THE JOURNAL OF GENE MEDICINE
J Gene Med 2008; **10**: 564–574.
Published online 27 February 2008 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/jgm.1170

RESEARCH ARTICLE

Plasmid growth hormone releasing hormone therapy in healthy and laminitis-afflicted horses – evaluation and pilot study

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Received: 2 November 2007
Revised: 3 December 2007
Accepted: 8 January 2008

Abstract

Background *In vivo* electroporation dramatically improves the potency of plasmid-mediated therapies, including in large animal models. Laminitis and arthritis are common and debilitating diseases in the horse, as well as humans.

Methods The effects of growth hormone releasing hormone (GHRH) on healthy horses and on horses with laminitis that were followed for 6 months after a single intramuscular injection and electroporation of 2.5 mg of an optimized myogenic GHRH-expressing plasmid were examined.

Results In the first study on six healthy horses, we observed a significant increase in body mass by day 180 compared to baseline ($P < 0.003$), and an increase in erythrocyte production (hematocrit, red blood cells, hemoglobin, $P = 0.03$). IGF-I levels were increased by 7% by day 120 ($P = 0.02$). A pilot study was performed on two horses with chronic laminitis, a vascular condition often associated with arthritis, with two horses with similar clinical disease serving as non-treated controls. Treated horses experienced an increase in weight compared to control horses that received standard care ($P = 0.007$). By 6 months post-treatment, treated subjects were rated pasture sound. Physical and radiographic evaluation demonstrated significant improvement with reduced inflammation and decreased lameness.

Conclusions These results demonstrate that a plasmid therapy delivered by electroporation can potentially be used to treat chronic conditions in horses, and possibly other very large mammals. While further studies are needed, overall this proof-of-concept work presents encouraging data for studying gene therapeutic treatments for Raynaud's syndrome and arthritis in humans.

Keywords arthritis; laminitis; growth hormone releasing hormone; gene therapy; plasmid; electroporation

Introduction

Laminitis is defined as the degeneration of the lamellae of the inner foot wall (hoof) resulting in the detachment of the distal phalanx (P3) from the inner wall. Considerable research relating to the pathophysiology of laminitis has been conducted over the past twenty years, with factors as varied as peripheral ischemia, bone and lamellar remodeling of different causes, arthritis and sepsis, as well as metabolic syndrome incriminated [1–3]. In many cases, laminitis can be secondary to arthritic conditions

such as injection of steroids in arthritic joints [4], sequelae to treatments of infectious arthritis [5], and as post-surgical complication following treatment of lameness resulting from osteoarthritis [6]. Conversely, chronic laminitic horses may also develop arthritic conditions as a consequence of the laminitis. Many compounds are being investigated for the control of symptoms of osteoarthritis in people and animals. Ideally, treatment should include analgesia, inflammation control, and chondroprotection [7–9]. A few of the newer therapies and pharmaceutical agents have been investigated in the horse as a model for human arthritis [10]. It has also been recently reported that there is a neuropathic component to the pain associated with laminitis [11] and therefore the need for a comprehensive therapy for both the joint problems and general health and welfare of the affected subject is critical [12].

In aging mammals, the growth hormone releasing hormone (GHRH) – growth hormone (GH) – insulin-like growth factor I (IGF-I) axis undergoes considerable decrement, with reduced GH pulsatile secretion and IGF-I production associated with a loss of skeletal muscle mass, osteoporosis, arthritis, increased fat deposition and decreased lean body mass [13,14]. Many studies have shown that the development of some of these changes can be offset by a GH or GHRH therapy [15] at low, physiologic concentrations [16]. It has also been shown *in vitro* that the production of hyaluronan and chondroitin sulphate proteoglycans is regulated by GH, IGF-I, and that these molecules may be of significant importance in the therapy of joint pathology [17,18]. A GHRH-based therapy that would address both the arthritic disease and the muscle wasting, while being safe and correlated with minimal or no adverse effects [19], would be a major step forward in the well-being and quality of life of patients.

Various viral and non-viral vectors have been developed in the field of gene therapy [20] each category exhibiting important associated unresolved problems, such as secondary toxicity, requirement for intra-articular delivery [21], weekly administrations of formulations that were difficult to manufacture under cGMP [22] or low gene transfer efficiency. In our current study, we have utilized a non-viral methodology, direct plasmid delivery followed by electroporation (EP), that proved in many studies in small and large mammals to be safe and efficient, achieving expression levels within therapeutic range for extended periods of time [23,24]. Plasmid-mediated GHRH supplementation by EP was previously shown to be effective in enhancing long-term quality of life of geriatric or cancer-afflicted dogs that showed signs of wasting prior to treatment [25] while maintaining GHRH levels and its downstream effectors (such as IGF-I) within physiologic range for at least 1 year, and avoiding adverse effects.

There are numerous recent reports of gene therapy application in small animal models of arthritis. In a mouse model of collagen-induced arthritis, EP of tumor necrosis factor alpha soluble receptor I variants reduced joint destruction [26]. An Adenovirus-mediated interleukin-13 (IL-13) administration in a rat adjuvant-induced arthritis model of rheumatoid arthritis was anti-angiogenic and showed potential for controlling pathologic neovascularization [27] and the expression of IL-10 in a collagen-induced arthritis mouse model by intramuscular EP significantly inhibited all the clinical and biological features of arthritis [28]. These studies demonstrate the potential for gene therapy in particular combined with EP in treating arthritis.

However, the efficacy of gene therapy, in particular plasmid therapies, in large animal models of arthritis is largely understudied. Therefore, we investigated the effects of GHRH on six healthy horses, as well as on horses with laminitis that were followed for 6 months after a single GHRH plasmid treatment delivered by constant current EP [29]. Based on our experience in other animal species, we hypothesized that the treatment would be well tolerated without any serious adverse events in the six healthy horses and that the animals with arthritis/laminitis symptoms treated with the GHRH plasmid would show improvements in clinical signs and general well-being. Our results show that administration of as little as 2.5 mg of an optimized myogenic GHRH-expressing plasmid directly injected into the skeletal muscle followed by EP did not cause local or systemic adverse effects in any of the horses and was sufficient to resolve the laminitis in the case study on afflicted horses. Future studies in a larger group of afflicted animals are needed, but this treatment regimen appears to be safe and effective in this large mammal model of arthritis/laminitis. Long-term studies using EP-mediated delivery of plasmids in large animal models of disease, showing its safe application, feasibility and positive clinical effects, are paramount for future human applications.

Materials and methods

DNA constructs

The eukaryotic expression plasmid, pAV0225 (Figure 1A), that encodes for the production of a synthetic mammalian GHRH(1–40)OH, was designed and prepared by assembly of overlapping, chemically synthesized oligonucleotides, according to specified sequence data (Aptagen, Inc., Herndon, VA, USA), and containing the expression cassette and elements required for replication and selection in *E. coli*, namely an optimized pUC origin of replication (pUC Ori) and a kanamycin resistance gene (Kan R). Expression of GHRH is driven by a synthetic myogenic promoter, namely SPc5-12 [30]. The GHRH cDNA transgene encodes for a synthetic GHRH molecule, similar to a shorter, 40 amino acids hydroxy version of the porcine/bovine or horse GHRH (partially cloned in our laboratory, Figure 1B), optimized using the Aptagen's Gene Forge® codon optimization and custom gene synthesis platform (Aptagen, Inc., 2190 Fox Mill Rd., Suite 300, Herndon, VA 20171, USA). The GHRH

DOI: 10.1002/jgm

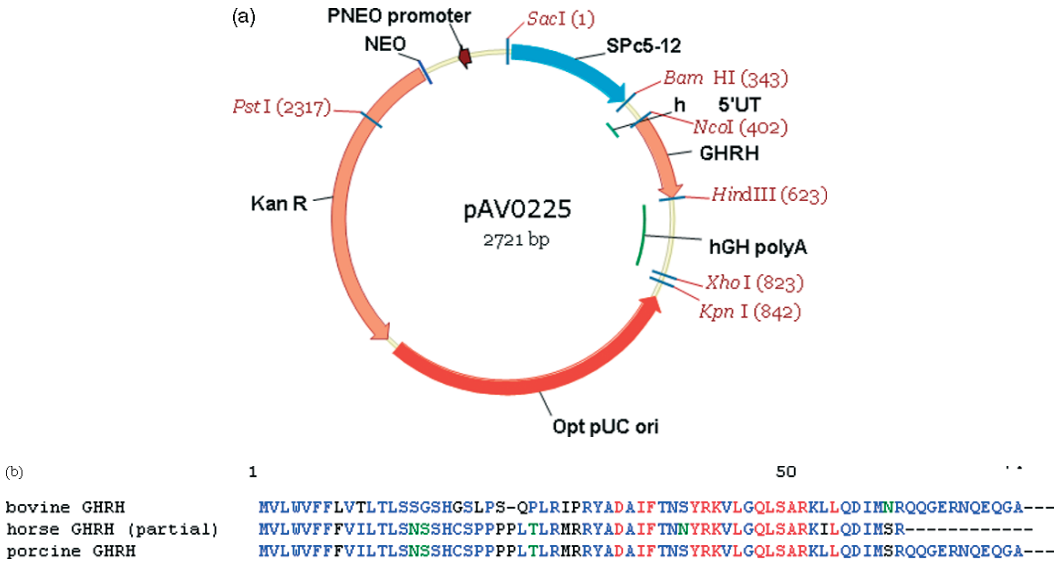

Figure 1. Maps and sequences. (a) Plasmid map of pAV0225 GHRH. (b) Synthetic consensus GHRH sequences. Bovine, horse and porcine sequences are shown

cDNA is followed by a 221 bp 3′ untranslated region and polyadenylation signal of GH.

Expression of GHRH

Cell culture

Dulbecco's modified Eagle's media (DMEM), fetal bovine serum (FBS), heat-inactivated horse serum (HIHS) and Pen-Strep (P/S) were obtained from Invitrogen (Carlsbad, CA, USA). FuGene for transfection was obtained from Roche (Indianapolis, IN, USA). Sol-8 muscle cells (ATCC Catalog #CRL-2174) were plated 24 h prior to transfection at a density of 6×10^5/100 mm plate in DMEM supplemented with 20% FBS, 1% penicillin/streptomycin (P/S). Cells were maintained in a humidified 5% CO_2, 95% air atmosphere at 37 °C. Just prior to transfection, media was changed to differentiation media (DMEM, 2% HIHS, 1% P/S). Cells were transfected with 4 µg plasmid DNA and 12 µl FuGene per 100 mm plate, according to the manufacturer's instructions. The media was replaced with fresh differentiation media 48 h after transfection. As the SPc5-12 promoter that drives the expression of GHRH in our plasmids is not well expressed in myoblasts, compared to differentiated muscle fibers, this step is obligatory in order to mimic the *in vivo* conditions in adult muscle fibers and to detect expression. Cells were harvested for RNA 96 h post-transfection.

GHRH radioimmunoassay (RIA)

GHRH concentrations in cells and culture media were measured with a heterologous human GHRH(1–44)NH2 RIA kit (Bachem Bioscience, King of Prussia, PA, USA). The sensitivity of the assay was 1 pg/tube. The intra-assay and inter-assay coefficients of variation were 3.8% and 6.8%, respectively.

RNA

Cells were extracted for RNA according to a protocol adapted from Chomczynski and Sacchi [31] as modified by Puissant and Houdebine [32], using acid guanidinium thiocyanate/phenol/chloroform extraction.

Northern blot analysis

Total RNAs (15 µg) were DNase I treated (Ambion, Austin, TX) and size separated on a 1.5% agarose/formaldehyde gel and transferred to Zeta-Probe GT Genomic Tested blotting membranes (Bio-Rad Hercules, CA, USA). The membranes were hybridized with cDNA probes ^{32}P-labeled by random priming (Ready-to-Go DNA labeling kit, Amersham, Piscataway, NJ, USA). The specific signal is detected using the GHRH probe, while the control for loading of RNA is the endogenous housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Northern analysis techniques involve the normalization of GHRH mRNA expression from transfected plasmid to GAPDH mRNA in order to correct for sample loading variability. The porcine-specific GHRH cDNA was used as a probe for GHRH expression; the control GAPDH probe was obtained from Ambion. Hybridization was carried out at 42 °C in Ultrahyb solution (Ambion, Austin, TX, USA) supplemented with denatured salmon sperm at 200 µg/ml. Membranes were washed twice, 5 min each, in 2X SSC, 0.1% SDS, and twice, 15 min each, in 0.1X SSC, 0.1% SDS, both washes at 42 °C. Blots were then exposed to a phosphor screen and analyzed on the STORM 840 (Amersham, Piscataway, NJ, USA) after overnight exposure.

Animals

The first 180-day study involved six normal adult mares (mean age 4.8 ± 1.06 years, range 4–7 years) using a

DOI: 10.1002/jgm

longitudinal self-controlled experimental design. Animals were of mixed breeds, with one Arab cross, four Quarter horse crosses and one pony mix. The study was performed between May 8 and November 18, during Texas summer months. Prior to and during this study, the mares were maintained in a 40-acre pasture and fed coastal hay and a 10% protein ration. All mares were fed separately. Weight was measured on the same calibrated scale. The mares had a mean body mass of 375.75 ± 38 kg, range 306–397 kg. General body condition, behavior, complete blood counts, clinical chemistries and selected endocrine parameters were used to assess the safety and effectiveness of the treatment. All mares were subjected to two control data sets collected day −30 and day 0. Each data set consisted of a physical examination, including determination of body mass and blood collection. Following treatment with 2.5 mg of GHRH plasmid injection followed by electroporation (EP), additional data sets were collected at days 14, 28, 60, 90, 120, 150, and 180. The second study was designed as a parallel and sequential controlled, randomized study utilizing four horses with chronic laminitis/arthritis conditions. The laminitis/arthritis subjects were naturally occurring chronic laminitis (at least 1 year in duration) patients that had developed arthritic symptoms either as a consequence of the laminitis or had a co-existing arthritic condition at the time the subject was first seen. Two subjects (one control and one treatment subject) also demonstrated a severe loss of body condition due to a combination of age and disease. The level of care was normal for animals afflicted with this condition, and both treated and controlled animals were maintained under identical conditions. Horses were followed for a control period of at least 90 days before being subjected to plasmid GHRH therapy. Prior to treatment, all horses were removed from all other treatments with the exception of systemic analgesics. Following treatment, subjects were followed for 6 months with clinical evaluations being completed at approximately 30-day intervals. Evaluation parameters consisted of (1) radiographic evaluation, (2) physical lameness assessment, (3) quantitative force plate evaluation defining voluntary limb load and load distribution profile, (4) body condition score, (5) body mass, (6) a complete blood count, and (7) a standardized chemistry panel. Animal experimentation was conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals and Texas A&M University IACUC.

Plasmid injection and electroporation

Animals were treated at day 0 with 2.5 mg pAV0225, followed by constant current EP using the optimized electroporator CELLECTRA™ device and electrodes, as described [29]. This dose of plasmid was previously shown to be effective in 500-kg cows [33]. The electroporator settings were 1 A intensity of the electric field, 5 pulses, 52 ms/pulse, 1 s interval between pulses.

Figure 2. Electroporation. Image of administration area of the GHRH-expressing plasmid by plasmid injection in the cranial aspect of the brachiocephalic muscle

Needles were 21 gauge, 1 inch in length, and completely inserted into the brachiocephalic muscle through the intact skin (Figure 2). The plasmid was formulated in a 1% poly-L-glutamate solution, as previously described [34]. The injection was performed under light anesthesia using xylazine hydrochloride (1.1 mg/kg) and ketamine (2.2 mg/kg) to avoid involuntary movements of the animals.

Complete blood counts and biochemical parameters

Whole blood from all horses was collected in EDTA and submitted for complete blood count analysis (Texas Veterinary Medical Diagnostic Laboratory, College Station, TX, USA) prior to treatment, and at the indicated days post-treatment. Hematology parameters included: erythrocyte counts, hematocrit, hemoglobin, total leukocyte count, and differential leukocyte counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils), platelet count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and fibrinogen. Serum samples were also collected. Serum was aliquoted for radioimmunoassay (RIA) and biochemical analysis and stored at −80 °C until just prior to analysis. Biochemical analysis occurred within 48 h after serum collection (Texas Veterinary Medical Diagnostic Laboratory, College Station, TX, USA). Serum biochemical endpoints included alanine aminotransferase, gamma-glutamyltransferase, creatine phosphokinase, total bilirubin, total protein, albumin, globulin, blood urea nitrogen, creatinine, phosphorus, calcium, and glucose. Cortisol, ACTH, T3, T4, and insulin assays were performed by an independent laboratory (Texas Veterinary Medical Diagnostic Laboratory, College Station, TX, USA). All samples were analyzed in the same assay.

DOI: 10.1002/jgm

Lameness assessment

Horses were given a physical lameness assessment, a gait analysis assessment and a quantitative force plate evaluation defining voluntary limb load and load distribution profile. Determination of limb-load distribution was accomplished by use of a custom-designed computerized system consisting of four independent force plates. The system allowed for quantification of mean load expressed as a percentage of body weight placed on each limb over a 5-min period, using a data sampling rate of 0.1 s. The standard deviation (SD) of limb load was used as an index of limb-load variation (i.e. frequency of load redistribution) and was referred to as the 'load distribution profile' (LDP) [35]. The higher the LDP value indicates an increase in weight shifting in the limb(s). Radiographic assessments of the hoof at days 0 and 180 were also used as a measure of pathological lesions.

RIA for IGF-I, IGF-BP2 and IGF-BP3

Serum IGF-I was measured using a heterologous human immunoradiometric assay kit following the manufacturer's protocol (Diagnostic System Labs, Webster, TX, USA). The kit employs an extraction step to cleave binding proteins. All samples were run in the same assay. The intra-assay variability was 3.6%. Cross-reactivity of human IGF-I antibody for horse IGF-I is 100%. IGF-BP2 and IGF-BP3 were measured using human immunoradiometric assay kits for the respective binding proteins and run according to the manufacturer's protocol (Diagnostic System Labs). All samples were run in the same assay. The intra-assay variability for IGF-BP2 was 7.5% and IGF-BP3 was 3.3%.

Statistics

The data in the above examples were analyzed using SAS statistics analysis package (Cary, NC, USA). Values shown in the figures are depicted as individual values or the mean of the individual values per group. Comparison was made with baseline measurements. Specific P values were obtained by analysis of variance with a Tukey post-hoc test. A P value <0.05 was set as the level of statistical significance.

Results

GHRH expression

GHRH mRNA expression from the optimized pAV0225 (Figure 1A) was analyzed by Northern blot, and a correct size band was identified. Expression of GHRH was measured *in vitro* after plasmid transfection in a mouse muscle-specific cell line (Sol8) in both culture media and cells using a GHRH-specific RIA as described in Materials and Methods. GHRH levels measured in media from cells transfected with the synthetic GHRH expression plasmid were significantly increased compared to GHRH produced by cells transfected with a mouse-specific GHRH expression plasmid used as a positive control (10.81 ± 0.90 vs. 7.51 ± 0.77 ng/ml, respectively, $P = 0.016$), and compared to sham transfected cells used as a negative control (0.4 ± 0.03 ng/ml).

Study 1 – Healthy horses

A longitudinal self-controlled experiment for this study was designed to determine if the GHRH-expressing plasmid delivered by direct intramuscular injection followed by EP was safe and effective in equine (Figure 2). All animals received the plasmid formulation in one administration in the brachiocephalic muscle. Animals were treated at day 0 with 2.5 mg of the GHRH-expressing plasmid (pAV0225), followed by EP using the optimized CELLECTRA™ constant current electroporator device and electrodes, as previously described [29,33]. Animals were injected in the contralateral muscle with vehicle only, to assess the impact of plasmid locally, at the EP site. General body condition, behavior, complete blood counts, clinical chemistries and selected endocrine parameters were used to assess the safety and effectiveness of the treatment at days 0, 14, 28, 60, 90, 120, 150 and 180.

Local or systemic adverse effects

None of the horses demonstrated significant tissue reactions at either the plasmid or control EP sites. Three horses exhibited soft tissue swelling at EP sites (both plasmid and vehicle control) at 24 h following treatment, of no more than 2 cm in circumference, which resolved within 2 days. None of the horses demonstrated any short- or long-term adverse systemic effects following treatment.

Body mass

For all biological parameters, baseline measurements were taken at day 0. Body weights increased in all animals during the 180 days post-treatment to 422.8 ± 19.0 kg from the baseline value of 383.64 ± 14 kg at day 0, $P < 0.003$. A distinct trend for increase in body mass occurred after 60 days post-treatment, and was significantly elevated from the baseline values by 90 days post-treatment.

Clinical parameters

Hematological parameters

Hematological parameters at all data points are included in Table 1. Hematocrit increased from 34.8 ± 4.2 to $41.6 \pm 2.3\%$ at the end of the study ($P < 0.01$); total red blood cell count increased from 7.7 ± 0.9 to

DOI: 10.1002/jgm

Table 1. Hemogram – Mean ± standard error of mean (SEM) for the six treated horses in study 1

Parameter	DAYS POST-TREATMENT									P value
	−30	0	14	28	60	90	120	150	180	
Hematocrit %	34.82 ± 1.71	36.90 ± 2.29	36.05 ± 1.74	37.17 ± 1.49	35.88 ± 2.66	37.02 ± 1.60	36.43 ± 2.16	41.13 ± 1.39	41.57 ± 0.095	<0.03 (D180)
Red cells million/μl	7.71 ± 0.35	8.12 ± 0.43	8.21 ± 0.37	8.52 ± 0.41	8.10 ± 0.60	8.15 ± 0.35	8.05 ± 0.55	9.12 ± 0.40	9.30 ± 0.31	<0.03 (D180)
Hemoglobin g/dl	12.68 ± 0.63	13.28 ± 0.76	13.25 ± 0.61	13.62 ± 0.55	13.32 ± 0.94	13.18 ± 0.59	12.85 ± 0.74	14.47 ± 0.47	14.63 ± 0.31	<0.03 (D180)
MCV	45.25 ± 1.31	43.78 ± 2.60	43.92 ± 1.03	43.80 ± 1.10	44.28 ± 0.88	45.48 ± 0.95	44.80 ± 1.00	45.30 ± 0.91	44.87 ± 0.94	NS*
MCH	16.48 ± 0.43	16.38 ± 0.43	16.18 ± 0.41	16.05 ± 0.48	16.47 ± 0.31	15.67 ± 0.55	16.03 ± 0.35	15.92 ± 0.30	15.78 ± 0.38	NS
MCHC	36.43 ± 0.30	36.08 ± 0.48	36.78 ± 0.18	36.63 ± 0.25	37.17 ± 0.18	35.58 ± 0.49	35.30 ± 0.15	35.20 ± 0.14	35.23 ± 0.21	NS
Total white cells/μl	9900 ± 443.4	11 250 ± 1137.1	9500 ± 413.1	10 117 ± 320.9	10 483 ± 617.8	10 750 ± 737.5	9850 ± 523.3	9300 ± 429.0	10 450 ± 447.0	NS
% Neutrophil	55.17 ± 2.18	60.17 ± 5.74	52.83 ± 3.79	55.00 ± 2.82	61.33 ± 3.16	65.17 ± 3.94	51.50 ± 2.69	53.83 ± 1.96	58.17 ± 3.26	NS
% Lymphocyte	34.50 ± 5.80	34.00 ± 4.91	41.67 ± 4.01	38.33 ± 2.79	32.83 ± 3.66	28.50 ± 3.96	40.67 ± 3.42	38.83 ± 1.72	35.50 ± 3.95	NS
% Monocytes	1.83 ± 1.33	2.50 ± 0.85	2.17 ± 0.48	1.67 ± 0.67	1.83 ± 0.60	2.50 ± 0.43	3.83 ± 0.31	3.17 ± 1.35	2.17 ± 0.83	NS
% Eosinosphils	3.67 ± 0.71	3.17 ± 0.87	3.33 ± 1.02	4.67 ± 0.92	3.67 ± 0.67	3.17 ± 0.48	3.83 ± 1.19	3.33 ± 0.56	3.33 ± 1.56	NS

*NS = not statistically significant.

9.3 ± 0.8 million/μl during the same 180-day evaluation period ($P < 0.03$); hemoglobin increased from initial control values of 12.7 ± 1.5 to 14.6 ± 0.8 ($P < 0.03$). Over the 180-day study, there were no significant changes in the leukocyte values and differentials; neutrophil levels were variable during study. The other hematological parameters were not different over the course of this study. All values were within normal physiological range for horses.

Clinical chemistries

Serum proteins

A significant decrease in the total serum protein ($P < 0.0001$), serum albumin ($P < 0.0001$), and globulins ($P < 0.0001$) was observed at 28 days post-treatment (Table 2), correlating to muscle mass deposition. These changes were within the normal laboratory values for these parameters and all values returned to baseline levels by day 180. The albumin/globulin ratio did not significantly change during the course of this study ($P = 0.2$).

Serum calcium and phosphorus

Serum calcium and phosphorus demonstrated a trend to decrease at days 14 and 28, with phosphorus still decreased at 60 days post-treatment; calcium did increase after day 28 to levels above the baseline. Repeated measures analysis indicated that these changes were significant ($P = 0.0001$) even though the values did not exceed the normal range expected for these electrolytes.

Blood glucose

The blood glucose concentration decreased at 14 and 28 days post-treatment, followed by a return to baseline values over the remainder of the study. Repeated measures analysis indicated that these changes were significant ($P = 0.0003$). The mean value at the 28-day sampling period (63.2 mg/dl) was below the normal range of the laboratory (75 mg/dl).

BUN, creatinine, total and direct bilirubin, serum enzymes

All these parameters were consistently within the normal range for horses, and not changed at the end of the study compared to baseline measurements.

Endocrine data

Cortisol and ACTH

Cortisol levels for the first and second baseline measurements were 6.0 ± 0.5 μg/dl and 6.2 ± 1.1 μg/dl, respectively (see Table 3). There appeared to be a trend for the cortisol serum levels to progressively decrease, initiated between 60 and 90 days post-treatment. The 180-day cortisol level was 2.7 ± 0.73 μg/dl. Repeated measures analysis indicated that the level at 180 days post-treatment

DOI: 10.1002/jgm

Table 2. Clinical chemistries – Mean ± standard error of mean (SEM) for the six treated horses in study 1

	DAYS POST-TREATMENT									
Parameter	−30	0	14	28	60	90	120	150	180	*P* value
Total protein g/dl	7.77 ± 0.21	7.93 ± 0.25	6.72 ± 0.40	6.45 ± 0.29	7.20 ± 0.26	7.98 ± 0.19	7.53 ± 0.11	7.53 ± 0.26	7.88 ± 0.27	<0.0001 (D28)
Albumin g/dl	2.72 ± 0.06	3.00 ± 0.06	2.35 ± 0.19	2.27 ± 0.11	2.53 ± 0.11	2.80 ± 0.05	2.82 ± 0.07	2.92 ± 0.05	2.72 ± 0.03	<0.0001 (D28)
Globulins g/dl	5.05 ± 0.19	4.93 ± 0.03	4.37 ± 0.24	4.18 ± 0.23	4.67 ± 0.21	5.18 ± 0.22	4.72 ± 0.18	4.62 ± 0.27	5.17 ± 0.29	<0.0001 (D28)
AG ratio	0.54 ± 0.02	0.62 ± 0.03	0.54 ± 0.03	0.47 ± 0.09	0.55 ± 0.03	0.55 ± 0.03	0.52 ± 0.10	0.64 ± 0.04	0.54 ± 0.03	NS
Calcium mg/dl	11.78 ± 0.14	11.92 ± 0.54	10.50 ± 0.63	10.28 ± 0.45	11.48 ± 0.26	12.23 ± 0.04	12.03 ± 0.21	12.57 ± 0.06	12.17 ± 0.17	<0.0001 (D28)
Phosphorus mg/dl	3.82 ± 0.26	4.17 ± 0.24	3.53 ± 0.18	3.28 ± 0.19	3.45 ± 0.16	4.40 ± 0.09	3.97 ± 0.17	4.45 ± 0.20	3.70 ± 0.17	<0.0001 (D28,60)
Glucose μg/dl	79.67 ± 6.50	82.67 ± 1.14	75.17 ± 7.05	* **63.17** ± 6.99	84.50 ± 4.87	73.17 ± 3.34	92.50 ± 4.23	84.67 ± 4.66	90.17 ± 2.41	0.0003 (D28)
BUN mg/dl	20.27 ± 1.03	21.65 ± 1.14	17.50 ± 1.39	18.02 ± 1.10	21.48 ± 1.09	22.53 ± 1.08	20.77 ± 1.41	18.65 ± 2.15	18.68 ± 0.74	NS**
Creatinine mg/dl	0.93 ± 0.05	1.00 ± 0.08	1.12 ± 0.09	1.02 ± 0.10	1.00 ± 0.08	1.13 ± 0.10	1.05 ± 0.10	1.18 ± 0.11	1.40 ± 0.13	NS
Total bilirubin mg/dl	0.48 ± 0.05	0.53 ± 0.04	0.35 ± 0.04	0.42 ± 0.02	0.45 ± 0.03	0.57 ± 0.08	0.50 ± 0.07	0.58 ± 0.07	0.60 ± 0.07	NS
Direct bilirubin	0.15 ± 0.02	0.17 ± 0.02	0.12 ± 0.02	0.13 ± 0.02	0.13 ± 0.02	0.17 ± 0.03	0.17 ± 0.02	0.15 ± 0.02	0.22 ± 0.02	NS
ALP U/l	192.00 ± 17.9	223.17 ± 12.3	170.00 ± 20.9	154.17 ± 15.7	181.67 ± 24.7	202.17 ± 14.8	182.83 ± 12.0	180.17 ± 11.7	211.83 ± 13.5	NS
CK IU/l	519.83 ± 53.6	546.33 ± 106.9	349.17 ± 54.8	370.17 ± 59.8	511.67 ± 96.4	549.83 ± 92.4	519.50 ± 57.0	549.67 ± 55.9	442.17 ± 52.4	NS
AST U/l	358.33 ± 24.1	360.50 ± 25.0	267.17 ± 32.6	222.17 ± 16.6	261.17 ± 19.4	309.67 ± 17.9	307.00 ± 23.0	308.50 ± 12.0	277.00 ± 14.3	NS
GGT U/l	12.17 ± 1.76	12.67 ± 1.61	12.67 ± 2.28	10.50 ± 1.41	12.17 ± 2.10	10.00 ± 1.46	11.67 ± 0.92	8.67 ± 1.31	12.17 ± 1.22	NS

*Below the normal laboratory range of 75 μg/dl.
**NS = not statistically significant.

Table 3. Endocrine parameters - Mean ± standard error of mean (SEM) for the six treated horses in study 1

	DAYS POST-TREATMENT									
Parameter	−30	0	14	28	60	90	120	150	180	p value
Cortisol μg/dl	6.02 ± 0.52	6.17 ± 1.10	5.52 ± 0.70	6.10 ± 0.47	6.45 ± 1.36	5.92 ± 0.34	5.15 ± 0.82	5.28 ± 0.52	2.68 ± 0.27	p = 0.005 (D180)
T3 μg/ml	0.32 ± 0.04	0.32 ± 0.02	0.26 ± 0.06	0.37 ± 0.08	0.58 ± 0.09	0.41 ± 0.10	0.44 ± 0.12	0.38 ± 0.03	0.39 ± 0.06	NS
T4 μg/ml	1.50 ± 0.11	1.61 ± 0.16	1.64 ± 0.10	1.70 ± 0.19	1.80 ± 0.15	1.81 ± 0.19	1.90 ± 0.19	1.95 ± 0.16	1.77 ± 0.08	NS
ACTH pg/ml	30.39 ± 4.15	55.78 ± 8.03	29.46 ± 1.44	34.48 ± 3.37	36.00 ± 2.32	44.37 ± 3.68	27.95 ± 2.00	33.87 ± 3.58	30.50 ± 2.52	NS
Insulin μU/ml	3.85 ± 2.14	5.13 ± 1.96	5.33 ± 1.67	9.48 ± 1.46	19.52 ± 5.63	6.58 ± 1.82	13.40 ± 5.07	8.40 ± 1.73	4.30 ± 2.55	NS
IGF-I ng/ml	ND	229.5 ± 50.7	ND	ND	224.7 ± 44.2	ND	245.6 ± 45.2	ND	222 ± 41.3	p < 0.02 (D120)
IGF-BP2 ng/ml	ND	22.13 ± 2.21	ND	ND	23.1 ± 0.86	ND	21.61 ± 2.62	ND	22.34 ± 1.17	NS
IGF-BP3 ng/ml	ND	5.28 ± 1.99	ND	ND	5.28 ± 0.85	ND	4.92 ± 1.83	ND	4.29 ± 1.60	p < 0.06 (D180)

NS = not statistically significant; ND = not determined.

was different from all other samples, $P = 0.005$. ACTH levels were variable before and following treatment, with a peak at second baseline.

T3 and T4
There were no statistically significant changes in the serum T3 and T4 following treatment, $P = 0.3$ and $P = 0.45$, respectively. Both parameters demonstrated a tendency to increase slightly following treatment.

Insulin
Insulin values were variable during the study, with peak values being present at 60 and 120 days post-treatment, and 180-day values were similar to baseline.

IGF-I and IGF-BPs
IGF-I levels were increased by 7% at day 120 post-treatment and regained baseline levels at day 180 (baseline: 229.5 ± 50.7 ng/ml; day 120: 245.6 ± 45.2 ng/ml, $P = 0.02$; day 180: 222 ± 41.3 ng/ml). IGF-BP2 levels showed no changes from baseline values (baseline 22.13 ± 2.21 ng/ml; day 120: 21.61 ± 2.62 ng/ml; day 180: 22.34 ± 1.17 ng/ml). A borderline decrease from baseline was seen with IGF-BP3 at day 180 (baseline 5.28 ± 1.99 ng/ml; day 120: 4.92 ± 1.83 ng/ml; day 180: 4.29 ± 1.60 ng/ml, $P = 0.06$).

Study 2 – Laminitis case reports

This case report study was designed as a parallel, sequential controlled, randomized study utilizing four horses with chronic laminitis/arthritic conditions. Two animals were treated at day 0 with 2.5 mg pAV0225, followed by EP using the optimized constant current CELLECTRA™ electroporator device and electrodes, as previously described [29], while two animals were used as untreated controls. Body weights, blood samples and lameness assessments were collected at days −12, 0, 30, 60, 90, 120, 150 and 180. Radiographs of the treated animals were taken at days 0 and 180.

Body mass

The average body mass of the treated horses increased significantly compared to baseline ($P = 0.007$) by the end of the evaluation period by almost 20 kg (435.5 ± 1.06 kg at day 0 and 455.5 ± 2.5 kg at day 180), while the control animals that received the same standard of care showed only a 10.5-kg weight increase during the same period of time (406.0 ± 52.0 kg at day 0 and 416.5 ± 32.5 kg at day 180).

Clinical parameters

Complete blood counts, biochemical and hormonal parameters all remained within the normal range with no significant changes during the 180-day evaluation.

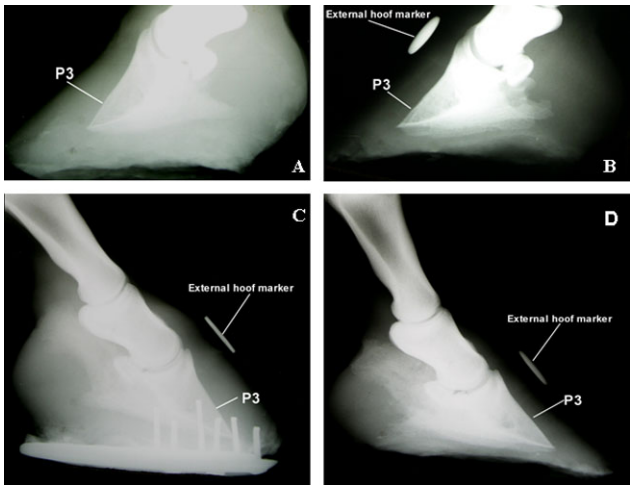

Figure 3. (a) Radiograph of the afflicted hoof of horse L1 at day 0. This illustrates rotation of distal phalanx (P3) and extensive inflammation of the lamellae tissue surrounding P3. (b) Radiograph of the afflicted hoof of horse L1 at day 180. Shows significant reduction of inflammation of the lamellar tissue surrounding the distal phalanx (P3) and P3 returned to normal position parallel to external hoof marker after GHRH treatment. (c) Radiograph of the afflicted hoof of horse L2 at day 0. This illustrates rotation of distal phalanx (P3) and extensive inflammation of the lamellae tissue surrounding P3. (d) Radiograph of the afflicted hoof of horse L2 day 180. Shows significant reduction of inflammation of the lamellar tissue surrounding the distal phalanx (P3) and P3 returned to normal position parallel to external hoof marker after GHRH treatment

Radiographs

Comparison of the radiographs from both GHRH-treated horses taken at day 0 and day 180 revealed a significant reduction of the inflammation of the lamella and a total correction of the rotation of P3 at day 180 (Figures 3a–3d).

Physical and force plate evaluation

Both horses had considerable improvement in lameness as assessed by physical examination during the 6-month period and were able to be removed from all previous medications (see Materials and Methods). The two horses had very different load distribution profiles (LDPs) at day 0 (data not shown), but were similar at approximately day 40 and both horses started to decrease around day 50, reaching a reasonably stable state at day 120.

Discussion

Among the different non-viral methods currently under investigation, *in vivo* plasmid delivery by EP has proven to be both efficient and simple, with significant therapeutic and vaccination applications [23,36]. This technique can be applied to almost any tissue of a living animal, including skeletal muscle, tumors, skin, liver, kidney, artery, eye, joints, brain, lung, and its possible uses

DOI: 10.1002/jgm

in gene therapy, vaccination, or functional studies are numerous, as previously reviewed [24]. In a previous study, we measured the tissue resistance of muscles in different species and determined that it can vary from subject to subject, from muscle to muscle within the same animal and between individual EP pulses [37]. We have used in this study an adaptive constant current CELLECTRA™ electroporation device which is able to measure the tissue resistance in real-time and adjust the voltage to account for the changes in resistance during EP, preventing heating of a tissue and consequently reducing tissue damage and pain [37,38]; this was confirmed in the treated horses which exhibited no local adverse effects related to EP or minimal local swelling which resolved within 2 days of treatment.

In these studies, we have used an optimized plasmid construct that included a synthetic backbone CpG depleted. The expression cassette was driven by a muscle-specific promoter [30] and contained a synthetic GHRH sequence optimized for mammalian expression, and based on a partial horse cDNA GHRH clone isolated in our laboratory (see Figure 1B), as well as the known porcine and bovine sequences. The increase in efficacy is evidenced by the relatively small quantity of plasmid that was delivered by EP in this very large species: 2.5 mg in a 450-kg animal translates to an effective dose of approximately 56 μg/kg. This is of relevance for human patients in terms of both efficacy and toxicity.

Gene therapeutic approaches involving molecules in the GHRH-GH-IGF-I axis have already been proposed for the treatment of arthritis, although to our knowledge this is the first proof-of-concept study demonstrating a beneficial effect on arthritis/laminitis of a therapeutic plasmid in a large mammal. Gene transfer of IGF-I into rabbit knee joints promotes proteoglycan synthesis without significantly affecting inflammation or cartilage breakdown [39]. However, the inoculation of an adeno-associated virus vector expressing IGF-I in rat knees damaged either mechanically or by collagen-induced arthritis did not result in any improvement [40]. It has been also shown that increased levels of IGF-binding-proteins in arthritis may result in the reduced availability of free IGFs that can bind to IGF receptors, and thus participate in the catabolic processes in rheumatoid arthritis and the development of cachexia and wasting in these patients [41]; the favorable changes in IGF-I and IGF-BP levels described in this current work, correlated with improved body weight and clinical status of the affected horses, confirm this hypothesis.

GHRH levels were not assayed in the *in vivo* studies as the commercially available assays involve steps of column purification with variable loss of material; many discrepancies exist between GH assays (also, in the case of GH, secretion is pulsatile) [42]. The increases that we have previously observed in serum GHRH levels post-treatment are in the physiological range, thus the differences cannot be readily detected; nevertheless, we were able to measure IGF-I levels throughout the 6-month evaluation of the healthy horses and show that its increase correlated with the described changes.

Significant increases in hematological values within physiological parameters were shown in study 1; similar changes have been seen in normal dogs [43] and cows [33] that received the plasmid-mediated GHRH treatment.

Insulin resistance has been identified as a risk factor for laminitis [44]; hence it was important to assess the effect of GHRH treatment on the endocrine system prior to treating horses with laminitis. All endocrine parameters were maintained within the normal range throughout these studies. The cortisol levels were lower at day 180. The difference in levels can be explained by the time of the year when samples were collected [45] – the first samples were collected between May and September, while the last sample was collected in November. In south Texas, during the summer season animals are under heat stress conditions and usually their cortisol levels adjust upwards [46]. After day 90, all six healthy horses experienced weight gains from baseline which varied from 24.6 to 72.7 kg. Horses in the 4–7 year age group may continue to grow, especially if actively exercised. However, these horses were on the pasture, and data in the literature suggests that weight gain is almost completed by 540 days of age [47] in a majority of circumstances and breeds. Furthermore, the approximately 10% body mass gain observed occurred over the course of the summer months in Texas when heat stress usually makes weight gain difficult. This confirms our findings from a different study, where we have shown that calves born to GHRH-treated heifers during periods of heat stress have increased weight gain compared to calves born to non-treated controls [48].

The GHRH-treated laminitis/arthritis-affected horses demonstrated a significant improvement in lameness status as the horses were no longer lame on physical examination at the walk and trot. The LDP of the treated and control horses was variable and it was difficult to assess a clear trend and distinction between the groups. This variability could be explained by continued mandatory treatment with analgesics for the control animals for humane purposes, while pain in the treated horses had decreased and these animals were able to be removed from treatment with analgesics [35]. In addition, their hooves were trimmed and one horse had corrective shoes to provide support for the hoof. One horse was also receiving hyaluronate sodium injections to address the arthritic symptoms. Six months following treatment, both subjects were rated as pasture sound and neither subject required systemic analgesics.

The most compelling data for response to treatment with GHRH were provided in the physical and radiographic evaluation of the feet, which demonstrated significant improvement, and lameness associated with the arthritic condition was no longer evident. One of the laminitis/arthritis control horses had to be euthanized prior to the completion of the study due to increasing lameness and marked loss of body condition. The second control subject was still lame at the end of the study and

DOI: 10.1002/jgm

demonstrated difficulty in maintaining body condition typical of the chronically affected laminitis condition. The CBC and chemistry profiles of both groups remained in the normal range throughout the study. Similar to study 1, there were no data that reflected a toxic or adverse effect of the treatment with the GHRH plasmid delivered by intramuscular EP.

It has been suggested that Raynaud's syndrome and laminitis are the same peripheral vascular disease in different species [49]. The fact that equine laminitis can be experimentally induced is of potential value in elucidating the exact pathophysiology of both diseases, and using a plasmid-mediated therapy could avoid frequent administration of newly identified compounds. Understanding the causes of laminitis and developing new therapeutic approaches in horses may allow translation into the corresponding human disease.

In the cases where laminitis is a consequence or a secondary condition to arthritis, a series of therapies have been proposed: lavage of the affected joints if septic, intra-articular administration of antibiotics, hyaluronidase or corticosteroids [50,51], arthroscopic debridement with or without partial synovectomy, systemic administration of antibiotics, anti-inflammatory or chondroprotective drugs. Reduced perfusion and vasoconstriction potentiated by treatment with glucocorticoids have also been implicated in the pathogenesis of laminitis [52,53]. Decrease in body weight, reduce force, and ultimately wasting are a secondary consequence of the disease.

Current therapies for laminitis include long-term non-steroid anti-inflammatory compounds which have the potential for adverse side effects. In addition, these drugs have to be administered on a daily basis, while the plasmid GHRH treatment is a one-time injection having long-term therapeutic effects, as evidenced by previous studies in other animal species [25,33]. The results of this preliminary study demonstrated that plasmid-mediated GHRH supplementation delivered by intramuscular injection followed by adaptive EP is a safe, effective therapy which can be used to treat laminitis/arthritis in horses. Larger controlled studies are needed to confirm these results and to move this technology towards human clinical trials.

Acknowledgements

The authors would like to particularly thank David Hood, Hoof Diagnostic and Rehabilitation Clinic, College Station, Texas, for providing the clinical assessment, maintenance and blood sampling the horses during the study, Amir Khan for the editorial correction of this manuscript and to Melissa A. Pope for performing the RIAs and for her technical input during this study. We acknowledge support for this study from VGX Pharmaceuticals, Immune Therapeutics Division (formerly ADViSYS, Inc., The Woodlands, TX, USA).

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DOI: 10.1002/jgm