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Preclinical Toxicology and Biodistribution Studies of Recombinant Adeno-Associated Virus 1 Human Acid α-Glucosidase

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Abstract

A biodistribution and toxicology study was performed to test the acute toxicities of intradiaphragmatic injection of a recombinant adeno-associated virus (rAAV) 2/1-human acid alpha-Glucosidase (h*GAA*) driven by a cytomegalovirus (CMV) promoter (rAAV1-CMV-h*GAA*) in New Zealand white rabbits and in the rodent Pompe disease model by injecting at the right quadriceps. Studies performed using fluoroscopy and AAV2-GFP demonstrated spread upon intradiaphragmatic injection, and the ability of AAV to infect and express acid α-glucosidase (GAA) throughout the diaphragm. For the preclinical study, 10 rabbits (5 male, 5 female) were divided into two groups, vehicle control (Lactated Ringer's) and test article (1.5×10^{12} vector genomes [vg] rAAV1-CMV-h*GAA*), and euthanized on day 21. After direct visualization, the left hemidiaphragm was injected at three locations. There was up to a 2,500-fold increase in circulating anti-AAV1 antibodies directed to the vector capsids. In addition, up to an 18-fold increase in antibodies against the GAA protein was generated. Injection sites maintained up to 1.0×10^{5} vg/μg genomic DNA (gDNA), while uninjected sites had up to 1.0×10^{4} vg/μg gDNA. Vector DNA was present in blood at 24 hr postinjection at up to 1.0×10^{6} vg/μg gDNA, followed by a decrease to 1.0×10^{3} vg/μg gDNA at euthanization on day 21. Nominal amounts of vector DNA were present in peripheral organs, including the brain, spinal cord, gonads, and skeletal muscle. Upon histopathological examination, fibroplasias of the serosal surface were noted at diaphragm injections sites of both groups. In addition, an increase in mononuclear cell infiltration in the diaphragm and esophagus in vector-dosed animals was found. Elevated creatine phosphokinase levels, an indicator of muscle repair, was observed in all animals postprocedure but persisted in vector-injected rabbits until euthanization. A follow-up study suggested that this was directed against the human transgene expression in a foreign species. Overall, this study demonstrates diffusion of vector throughout the diaphragm after localized injections.

Introduction

Pompe disease (also glycogen storage disease type II acid maltase deficiency) results in a deficiency of the lysosomal enzyme acid α-glucosidase (GAA). The deficiency results in lysosomal and cytoplasmic glycogen accumulation in almost all tissues, including cardiac and skeletal muscles and phrenic motoneurons (Hirschhorn, 2001; DeRuisseau *et al.*, 2009). There are a broad range of clinical phenotypes that present at different ages that correlate to the amount of residual GAA activity (Raben *et al.*, 2002). The most severe form, classic infantile onset Pompe disease, most often results from a null or severe missense mutation resulting in complete or near-complete lack of GAA (Byrne *et al.*, 2011). Patients with this form of the disease present clinical symptoms within the first few months of life that include rapidly progressing skeletal muscle weakness, cardiomyopathy, and decline in ventilator function. Without medical intervention, these patients die because of cardiorespiratory within the first year of life (Kishnani *et al.*, 2006).

In 2006, enzyme replacement therapy (ERT) in the form of recombinant human acid alpha-Glucosidase (rhGAA) was approved as a treatment for Pompe disease. While ERT has been shown to improve symptoms in the most severe form of the disease, it has not been as successful at correcting the high rate of cardiorespiratory failure in these patients

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(Nicolino *et al*., 2009). Immune responses to rhGAA, especially in patients with a complete lack of endogenous GAA, along with complications arising from the frequent intravenous administration of the enzyme negatively, affect the efficacy of this treatment option (Kishnani *et al*., 2010). A more comprehensive treatment option is required for patients who do not fully respond to ERT.

Gene replacement therapy with recombinant adeno-associated viral (rAAV) vectors is an attractive treatment option because of its nonpathogenic properties, specific tissue tropism of unique serotypes, and stable gene transfer caused by persistent episomal vector genomes (Van Vliet *et al*., 2008). Studies have demonstrated that rAAV1 vectors transduce skeletal muscle more efficiently than rAAV2 vectors, and do not elicit a humoral immune response to the transgene protein (Chao *et al*., 2001; Mah *et al*., 2007). Currently, more than 90 clinical trials have begun using rAAV vectors (Ginn *et al*., 2013).

The $Ga^{-/-}$ mouse model has allowed for the study of not only the biochemical consequences of rAAV-*GAA* gene therapy but also the physiological improvements it imparts as well. The $Gaa^{-/-}$ mouse demonstrates many phenotypes of Pompe disease, including glycogen accumulation in the skeletal muscle, diaphragm, and heart; electrophysiological abnormalities; decreased cardiac function; increased left ventricular mass; and a decline in ventilatory function (Raben *et al*., 1998, 2003; Mah *et al*., 2007; DeRuisseau *et al*., 2009).

Our group has demonstrated that intramuscular delivery of rAAV1-*GAA* in $Gaa^{-/-}$ mice led to eight-times normal GAA activity with concomitant glycogen clearance and improved skeletal muscle contractility (Fraites *et al*., 2002). Gel-mediated direct delivery to the diaphragm of rAAV1-h*GAA* resulted in ~85% of normal GAA activity and clearance of glycogen from the diaphragm tissue, as well as increased diaphragm contractility and partially restored ventilatory function (Mah *et al*., 2010). In addition, delivery of rAAV1-cytomegalovirus (CMV)-h*GAA* to the diaphragm also resulted in more robust phrenic nerve efferent activity in $Gaa^{-/-}$ mice compared with the untreated group. This phrenic nerve activity may be more important than previously thought as demonstrated by the recent finding that glycogen accumulation in the central nervous system (CNS) and phrenic motor neurons may play a pivotal role in the attenuated ventilatory function seen in Pompe disease (DeRuisseau *et al*., 2009; Li *et al*., 2013).

These proof-of-concept studies using intramuscular diaphragm delivery of rAAV1-CMV-h*GAA* were the basis for the initiation of a preclinical study. A biodistribution and toxicology study was performed to test the acute toxicities of direct intradiaphragmatic injection of an rAAV2/1-h*GAA* driven by a CMV promoter (rAAV1- CMV-h*GAA*) in New Zealand white rabbits. Additionally, a complementary biodistribution and toxicity study in *Gaa* (129) $^{6neo/6neo}$ knockout mouse quadriceps at doses of 3.0×10^{9}, 3.0×10^{10}, and 3.0×10^{11} vector genomes (vg) was performed and is summarized in the Supplementary Results (Supplementary Data are available online at www.liebertonline.com/humc). For the large-animal preclinical study, 10 rabbits were divided into two dose groups—vehicle control and test article (1.5×10^{12} vg rAAV1-CMV-h*GAA*)—and euthanized on day 21. An additional study was performed to further elucidate the cause of the elevated creatine phosphokinase (CPK) levels found in the primary toxicology study at euthanization. This study consisted of two experimental arms, one vehicle control and one test article (4.6×10^{12} vg rAAV1-CMV-h*GAA*), of three female rabbits each. The rabbits were injected intramuscularly into the diaphragm through a less-invasive thoracoscopic approach, which is identical to the route of clinical vector administration. CPK assessments were performed at baseline, at postinjection, and on days 1, 3, 6, 9, 15, 31, and 45. Overall, these studies demonstrate diffusion of vector throughout the diaphragm after localized injections in large animals. Rodent studies confirm a biodistribution profile similar to other AAV1 intramuscular clinical programs.

Results and Discussion

Clinical trial

The currently ongoing clinical study is an open-label, single-center, sequential, two-arm, phase I/II study evaluating the safety and potential activity of a single administration of rAAV1-CMV-h*GAA* vector injected intramuscularly into the diaphragm for the purpose of diaphragm and motor neuron transduction. Three subjects per dose level (1.0×10^{12} and 5.0×10^{12} vg) will have the total dose distributed over six injection sites. Each arm of three patients are enrolled sequentially, and enrollment into the next higher dose level was dependent on the assessment of dose-limiting toxicity. The study population consists of 6 subjects, male or female, 2–12 years of age, who have been diagnosed with Pompe disease, received ERT, and have progressed to invasive ventilator dependence. Safety is the primary outcome and is assessed by measurement of changes in serum chemistries and hematology, urinalysis, blood assay for vector genomes, immunologic response to GAA and AAV, as well as reported subject history of any symptoms or adverse events.

Objectives and study design

The objective of this study was to determine the potential toxicity of AAV serotype 1 expressing the h*GAA* gene driven by a CMV promoter (rAAV1-CMV-h*GAA*) after intradiaphragmatic injection in New Zealand white rabbits.

Table 1. Summary of Primary Toxicology and Biodistribution Dose Groups in Rabbits

Group number	*Number of rabbits (rabbits/sex)*	*Substance*	*Total dose (vg)*	*Total dose volume (μl)*	*Dosing regimen*	*Route*	*Observation period*
1	3M/2F	Lactated Ringer's	—	600	3 sites, 1 time	IM-Dia	21 days
2	3M/2F	rAAV1-CMV-h*GAA*	1.5×10^{12}	600	3 sites, 1 time	IM-Dia	21 days

Dia, diaphragm; IM, intramuscular; vg, vector genomes.

Table 2. Testing Schedule of the Primary Toxicology and Biodistribution Study

Tests performed in rabbits	*LRS-treated group*	*rAAV1-CMV-hGAA (1.5×10^{12} vg/ml) group*
Organ histopathology	Day 21	Day 21
Clinical pathology		
CBC	Day 0, 21	Day 0, 21
Serum chemistry	Day 0, 21	Day 0, 21
Biodistribution		
Blood	Day 0, 1, 21	Day 0, 1, 90
Tissue	Day 21	Day 21
Body weights	Day 0, 1, weekly, 21	Day 0, 1, weekly, 21
Clinical observations	Daily	Daily

CBC, complete blood counts.

The study comprised two experimental groups, each arm consisting of five rabbits, which included three males and two females at the euthanization time point of 21 days (±2 days; Table 1). The rabbits were injected intramuscularly within the diaphragm at three sites with either Lactated Ringer's (vehicle control group) or rAAV1-CMV-h*GAA* at a total dose of 1.5×10^{12} vg (3.75×10^{11} vg/kg). The primary safety endpoints of this study (Table 2) were the histopathologic examination and detection of vector genomes in the blood, spleen, kidney, jejunum, pancreas, lung, cerebellum, medulla oblongata, spinal cord, heart, mesenteric lymph nodes, liver, distal esophagus, skeletal muscle left quadriceps, diaphragm muscle distant from injection site, and diaphragm muscle near injection site ($N=3$). Clinical assessments were also measured by complete blood counts (CBC) and serum chemistry analysis, and circulating antibodies to the AAV1 capsids and h*GAA* transgene were measured by enzyme-linked immunosorbent assay. An additional rodent study in the *Gaa* $(129)^{6neo/6neo}$ knockout mouse quadriceps is summarized in Supplementary Table S1 (Supplementary data are available online at www.liebertonline.com/humc), and biodistribution and toxicity measurements were nearly identical to the large-animal study.

To support future clinical trials where the route of injection would be the diaphragm, we needed to use a large-animal model with a diaphragm thickness that was feasible for injection. The diaphragms of mice and rats are too thin; therefore, we used the rabbit as a model for this purpose. The thickness of a rabbit diaphragm is 2–3 mm and allows for a 26-gauge needle or smaller to be placed between the muscle layers.

Summary of data

Route of injection validation and vector spread. Before initiation of the primary toxicology and biodistribution study, it was necessary to evaluate the feasibility of directly injecting the diaphragm (2–3 mm thickness) of the rabbit model by contrast injection and visualization by X-ray. Injection of contrast alone indicated that a liquid substance had the potential for dispersing beyond the initial site of injection (data not shown). To assess the ability of AAV-GFP to distribute throughout the rabbit diaphragm tissue and express the transgene (GFP), 3.3×10^{9} vg were injected into three sites by direct visualization. Twenty-one days postadministration, the animals were euthanized and diaphragm was sectioned and stained for GFP (Fig. 1). Identical sections were also assayed for vector genomes by quantitative polymerase chain reaction (PCR). GFP could be detected at the injection sites (Fig. 1, sections C, F, and I) and minimally in adjacent sites (Fig. 1, section A). Vector genomes were detected at the sites of injection, up to 20,000 copies/μg genomic DNA (gDNA), and throughout the diaphragm at reduced copy numbers as the distance between the injection site and tissue sample increased.

Primary toxicology and biodistribution study. Relevant tests and results used to evaluate the safety of the AAV1-CMV-h*GAA* vector are presented in the following section. Expected, nondetermining, and rodent results are further outlined in the Supplementary Results.

Histopathology findings: All 10 animals survived to the scheduled necropsy time point. There were gross findings on the diaphragm of multiple animals, which were noted as possible scar tissue. This finding was noted in two of the vehicle group males, one vehicle group female, and one AAV1-CMV-h*GAA* group female. In some animals, this gross finding was noted at more than one injection site. In all cases, this gross finding was correlated with fibroplasia of the serosal surface of varying severity. This fibroplasia occasionally included dislodged skeletal muscle myocyte fragments and suture material.

Microscopically, the three injection sites in the diaphragm of each animal had similar findings. There was an increase in severity of mononuclear cell infiltration, degeneration of skeletal muscle, and regeneration of skeletal muscle in the AAV1-CMV- h*GAA*-treated animals compared with the

Table 3. Summary of Vector-Related Histopathology Findings in the Diaphragm

Diaphragm #1, ventral	
Infiltration, mononuclear Cell	++(2), +++(3)
Degeneration, muscular	++(3), +++(2)
Regeneration, muscular	+(1), ++(3)
Diaphragm #2, mid	
Infiltration, mononuclear Cell	++(1), +++(3), ++++(1)
Degeneration, muscular	++(1), +++(3)
Regeneration, muscular	+(1), ++(1)
Diaphragm #3, dorsal	
Infiltration, mononuclear Cell	++(2), +++(2), ++++(1)
Degeneration, muscular	++(5)
Regeneration, muscular	++(1)
Esophagus	
Infiltration, mononuclear cell; tunica muscularis	+(2), ++(1)
Degeneration; tunica muscularis	+(2)
Regeneration; tunica muscularis	+(1)

+, minimal; ++, mild; +++, moderate; ++++, marked (number of subjects).

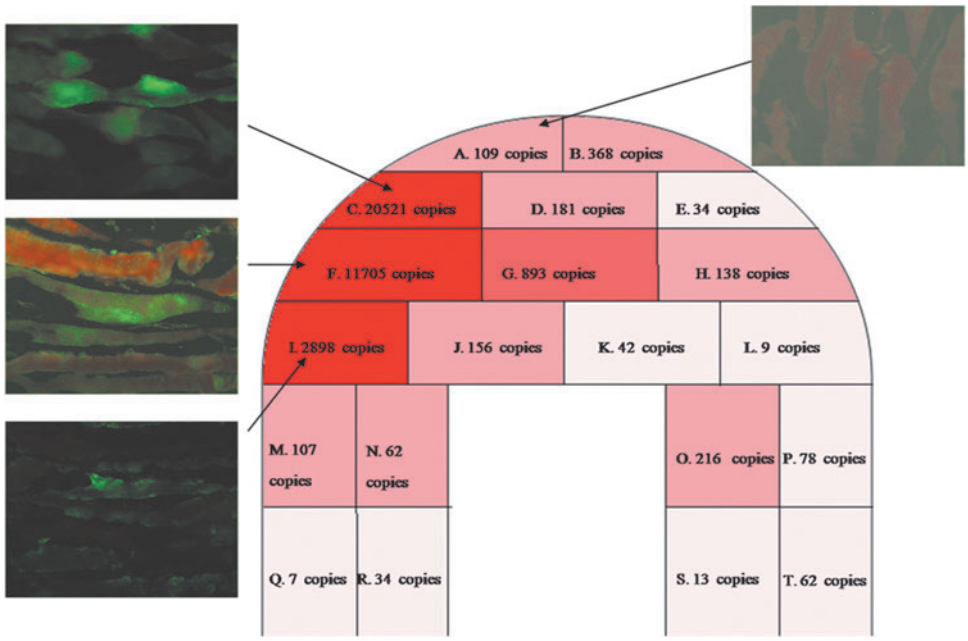

FIG. 1. Ease of injection and vector spread throughout the rabbit diaphragm. A rabbit was injected at three sites (sections C, F, and I) by direct visualization with 3.3×10^{9} vg per site with AAV1-GFP. Three weeks after administration, samples were taken from 20 sites for GFP detection by immunohistochemistry and vector genomes by real-time qPCR. Substantial GFP was detected at the sites of injection and waned in distal sites. Vector genomes were detected in the highest copy number at the sites of injection and minimally at distal locations. Overall, the number of vector genomes decreased as the sampling moved further from the sites of injection (decrease in blush). AAV, adeno-associated virus; qPCR, quantitative polymerase chain reaction; vg, vector genomes.

vehicle control group (Table 3). These findings were considered to be related to the test article. Fibroplasia of one of the serosal surfaces was present in at least one injection site in all animals, regardless of treatment group (Fig. 2B and C). The fibroplasia was relatively exuberant, but this degree of response has been seen in rabbits (Laje *et al.*, 2008; Otto *et al.*, 2008). There was no overall difference in severity between groups, and this finding was considered secondary to trauma and not related to administration of test article (Table 4).

In the esophagus, there was an increase in incidence of mononuclear cell infiltration in the outer longitudinal muscle layer of the tunica muscularis in the AAV1-CMV-h*GAA*-treated group (two out of five subjects; Fig. 2A). The similarities in the findings in the esophagus and the injection sites support the conclusion that this finding may be test article related.

Clinical pathology: Clinical chemistry and hematology results produced equivocal parameters for all measured values between groups and when comparing baseline and euthanization (day 21) values. Of note, results for CPK increased from baseline to euthanize in the vector and control

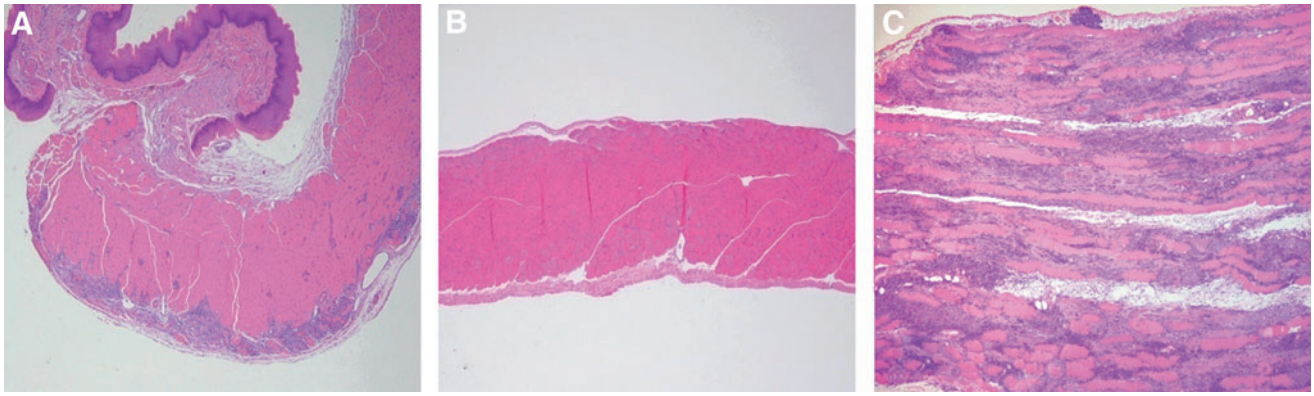

FIG. 2. Inflammatory cells in the diaphragm and distal esophagus. At euthanization on day 21, inflammatory cells were seen on the distal esophagus **(A)** and presented as mononuclear cell infiltrations in the outer longitudinal muscle layer of the tunica muscularis of the esophagus. In the diaphragm **(C)**, at the sites of injection, an increase in severity of mononuclear cell infiltration, degeneration of skeletal muscle, and regeneration of skeletal muscle was observed in comparison to uninjected diaphragm muscle **(B)**.

Table 4. Summary of Treatment-Related Histopathology Findings in the Diaphragm

	LRS	*AAV1-CMV-hGAA*
Diaphragm #1, ventral		
Fibroplasia, serosa	+(2), ++(1), +++(1), ++++(1)	+(2), ++(1)
Fibrosis	+(1)	+(2)
Granuloma, foreign body	—	+(1)
Diaphragm #2, mid		
Fibroplasia, serosa	++(3), ++++(1)	+(1), +++(2)
Degeneration, muscular	+(1)	—
Diaphragm #3, dorsal		
Fibroplasia, serosa	+++(2), ++++(1)	+(1), ++(3)
Infiltration, mononuclear cell	+(1)	—
Fibrosis	+(1), ++(1)	—
Lung		
Fibrosis, pleural	++(2)	++(1)

groups—indicative of muscle damage and stress to the diaphragm and/or latissimus dorsi and serratus ventralis thoracic muscles. There was a moderately higher increase in CPK results in vector-injected subjects at euthanization (2,239 ± 1,388 U/liter) compared with controls (976 ± 543 U/liter) (normal range 141–579 U/liter). This finding could potentially be related to the vector and/or transgene.

Vector dissemination in the diaphragm: In the diaphragm, vector genomes were localized to the three injection sites. Each site (Fig. 3, injection sites #1–#3) depicted represents the same area of the diaphragm for each rabbit. By group mean, each injection site maintained at least 1.0×10^5 vector copies per μg gDNA at the time of euthanization. An additional sample taken from the contralateral side of the diaphragm, away from the sites of injection, showed the presence of vector genomes of at least 1.0×10^4 vg/μg gDNA, an indication of vector spread throughout the organ.

Evaluation of increased CPK. During the toxicology study, elevated serum CPK persisted in both the vector-treated and control groups. To differentiate elevated CPK values because of surgical procedures from local vector-related inflammation, a further study was initiated with the use of fluoroscopy. Two cohorts of three female rabbits were studied in which the surgical approach was modified to better represent the expected clinical administration procedure. This included a less invasive endoscopic procedure requiring one minor incision and three needle punctures through the chest wall. In addition, injection sites were not marked by suture, but recorded by fluoroscopy (Fig. 4). All vector-treated animals received a higher dose than what was used in the initial toxicology study, 4.6×10^{12} vg AAV1-CMV-h*GAA* ($\sim1.15\times10^{12}$ vg/kg), distributed over three sites or Lactated Ringer's (vehicle). Serum samples were collected for CPK analyses at baseline, at 1–3 hr post-procedure, and on days 1, 3, 6, 9, 15, 21, 31, and 45.

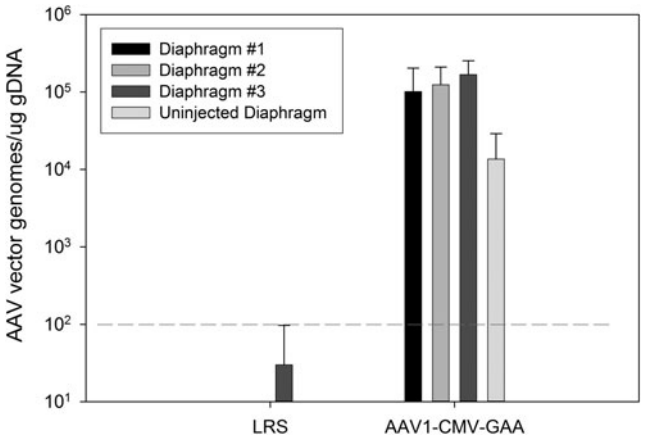

FIG. 3. Real-time PCR for vector genomes in the diaphragm. The total copies of vector DNA/μg gDNA detected in the diaphragm are presented. The positive cutoff limit, $\geq$100 vg/μg gDNA, is presented by the horizon trend line (dashed). gDNA, genomic DNA.

All animals survived the procedure without complication. From the data shown in Fig. 5, there is no significant difference between the vector- and vehicle-control-treated animals on day 1 ($p=0.313$) and day 15 ($p=0.171$) at the time of peak CPK levels. Both groups of animals had elevated CPK levels immediately following the procedure, which peaked at 24 hr and was resolved by day 6. By day 15, the vector-treated group showed a second elevation in CPK values, which resolved by day 45 to equivalent levels of the control group. These data delineate the time course of CPK changes that were not revealed during the primary study because of sampling time points and further show the safety and resolution of toxicity indicators over time.

Conclusions

The primary safety endpoints of this study were the histopathological examination of tissues and detection of vector genomes in the blood and peripheral organs. Humoral immune responses were detected by the presence of antibodies to AAV1 and hGAA in the serum. CBC and serum chemistry analysis were performed as a measure of acute toxicity as a result of the procedure, vehicle, or vector. No clinical observations of adverse reactions were noted from time points around the procedure until euthanization. There was up to a 2,500-fold increase in circulating anti-AAV1 antibodies directed to the vector capsids. In addition, up to an 18-fold increase in antibodies against the GAA protein was generated. Injection sites maintained up to 1.0×10^5 vg/μg gDNA,

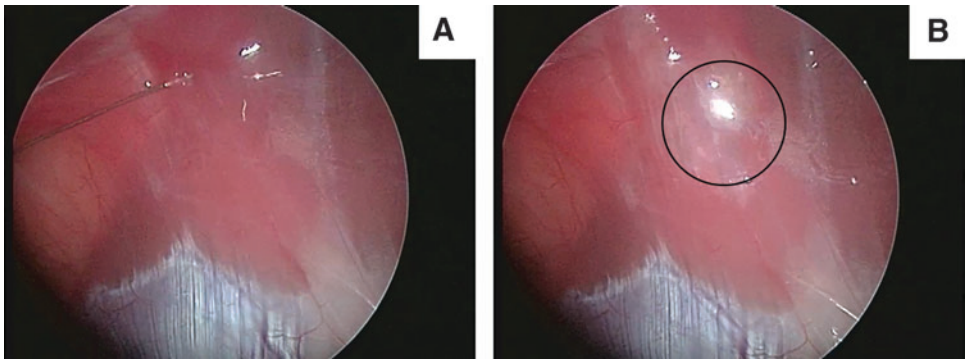

FIG. 4. Endoscopy-guided vector administration in the rabbit diaphragm. **(A)** Endoscopy was used from the thoracic side of the diaphragm to guide the injection needle through the chest wall. **(B)** Confirmation of vector administration by the formation of a bleb (circle) and potential leakage.

whereas uninjected sites had up to 1.0×10^4 vg/μg gDNA. Vector DNA was present in blood at 24 hr postinjection of up to 1.0×10^6 vg/μg gDNA followed by a decrease to 1.0×10^3 vg/μg gDNA at euthanization on day 21. Nominal amounts of vector DNA were present in peripheral organs, including the brain, spinal cord, gonads, and skeletal muscle. Upon histopathological examination, fibroplasias of the serosal surface were noted at diaphragm injections sites of both groups. In addition, an increase in mononuclear cell infiltration in the diaphragm and esophagus was seen in vector-injected animals. Elevated CPK levels, an indicator of muscle repair, were observed in all animals postprocedure, but persisted in vector-injected rabbits until euthanization. A follow-up study suggested that this elevation was biphasic, with the second CPK elevation corresponding to a cytotoxic cellular response being directed against the human transgene expression in a foreign species and therefore elevated CPK values are not expected during the clinical trial. This conclusion was further supported by the finding of significant anti human GAA antibodies at euthanization. Overall, this study demonstrates that diffusion of vector throughout the entire diaphragm is possible after a few localized injections.

In regard to the clinical trial, an evaluation and potential adjustment to the volume and rate of injection in human subjects is warranted by the toxicology data. In addition, we would expect to observe the presence of vector genomes in circulation for at least 21 days postinjection, as well as a humoral immune response to the AAV1 vector capsids. However, as opposed to the response seen in rabbits, we would not expect to see an increase in anti-hGAA antibodies in humans.

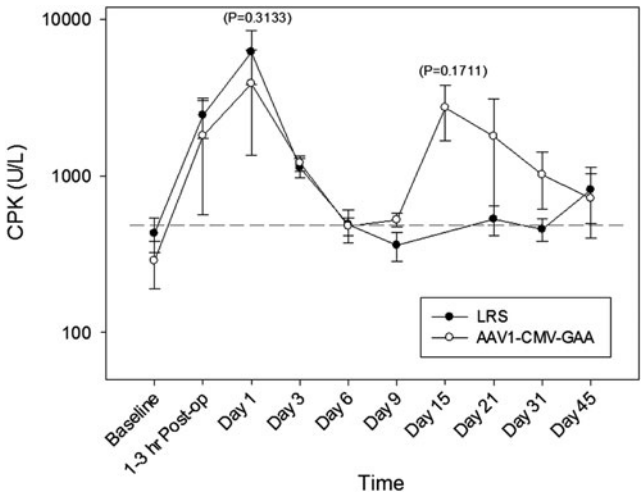

FIG. 5. CPK levels in rAAV1-CMV-h*GAA* unilaterally diaphragm-injected rabbits. Serum CPK is plotted as groups mean ± standard deviation for each group and upper limit of the normal reference range indicated (dashed trend line). Significance was determined on days 1 and 15 by unpaired *t*-test. CPK, creatine phosphokinase.

Two similar toxicology studies and subsequent initiated clinical trials have used the AAV1 vector as a method to introduce transgenes into muscle tissue. Flotte *et al.* (2007) aimed to transduce skeletal muscle tissue with an AAV1-*AAT* vector for the expression of alpha-1 antitrypsin (AAT). Preclinical data from mice and rabbits showed a similar biodistribution profile in the blood and peripheral tissues. There were no histopathologic observations attributed to vector alone. Furthermore, both studies showed no vector-related changes in CBCs or clinical chemistries showing a no-effect conclusion of hepatic or renal toxicity. Finally, although not a current concern in the proposed ongoing clinical trial for Pompe disease, the potential for vertical transmission of the vector, shown through real-time PCR of fractionated semen samples from rabbits, demonstrated a very low likelihood of this occurring. Another clinical study for limb girdle muscular dystrophy using an AAV1-alpha-sarcoglycan (AAV1-α*SG*) was initiated as a result of additional toxicity studies (unpublished) in C57Bl/6 and αSG knockout mice (Mendell *et al.*, 2009). These animals were euthanized on 30, 60, 90, 180, and up to 540 days postinjection in the quadriceps. The primary goal of the study was observing chronic toxicities and carcinogenicities from the spread of vector to peripheral organs. These studies concluded that no histopathology findings were related to the vector.

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Author Disclosure Statement

B.J.B. and the University of Florida have a financial interest in the use of AAV therapies and own equity in a company (AGTC, Inc.) that might, in the future, commercialize some aspects of this work. The other authors declare no conflicts of interest.

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