beneficial effects in horses with acute or subacute forms of lesions, primarily in the forelimb.

Keywords: proximal suspensory desmitis, plasmid DNA, bone morphogenetic protein 2, vascular endothelial growth factor.

List of Abbreviations

AAEP – American Association of Equine Practitioners'
BMP2 – bone morphogenetic protein 2
CSA – cross section area
DNA – deoxyribonucleic acid
DP – dorsoplantar thickness
GAGs – glycosaminoglycan's
LM – lateromedial width
MSCs – mesenchymal stem cells
pDNA – plasmid DNA
PSD – proximal suspensory desmitis
PSL – proximal part ligament
SL – suspensory ligament
TGF-β – transforming growth factor-β
VEGF164 – vascular endothelial growth factor 164

Introduction

The suspensory ligament (SL), also called the third interosseous muscle in the horse is a strong fibrous connective structure that can be divided into three separate regions: the proximal part (PSL), the body, and the medial and lateral suspensory branches (Dyce *et al.*, 2002). The anatomy of the SL in the forelimbs and hindlimbs is similar, but substantial differences exist between them. In the forelimb, the PSL originates from the palmar carpal ligament and outpouchings of the carpometacarpal joint. The forelimb PSL has two distinct medial and lateral lobes, which rapidly fuse. In the hindlimb, the PSL is confined rigidly by the plantar aspect of the third metatarsal bone. In comparison to the forelimb, the medial and lateral lobes of the PSL in hindlimbs are less distinct and are rigid as they are covered with the deep laminar plantar metatarsal fascia of the retinaculum flexorum (Denoix, 1994). From a histological point of view, the SL is a structure that is neither a tendon nor a ligament, but a more complex ligamentous structure that in an evolutionary

sense originates from muscle (Van de Lest, 2010). The SL is composed of predominantly tendinous (collagenous) tissue, with infrequent fibroblasts embedded in the collagen matrix peripherally, surrounding two central muscle bundles and a small amount of adipose tissue (Van de Lest, 2010). The amount of muscle tissue within the body of the SL changes in response to training and varies between breeds, for example, Standardbred horses have a higher proportion of muscle tissue than Thoroughbreds (Souza al., 1991). In the SL of the hindlimb there is more muscle content than observed in the forelimb (Soffler & Hermanson, 2006). This was determined as being approximately 95% type I muscle fibers, which contract slowly and have a high oxidative potential, and less than 5% type II muscle fibers which consume a lot of energy and tire more quickly (Soffler & Hermanson, 2006). Despite this knowledge, surprisingly little is known about the biochemical composition and histomorphological structure of the proximal part of the equine SL, notwithstanding its clinical importance. It serves as connective tissue between the body of the SL and periosteum of the metacarpus/metatarsal bones and can be designated as fibrous enthesis (Apostolakos *et al.*, 2014). Fibrous enthesis is an interfacial zone of attachment with a specific fibrous connective structure, where the type I collagen (the prevailing collagen in the body of the tendons or ligaments) partially disappears and is replaced by type II collagen, via perforating mineralized collagen fibers. During enthesis of the equine PSL, the content of glycosaminoglycan's (GAGs) and collagen, as well as the cellularity content, is lower than in the body and branches (Van de Lest, 2010). The lower cellularity in the PSL may result in reduced metabolic activity in the area, and the lower GAG content indicates that this part of the SL is predominantly exposed to tensional, uni-axial forces (Roberts *et al*., 2019). It is assumed that permanent transmission of loads from the SL to the bone is essential for enthesis development and healing. The development and function of human enthesis requires the influence of various growth factors, primarily vascular endothelial growth factor (VEGF) and members of the transforming growth factor-β (TGF-β) superfamily (Apostolakos *et al.*, 2014; Roberts al., 2019; Robbins al., 1997). This not only includes a family of regulatory proteins, such as the TGF-β subfamily, but also includes bone morphogenetic proteins (e.g., BMP2 and BMP4). Based on the available literature, biochemical studies such as these have not been conducted on the equine PSL.

The function of the SL is to provide support for the fetlock joint during the stance phase of the stride (preventing hyperextension), to limit palmar flexion and to store elastic energy during locomotion (Dimery *et al.*, 2009). Poor stretch capability and maximal energy storage of the SL implies maximal deformation, which contributes to the high prevalence of injury. Based on several retrospective studies, injury of the proximal region of the suspensory ligament (proximal suspensory desmitis, PSD) is one of the most common causes of chronic lameness, especially in competing dressage horses (Kovac *et al.*, 2002; Murray *et al.*, 2010; Kovac, 2017; Gruyaert *et al.*, 2020). The main reason for this is due specifically to the load on the limbs that these sport horse's encounter. The static and dynamic conformations also play important roles in the incidence of the equine PSD. There is a strong correlation between straight hock conformation, hyperextension of the metatarsophalangeal joint, and incidence of PSD in hindlimbs (Dyson *et al.*, 2017).

Although injuries to the branches and the body of the equine SL can normally be treated successfully, completely different possibilities exist for the successful treatment of PSD (Dyson *et al.*, 2003). Many treatment methods have been used for the management of acute and chronic PSD, including conservative, regenerative, physical and chirurgical methods (Kovac, 2017). Current options, include box rest, controlled exercise, correction of foot imbalance and using an egg-bar shoe to reduce extension of the fetlock (Dyson *et al.*, 1995), administration of corticosteroids (Dyson *et al.*, 2003), hyaluronate (Dyson *et al.*, 1995), use of mixture according to Dr. Müller-Wohlfahrt (Dyson *et al.*, 2000), bone marrow aspirate (Siedler *et al.*, 2003), platelet rich plasma (Maleas *et al.*, 2021), growth factors and mesenchymal stem cells (Vandenberghe *et al.*, 2015), and appliance

of the extracorporeal shock wave therapy or radial pressure waves therapy (Schramme M.C. & Smith *et al.*, 2004; Lischer *et al.*, 2006). Treatments also include surgical methods, for example the osteostixis of the palmar/plantar aspect of the proximal third metacarpal/metatarsal bones (Launois *et al.*, 2003; Crowe *et al.*, 2004), fasciotomy with neurectomy of the deep branch of the lateral plantar nerve (for hindlimbs) or excision of the deep branch of the lateral palmar nerve for forelimbs (Dyson *et al.*, 2012; Guasco *et al.*, 2013). Unfortunately, these methods are more often unsuccessful in achieving full recovery (especially in chronic cases) and thus there is no gold standard for treating equine PSD.

The aim of this novel equine medicine pilot study was to provide clinical proof that a single intralesional injection of plasmid DNA, containing species-specific *bmp2* and *vegf164* cDNA genes, resulted in clinical and ultrasonic detectable effects in the treatment of subacute and chronic damage to the proximal region of the suspensory ligament.

Materials and Methods

Study Design

Five horses with naturally occurring PSD lesions were enrolled onto the study from 2017 to 2020 at the Equine Clinic – New Century of the Moscow State Academy of Veterinary Medicine and Biotechnology, Moscow. Three horses had forelimb PSD, with the remaining two horses presenting with hindlimb PSD. The horses engaged were required to meet the following criteria prior to acceptance: (1) subacute and chronic form of PSD in adult horses, which was confirmed with diagnostic anesthesia and ultrasonographic abnormalities of the PSL; (2) normal radiographic appearance of the proximal third metatarsal bone and the tarsometatarsal and distal intertarsal joints, and negative intra-articular anesthesia of tarsometatarsal joint; (3) normal radiographic appearance of the carpometacarpal joint and absence of other orthopedic diseases; (4) no previous topical medication applied to the PSL.

The owner reported duration from horse injury until treatment in the equine clinic ranged from 15-110 days (average 52.4 ± 31.57; Table 1). Mean duration of PSD lameness in the forelimb was 36.6 days and for hindlimb it was 76 days. In relation to the breeds of the horses investigated, two were Trakehner, two were Andalusian, and one was a Russian Saddle Horse. The study included four geldings and a stallion. The average age of the horses was 11.2 years (± 1.92), ranging from 9-14 years. The horses involved were mainly used for dressage (four cases), but one was used as a pleasure/riding horse (Table 1). On the basis of anamnesis data, before using gene therapy horses 1 and 3 were treated unsuccessfully (by independent veterinarians under standard veterinary care), with stall rest combined with a controlled rehabilitation program and administration of the non-steroidal antiphlogistics. Additionally, application of extracorporeal shock wave therapy was administered to treat three of the horses (1, 4 and 5) which resulted in temporary improvement followed by a relapse in lameness.

Each horse with PSD was considered as a separate case. Prior to treatment, all horses underwent clinical examinations (lameness grade), and the signs of inflammation in the palmar metacarpal/plantar metatarsal region (skin surface temperature, swelling, and painful sensitivity to palpation) were also monitored. Lameness diagnosis was performed and scored from 0 to 5 using the American Association of Equine Practitioners' (AAEP) scale, where 0 = no lameness, 1 = mild lameness, not consistently apparent at a trot, 2 = obvious lameness, consistently apparent at a trot, 3 = marked lameness, 4 = severe lameness, and 5 = non-weight bearing lameness. Lameness was evaluated with horses walking and trotting in hand in a straight line, and by lunging on hard and soft surfaces. Subsequently, distal and proximal flexion tests were performed. Where PSD presented in the hindlimbs, the Churchill hock test was also conducted. All the observations and determinations were made by the same veterinarian (M.K.) and were always made on the same ground surface.

The standard technique of diagnostic anesthesia was used to show the location of pain and to confirm the diagnosis of PSD (Bassage &

Table 1

Description, clinical history and diagnostic data of the horses prior to treatment with plasmid DNA encoding *bmp2* and *vegf164* genes

Horse no.	Gender	Breed	Age (years)	Discipline	Reported duration of injury until treatment (days)	Veterinary diagnosis	Lameness grade before treatment (AAEP scale 0-5)
1	Gelding	Andalusian	14	Dressage	40	PSD of left forelimb	2
2	Gelding	Trakehner	10	Dressage	15	PSD of right forelimb	3
3	Stalion	Russian Saddle Horse	9	Dressage	55	PSD of left forelimb	1
4	Gelding	Trakehner	11	Pleasure/ riding horse	110	PSD of left hindlimb	2
5	Gelding	Andalusian	12	Dressage	42	PSD of right hindlimb	2

Ross, 2003). Where included, it was either via infiltration of the origin of the suspensory ligament or perineural injection with mepivacaine hydrochloride 2%, over the palmar/plantar metacarpal or metatarsal nerves (high four-point block). Horses included in the study, required a minimum of a one grade lameness score improvement following local anesthesia.

Ultrasonographic Examination

The palmar/plantar regions of the proximal metacarpus/metatarsus were evaluated ultrasonographically using a Logiq 5 (GE Healthcare, United States), with a 7.5-MHz linear-array transducer with standard settings for flexor tendons. Prior to ultrasonographic examination all limbs were clipped on the palmar/plantar aspect from the distal carpus/tarsus to the mid cannon bone. The clipped area was scrubbed for 1 min using soap, subsequently cleaned with alcohol and covered with ultrasound gel (Sonosid). In the forelimbs, ultrasonographic examination was performed from the palmaromedial aspect of the carpus and the palmar aspect of the distal carpal and proximal metacarpal regions. In the hindlimbs, examinations of the distal tarsal and proximal metatarsal regions were performed from the plantar and plantaromedial aspects. In order to exclude the presence of other lesions, the flexor tendons in both of the forelimbs and hindlimbs also underwent ultrasonographic scanning, inclusive of the SL branches.

In this study the horses were analyzed for fiber alignment and areas of echolucency in the PSL (Dyson & Genovese, 2003). A grading system was used in accordance with Crowe *et al.* to categorize the grade (mild, moderate, severe) of each lesion in the proximal suspensory ligament (Crowe *et al.*, 2004). Where noticeable, a distinct dorsal border and a poor longitudinal fiber pattern was categorized as a mild grade. In the applicable horses a focal hypoechogenic region and loss of longitudinal fiber pattern was categorized as a moderate grade. The severe grade was applied to horses where the proximal part was enlarged with markedly hypoechogenic pattern and/or severe enthesiophyte formation accompanied with an absence of longitudinal fiber pattern. Transverse and longitudinal scans of full weight bearing fore- and hindlimbs were used to undertake histomorphometric measurements of the PSL: cross section area (CSA), dorsoplantar thickness (DP) and lateromedial width (LM). The outlining border of the PSL was used to measure the CSA. The dorsoplantar thickness was measured at the most distal end where the fibres attached to the proximal third metatarsal/meta-

carpus bones. The lateromedial width was measured at its greatest possible distance.

Digital Radiographs

Digital radiographs were also performed both before and 12 months after treatment with lateromedial and dorsopalmar/dorsoplantar projections of the proximal metacarpal or metatarsal regions captured. The radiographs were evaluated to detect any changes in the metacarpal/metatarsal bones at the origin of the suspensory ligament and in order to exclude the presence of other pathological conditions, such as osteoarthritis of the tarsometatarsal/ carpometacarpal joints.

Generation of Plasmid Construct

In this study, a plasmid DNA (pDNA) construct was made containing the horse species-specific codon optimized sequences encoding *bmp2* and *vegf164* as described previously (Litvin *et al.*, 2016). Briefly, starting coding sequences of horse *vegf164* (NCBI Reference Sequence: NM_001081821.1) and *bmp2* (NCBI Reference Sequence: XM_001493895) were codon optimized using algorithm "Optimum Gene" (GenScript, USA), synthesized de novo, and cloned into pUC57 (GenScript, USA). From pUC57 the codon optimized coding sequence was then cut and cloned into pBudCE4.1 (Invitrogen, USA; pBudCE4.1 Mammalian expression vector. Thermo-Fisher Scientific. https://tools.thermofisher.com/content/sfs/vectors/pbudce4_1_map.pdf.). The pDNA construct was a high copy number plasmid with two independent expression cassettes under the control of two different eukaryotic promoters: CMV and EF-1α.

Functional Activity of Plasmid Construct in vitro

For transfection of the HEK293FT cells and equine mesenchymal stem cells (MSCs) with pBud-ecVEGF-ecBMP2 the transfection agent "TurboFect" (ThermoFisher Scientific) was used according to the manufacturer's protocol. Efficiency of transfection was determined in HEK293FT cells using SDS-PAGE western blotting of cell lysates 48 h after transfection and performed using rabbit antibodies against BMP-2/4 (sc-9003, Santa Cruz, USA), and VEGF (sc-152, Santa Cruz, USA) at a dilution of 1:250, and secondary rabbit IgG antibodies conjugated with horseradish peroxidase at a dilution of 1:2000. Visualization of the immune precipitate was carried out using chemiluminescent substrate Clarity Western ECL Substrate (Bio-Rad, USA). The results were visualized using a BioRad ChemiDOC.

The osteogenic activity of the pDNA was evaluated by transfection of equine MSCs. The equine MSCs were obtained from a fat tissue horse biopsy as described previously (Zakirova *et al.*, 2021). Immunophenotyping of the equine MSCs was performed using antibodies against Thy-1 (Biolegend, clone 5E10, USA), CD44 (Biolegend, clone IM7, USA), and CD73 (Biolegend, clone AD2, USA) in accordance with the manufacturers' protocols. The immunophenotyping results were evaluated using a flow cytofluorimeter Guava EasyCyte 8HT (Millipore, USA). After that genetically modified and native MSCs were cultured in 6-well plates until they reached 80% confluence in a growth medium which consisted of αMEM (PanEco, RF), 10% fetal bovine serum (PanEco, RF), 7 mM of L-glutamine in (Sigma-Aldrich, USA) and penicillin-streptomycin (PanEco, RF). The plates containing genetically modified cells (n = 5) and some of the native MSCs (n = 5) were further cultivated in growth medium. Other native MSCs (n = 5) were cultivated in osteo-induction medium (Gibco, USA). All three groups received fresh media every 3 days for 21 days. Osteogenic differentiation in vitro was detected using Alizarin red S (Sigma-Aldrich. USA) staining of samples as described previously (33). Alizarin red S was used to evaluate cell calcium deposits in culture. The dye was extracted from the samples using 10% acetic acid for 15 min on shaker. An optical density of each of the solutions was detected using an Infinite M200 pro (TECAN, Austria) at 405 nm.

Preparation of the Drug and Equine Administration

The plasmid DNA pBud-ecVEGF-ecBMP2 was dissolved in 5 ml of a sterile 0.9% NaCl

solution to a final concentration of 1 mg/ml. This solution was stored in a fridge at +4°C overnight, with occasional gentle stirring and rotation. Immediately prior to administration into the PSL, the pDNA solution was warmed to +37°C. Before treatment, the horses were sedated with detomidine (0.01–0.03 mg/kg intravenously). Approximately, 3.5 ml total volume of the pDNA solution was drawn into a syringe and under aseptic conditions and sonographic guidance the solution, whilst the limb was elevated, was slowly injected using a 22-gauge needle into the PSL site of injury. The needle was inserted lateral to abxial margin of the SDF tendon and axial to fourth metacarpal/metatarsal bones. The full volume was injected into the most hypoechoic areas of tissue damage and into the adjacent normal tissues. pDNA was administered on only one occasion per animal. Following the injections, a bandage was applied, and a 2-day box confinement was instructed.

Horse Rehabilitation and Evaluation Following Plasmid DNA Gene Therapy

The horses were hospitalized for seven days after the procedure to enable observation and were then discharged. Special attention was given to detecting any possible clinical signs of local immunological reactions, including increases in temperature, tissue swelling and lameness. Approximately, two weeks after treatment, all of the horses underwent foot dorsoplantar (long toe, narrow heels, overgrown heels) and mediolateral imbalance corrections (unilateral overgrown/concave hoof wall) and wide egg-bar shoe application at the start of rehabilitation.

The rehabilitation plan was individually tailored according to the severity of the injury and in relation to the results obtained from the clinical and ultrasonographic evaluations conducted throughout the healing process. A controlled exercise program was initiated. After stall rest for the first two days after treatment, the animals were maintained at a low-level of exercise activity (hand walking) for a 3-week period. Then, once symptom-free, after a warm-up walk, most of the horses were trotted for 1–3 min on a firm flat ground mainly in straight lines, with increased intensity and time duration each week until they entered a complete training protocol which included walking, trotting and galloping. Each component of the exercise program was shortened or lengthened on the basis of ultrasonography evidence of healing progression and severity of lameness. The specific exercise program 20 weeks after the administration plasmid DNA was usually determined mostly by the owner/trainer to reflect the normal workload of the horse.

Clinic and ultrasound examinations to evaluate the regeneration rate of the PSL injury recovery, and to confirm the safety of this gene therapy in horses, were performed for all treated horses. They were conducted before the treatment (day 0), every 30 days in the first 2 months after treatment, then every 60-90 days until the end of the study. At the 12-month follow-up stage the lameness grade, time required to return to pre-injury activity level, and ultrasonography results were also evaluated, then re-evaluated against the previous timepoints. The treatment was considered to have been successful when the horse returned to its pre-injury training or competition level, without a relapse of the injury during the follow-up time.

Ethics

Plasmid vectors were created in accordance with the human standards set by the United States Food and Drug Administration (FDA, 2007, 2008), and the Committee for the Medicinal Products for Human Use in the European Medicines Agency (EMA, 2001, 2006, 2007). The Institutional Review Board of the Kazan Federal University approved this study (protocol No. 3; date 05.05.2015) in addition to the institutional committees of Moscow State Academy and the University of Nottingham, all national guidelines were adhered to. The horses presented at the clinic with naturally occurring injuries and informed consent was given by owners.

Injections and care were given in accordance with standard veterinary practice recommendations and undertaken by qualified clinicians with additional health and welfare checks and

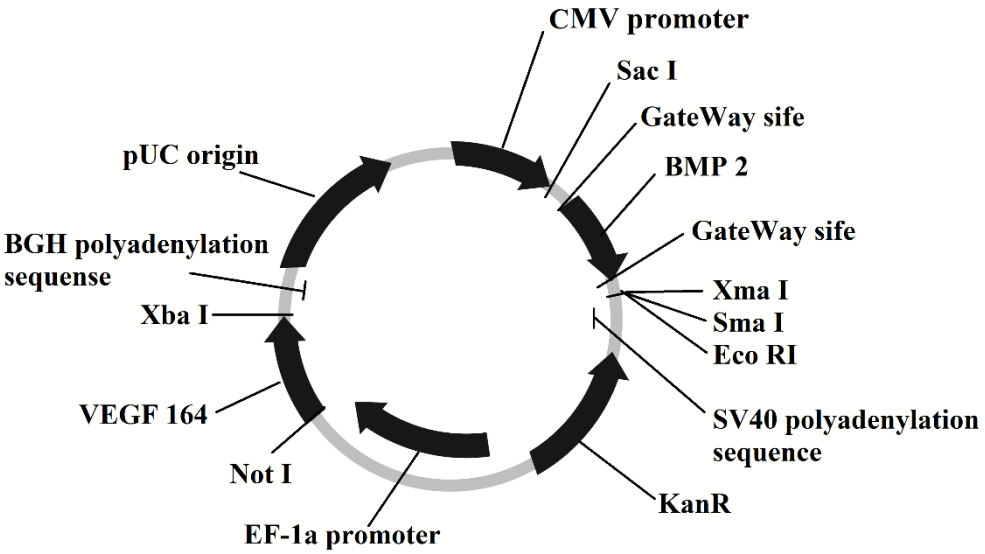

Fig. 1. Map of recombinant plasmid pBUDK-ecVEGF164-ecBMP2, contained species-specific genes of horse

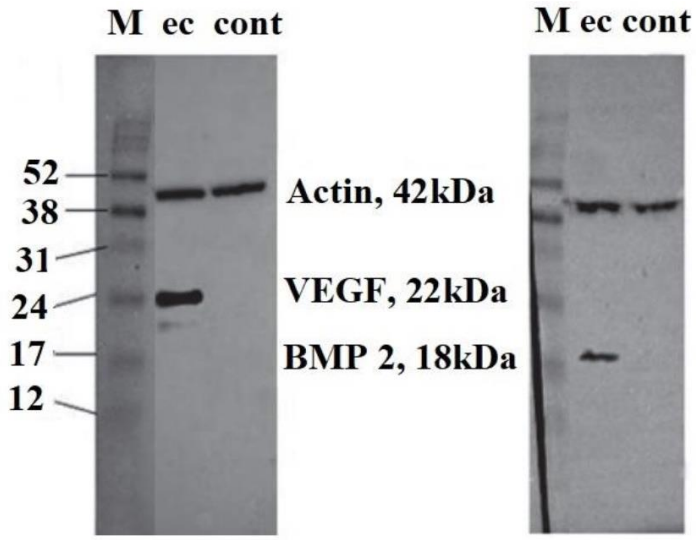

Fig. 2. Analysis of the biosynthesis VEGF164 and BMP2 proteins via immunoblotting in HEK293FT cells after transfection. Electrophoresis in 12% SDS-PAGE gel was performed using the Laemmli system. Antibodies against actin, VEGF and BMP2 proteins were used. Bands correspond to human actin (42 kDa), horse VEGF164 (22.3 kDa) and horse BMP2 (18 kDa). M – molecular weight protein marker (GE LifeSciences RPN756E); ec – HEK293FT cells transfected with pBUDK-ecVEGF164-ecBMP2; cont – non-transfected control cells

Table 2

Analysis of extensive mineralization and calcium depositions in samples with osteo-induction medium and genetically modified MSCs

Group	Native MSCs (normal growth medium)	Native MSCs after osteoinduction (osteo-induction medium)	Genetically modified MSCs (normal growth medium)
Optical density	0.064 ± 0.002	0.243 ± 0.005*	0.237 ± 0.009*

Note: *The result was significant at a level of $p < 0.05$ in comparison with native MSCs grown in the normal growth medium

clinical observations undertaken. Anatomical nomenclature followed the Nomina Anatomica Veterinaria 2017 (ICVGAN, 2017).

Statistical Analysis

Statistical processing of the results was carried out using primary statistical analysis methods, the results of which are presented in the form M ± m, where M represents the arithmetic mean value for the sample, and m the standard deviation. Secondary statistical data processing was performed using the nonparametric Wilcoxon-Mann-Whitney test. Differences were considered significant at $p < 0.05$.

Results

Functional Activity of Plasmid Construct in Vitro

The equine pDNA was successfully constructed, the map is shown in Figure 1. Recombinant proteins VEGF164 and BMP2 biosynthesis in transfected HEK293FT cells were confirmed by immunoblotting (Fig. 2).

The cells isolated from the horse adipose tissue expressed MSCs markers: 99% expressed Thy-1, 83% CD44, and 94% CD73. Extensive mineralization and calcium depositions were observed in the samples cultured in osteo-induction medium and additionally within the genetically modified MSCs after 21 days of differentiation as evidenced by high optical density following Alizarin Red S staining. This was not observed in the native MSCs grown in the normal growth medium (Table 2).

Evaluation of PSD in the Horses before Treatment

Prior to treatment, on day 0, the degree of lameness varied among the affected horses from mild to moderate and was rarely severe. The mean lameness score before administration of the pDNA was 2.0 ± 0.69 (Table 3).

Lameness was usually worse on soft ground, especially in the affected limb when it was on the outside of a circle. This was noticeable in each horse with PSD affecting a forelimb, but only observed in one of the two horses with PSD affecting a hindlimb. Responses to the flexion test before treatment ranged from mild to moderate. Prior to treatment, pain whilst pressing in a non-weight bearing position on the PSL was noted in four of the five horses.

Prior to treatment, three horses showed ultrasonographic signs of PSD to a moderate degree and two horses had a severe degree of PSD. Focal or diffuse areas of reduced echogenicity and reduced strength regarding fiber pattern, were found in all of the horses studied prior to treatment. Most of the horses with PSD had detectable primary enlargement of the cross-sectional area of the PSL prior to the treatment (Table 3). Horse 1 exhibited an irregularity of the palmar cortex of the third metacarpal bone. Prior to treatment, horse 4 had a severe longitudinal injury and irregularity of the plantar cortex of the third metatarsal bone with little mineralization (or entheseous new bone) on the plantar aspect of third metatarsal bone. None of the horses had significant abnormalities showing during the ultrasound in their contralateral limb. Horse 5 did have had ultrasonographic signs of scarring in branch of the SL in the contralateral non-lame limb on day 0.

Three types of radiographic abnormality of the proximal aspect of the third metacarpal (metatarsal) bone were identified prior to treatment: moderate irregular sclerosis of the trabecular pattern in the dorsopalmar (dorsoplantar) view, extending from the subchondral bone plate a variable distance distally (in horses 3 and 4) and disruption of the normal trabecular pattern and thickening of the cortex (horses 3, 4 and 5). Horses 1 and 2 exhibited no radiographic abnormalities in the PSL.

Evaluation of PSD in Horses after Treatment

None of the plasmid DNA treated horses encountered side effects in the form of local hyperthermia, swelling, ossification or any periarticular soft tissue damage during the observation period. By the 30th day post-treatment no pain was noted (as determined by digital pressing at the injury level) in horses 2, 4 and 5, and by day 60 post-treatment none of the treated animals exhibited pain upon digital pressing at the lesion in the palmar metacarpal/plantar metatarsal region, or in response to the flexion test.

Table 3

Degree of horse lameness after treatment

		Day							
		0	**30**	**60**	**90**	**120**	**180**	**300**	**360**
PSD forelimb	Horse 1	2	0	0	0	0	0	0	0
	Horse 2	3	1	0	0	0	0	3	1
	Horse 3	2	0	1	0	0	0	0	0
	Mean ± SD	2.3±0.7	0.3±0.5*	0.3±0.5*	0±0*	0±0*	0±0*	1.0±1.7	0.3±0.5*
PSD hindlimb	Horse 4	2	1	0	0	0	1	0	1
	Horse 5	2	2	1	1	1	2	1	2
	Mean ± SD	2.0±0.0	1.5±0.7	0.5±0.7	0.5±0.7	0.5±0.7	1.5±0.7	0.5±0.7	1.5±0.7

Note: *The result was significant at $p < 0.05$ in comparison to degree of lameness prior to treatment.

Horse 1 with PSD of the left forelimb achieved the best result following treatment. It was sound after 30 days and returned to its pre-injury, sport activity level within 2–6 months. Horse 1 began to carry out a full load for longer after 1 year, successfully participated in competitive dressage tournaments, and has continued to do so up until the present day (at the time of manuscript submission). In addition, horse number 3 showed similar outcomes.

Horse number 2 presented with right forelimb PSD and following treatment lameness was no longer detected at day 60, and it was able to carry out a full load at 9 months and returned also to sporting activity. In the later months it then presented with lameness again as the ground impact damaged the area and PSD reoccurred, in addition a lesion in the lateral suspensory branches of same limb was noted.

Horses 4 and 5 presented with hindlimb PSD. Following treatment lameness did not decrease to the same degree as observed in the three horses with forelimb PSD. Horse number 4 with PSD in its left hindlimb had no lameness after 60 days and began to carry out a full load until month 5 at a lower level than pre-injury, but after this point lameness was detected again due to ground relapse damage causing PSD in the same limb. Lameness was not always apparent at a working trot in a straight line and was sometimes observed when lunging on hard and soft surfaces at a medium trot. After treatment in horse 5, which started with a chronic form of PSD in the right hindlimb, an improved lameness score was observed. Throughout the observation period this reduced level of lameness continued but the horse became comfortable at pasture.

The age and gender of horses, and duration of lameness prior to treatment did not appear to influence clinical outcomes after gene therapy but it should be noted that larger studies would enable a more in-depth analysis of these factors. The best outcomes were observed in horses which had PSD for 40 and 55 days, followed by the horse which had been affected by 15 days, followed by those at 110 and 42 days. In addition, location of the PS damage before treatment had an impact, with forelimb PSD showing better recovery rates than those affected in the hindlimb.

Ultrasound characteristics in the horses with forelimb PSD started to improve at the checks performed 30 days after treatment in relation to both echogenicity and the fiber alignment scores, and this healing process was constantly maintained in later follow-ups (Fig. 3). According to our observations, in horses 4 and 5 with hindlimb PSD there was no substantial improvement in these factors in the first 90 days after treatment. In horses with recurrent lesions and lameness, the echogenicity and fiber alignment score deteriorated, as would be expected, again.

After treatment no significant changes ($P > 0.05$) were found during the follow-up period

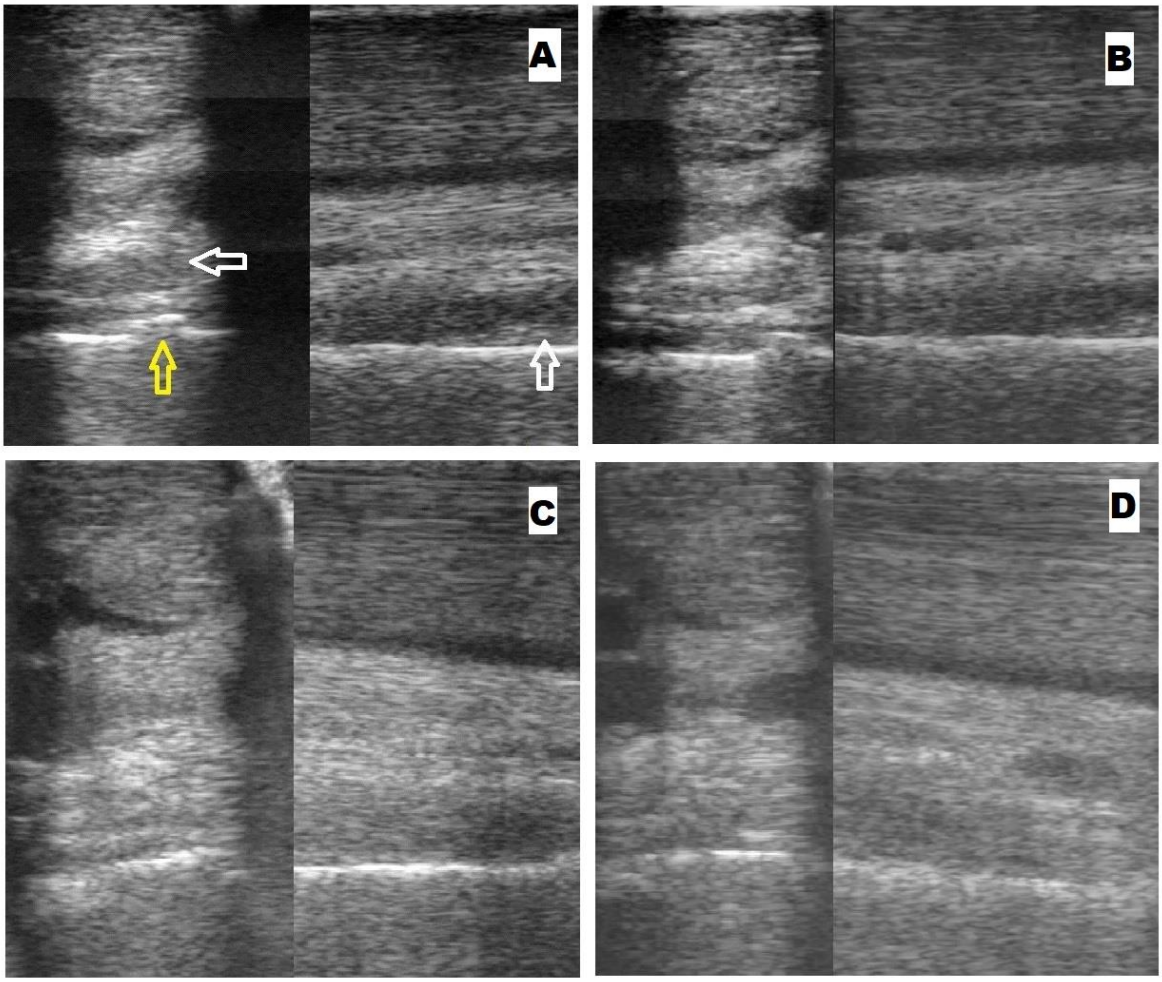

Fig. 3. A) Ultrasound images before and after administration of plasmid DNA encoding the BMP2 and VEGF164 genes. Transverse and longitudinal images of the metacarpal region of horse number 1 before treatment. The suspensory ligament shows a slight overall diffusely hypoechogenic reduction with a disruption of the fiber pattern (white arrow). Focal bone irregularity can be identified at the palmar cortex of the third metacarpal bone (yellow arrow). B, C, D) Ultrasound images on days 30, 90, and 300 respectively from horse 1 following administration of the plasmid DNA containing *bmp*2 and *vegf*164 genes

Table 4

Ultrasonographic results in horses with PSD before and after treatment with plasmid DNA encoding *bmp2* and *vegf164* genes

Number of days post treatment	Ultrasonographic parameter	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5
Day 0	DP (mm)	8	8	9	10	12
	LM (mm)	19	20	21	17	18
	CSA (cm^2)	1.67	1.86	1.93	2.05	2.3
Day 30	DP (mm)	8	7	8	10	11
	LM (mm)	16	17	18	16	16
	CSA (cm^2)	1.56	1.70	1.84	2.12	2.18
Day 60	DP (mm)	7	8	8	11	12
	LM (mm)	15	17	18	17	15
	CSA (cm^2)	1.38	1.57	1.74	1.77	2.09
Day 120	DP (mm)	9	7	9	10	11

The end of Table 4

Number of days post treatment	Ultrasonographic parameter	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5
	LM (mm)	17	15	18	15	17
	CSA (cm^2)	1.42	1.55	1.81	1.94	2.18
Day 180	DP (mm)	8	7	7	11	10
	LM (mm)	16	15	16	16	15
	CSA (cm^2)	1.39	1.64	1.51	1.84	2.11
Day 300	DP (mm)	7	8	8	9	12
	LM (mm)	18	16	16	15	16
	CSA (cm^2)	1.35	1.47	1.78	1.98	2.06
Day 360	DP (mm)	7	9	8	10	11
	LM (mm)	17	17	18	16	15
	CSA ($cm^{2)}$	1.32	1.67	1.34	1.66	1.86

Note: DP - dorsoplantar thickness; LM - lateromedial width; CSA - cross-sectional area. No statistically significant differences were observed ($P > 0.05$).

in the histomorphometric parameters which included dorsoplantar thickness, lateromedial width and cross-sectional area (Table 4, Fig. 2). In comparison to day 0, during the follow up periods after treatment there were no noticeable radiographic changes relating to the proximal aspect of the third metacarpal (metatarsal) bones in any of the horses.

Discussion

Gene therapies primarily focus on treatment for hereditary diseases, but in addition offer new possibilities for clinical management of orthopedic disorders through to genetic enhancement of production of specific growth factors, not only in humans (Tang *et al.*, 2016), but also in veterinary medicine (Soboka al., 2016; Levings *et al.*, 2020; Aimaletdinov *et al.*, 2020). Once the gene has been administered successfully and has inserted into the genome, the recipient's tissue constantly synthesizes the inserted protein(s) which can provide continuous stimulation of regeneration.

Recently, we successfully used gene therapy by injecting pDNA encoding species-specific *vegf164* and *fgf2* into ten horses with severe injuries to the branch of the suspensory ligament and superficial digital flexor tendon (Kovac *et al.*, 2017; Kovac *et al.*, 2018; Zakirova *et al.*, 2020). This method resulted in a more rapid recovery, within just three months, and return of the animals to their preinjury level of sports load in comparison with other conventional methods. As mentioned above the restoration of entheseal damage, due to specific histological and biochemical features is almost always more difficult to obtain than restoration of tendon and ligament lesions. Enthesopathy healing is a long process and is dependent upon the localization, severity, and size of the lesion. To date, direct gene therapy to treat equine PSD has never been employed, making the present study novel. Thereby, the present pilot study was designed to evaluate the clinical effects of direct gene therapy using pDNA containing species-specific *bmp2* and *vegf164* coding sequences to treat PSL injury. The pDNA construct was synthesized de novo and was designed on the basis of the pBudCE4.1 vector. When forming the pDNA containing species-specific *bmp2* and *vegf164* coding pDNA sequences, the osteoinductive features of equine MSCs were also investigated. MSCs have multipotent activities, including differentiation towards bone tissue, when osteoinductive growth medium was added (Qin *et al.*, 2014). Delivering genes leads to specific differentiation of MSCs, and the *bmp* genes are most commonly used to promote bone formation activity in these cells (Qin *et al.*, 2014). Our *in vitro* results showed that genetically modified equine MSCs grown in normal growth medium showed osteogenic commit-

ment. They as additionally exhibited extensive mineralization and calcium depositions similar to non-transformed cells cultivated in osteoinduction medium (Zakirova *et al.*, 2020).

In the present study, *vegf164* was chosen to restore entheseal damage for several reasons. Primary, it is one of main growth factors for tissue repair, with its major action relating to promotion of normal vasculogenesis and angiogenesis, whilst concomitantly reducing accumulation of fibrosis (Tang *et al.*, 2016). As equine PSD often accompanies damage to the periosteum of metasarpus/metatarsus bones, *bmp2* was chosen to compliment *vegf164*. BMP2, like other bone morphogenetic proteins, plays an important role in the development of bone and cartilage, induces neovascularization, and stimulates the process of stem cell differentiation into osteoblasts in both human and animal models (Krishnakumar *et al.*, 2017). Having realized the osteoinductive properties of the BMPs and having identified their genetic sequences, recombinant gene technology has since been used to produce BMPs for clinical application. Commonly this has been used to treat cases in which fracture healing is compromised, as well as to enhance the integration of grafts in bones or tendons (Mukhametov *et al.*, 2021).

According to our observations no systemic adverse reactions were observed as a result of the application of the plasmid DNA. Direct gene therapy did, however, achieve clinical improvements in lame horses. There was a significant decrease in the lameness scores 30 days after treatment, which continued during the follow-up periods. These improvements were primarily noticeable in horses with subacute forms of PSD in forelimbs, but not in horses with a chronic form of PSD in the hindlimb. Recurrent injury was noticed primarily in the horses with hindlimb PSD rather than those affected in the forelimb. The long-term outcomes after one year, suggested that this novel gene treatment had a positive healing effect but only in PSD relating to the forelimbs, as most treated horses were able to return to their preinjury level of sports load. However, both the time of onset of this effect and its duration remain unclear and must be evaluated further. Although this effect was statistically significant, it is not possible to attribute the decrease in lameness to the beneficial effects of gene treatment alone, since horses with PSD may improve by instituting box rest for few weeks and controlled walking exercise for three months [50]. It must be noted though that all of the horses in this trial had previously received conventional treatment (by independent veterinary professionals) which had not been successful.

It is essential to discuss the prognosis of PSD in the fore- and hindlimbs separately. In the forelimbs, the prognosis for acute PSD is excellent with conservative treatment (Dyson & Genovese, 2003). Prognosis is generally poorer in horses with hindlimb lameness, where lameness has persisted for a longer duration, and where more severe radiographic and ultrasonographic abnormalities are detected (Dyson, 2000). Despite the promise of regenerative medicine methods, such as the use of platelet rich plasma, autologous bone marrow and adipose tissue derived mesenchymal stem cells, this has at best achieved PSD recovery rates of no more than 70% (Maleas & Mageed, 2021). This phenomenon may be explained by anatomical differences between the fore- and hindlimbs. It has been hypothesized, that the PSL in hindlimbs is more constricted by periligamentous tissue in the hindlimb (a compartment-like syndrome), with pressure on the adjacent plantar metatarsal nerves (Dyson *et al.*, 2017). Additionally, in hindlimbs, the adhesions (restrictive bands of scar tissue) often occur between the PSL and the surrounding tissue which can complicate healing. Other words, lameness in horses with hindlimb PSD are affected by mechanical factors, and not only with inflammatory process. One should also emphasize that the disappearance of lameness following treated tendinitis or desmitis in a horse does not mean absolute tissue regeneration. Ultrasonographic examinations conducted after treatment in the present study showed that echogenicity and fiber alignment scores also started to improve 30 days post treatment. In horses with PSD of the hindlimbs echogenicity was increased, but uniform echogenicity was never restored. Serial ultrasonographic examinations

revealed no significant changes in PLS parameters occurred, which included CSA, dorsopalmar thickness and lateromedial width. It is possible that changes to these parameters, in cases of chronic damage, remain irreversible. It must be considered that there are difficulties when undertaking ultrasonographic evaluations of the origin of the suspensory ligament. Measurements of the dimensions relating to the PSL should be used with caution, as measurements of the lateromedial width and CSA are known to be highly variable (Zauscher *et al.*, 2012; Werpy *et al.*, 2013). In particular, the CSA measurement, considered to be an extremely valuable tool when diagnosing changes in the PSL, previously showed substantial differences between ultrasonographic and magnetic resonance images of the PSL and postmortem histological sections (Zauscher *et al.*, 2012; Werpy *et al.*, 2013). Thereby, presently, there is not ideal pattern or protocol for determining the accuracy of the ultrasonographic measurements (Whitcomb *et al.*, 2004). Because of a lack of previous reports relating to treating equine PSD with gene therapy, we cannot compare our results with results of other treatments. Previous studies, looking at differing genes and tissues can be compared to a degree as the same methodologies were applied. The previous studies compared the clinical and ultrasonographic results using plasmid DNA encoding the *vegf164* and *fgf2* genes for the treatment of injuries within the suspensory ligament branch and superficial digital flexor tendon (Kovac *et al.*, 2017; Kovac *et al.*, 2018). Compared to these previous reports, the present gene therapy outcomes were inferior in terms of the speed of the healing process and the rates of improvement in lameness overall. One reason for this observation, might be that *fgf2* offers more influence on the process of regeneration, primary through promotion of collagen production and expression of a series of other growth factor genes (Tang *et al.*, 2016). In addition, the tissues involved in the *vegf164* and *fgf2* studies may offer a more amenable tissue with regards to regeneration and/or healing. The present study does suggest that the transfer of *bmp2* and *vegf164* genes into the equine PSL has a beneficial effect in horses with acute or subacute forms of lameness which present primarily in the forelimb, and that this treatment significantly enhances healing capacities over a critical time period. A limitation of the present clinical study was the low number of cases and the fact that the investigation did not determine the exact mechanism of action of the direct gene therapy in relation to healing the injured equine ligaments. Both the time of onset of this positive effect and its duration should be evaluated further. Future methods could include a histological examination and immunohistochemistry of biopsy materials, thorough analysis of the tissue-specific expression of the relevant genes, biochemistry analysis of the pathological process, and further advanced diagnostic imaging. To determine the mechanisms and to further investigate the effects of direct *bmp2* and *vegf164* gene therapy on injured ligament healing cannot be fully evaluated without involving a large number of experimental animals alongside a double-blind study, but this preliminary study indicates that this is a promising area of research.

Conclusions

This clinical study was the first to use direct gene therapy to restore moderate and severe injuries of the proximal suspensory ligament in horses. Three horses had a subacute form of PSD in the forelimbs and two horses had a chronic form of hindlimb PSD. Plasmid DNA encoding two therapeutic species-specific growth factors, *bmp2* and *vegf164*, was injected at the site of injury of the proximal suspensory ligament. The gene treatment was followed by box rest and a controlled exercise program lasting 12 weeks. The effects of the treatment were evaluated with the use of clinical observation and ultrasound imaging for a period of 12 months. None of the treated horses encountered negative side effects following injection of the plasmid DNA. The direct gene therapy achieved clinical improvement in the previously lame horses. There was a significant decrease in lameness scores by day 30 after treatment, which continued during the follow-up period. These results were primarily in horses

with a subacute form of PSD in the forelimb, but not in horses with a chronic form of hindlimb PSD. The ultrasound characteristics relating to PSD started to improve in most of the horses at the checks performed 30 days after treatment using both echogenicity and the fiber alignment scoring. No changes were observed in parameters such as CSA, dorsopalmar thickness or lateromedial width in the observation period. This study showed that transfer of *bmp2* and *vegf164* into the equine PSL had beneficial effects in horses with acute or subacute forms of lesions, primarily in the forelimb.

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