Peripheral transvenular delivery of adeno-associated viral vectors to skeletal muscle as a novel therapy for hemophilia B

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Abstract

Muscle represents an important tissue target for adeno-associated viral (AAV) vector-mediated gene transfer of the factor IX (FIX) gene in hemophilia B (HB) subjects with advanced liver disease. Previous studies of direct intramuscular administration of an AAV-FIX vector in humans showed limited efficacy. Here we adapted an intravascular delivery system of AAV vectors encoding the FIX transgene to skeletal muscle of HB dogs. The procedure, performed under transient immunosuppression (IS), resulted in widespread transduction of muscle and sustained, dose-dependent therapeutic levels of canine FIX transgene up to 10-fold higher than those obtained by intramuscular delivery. Correction of bleeding time correlated clinically with a dramatic reduction of spontaneous bleeding episodes. None of the dogs (n=14) receiving the AAV vector under transient IS developed inhibitory antibodies to cFIX; transient inhibitor was detected following vector delivery with no IS. The use of AAV serotypes with high tropism for muscle and low susceptibility to anti-AAV-2 antibodies allowed for efficient vector administration in naïve dogs and in the presence of low-titer but not high-titer anti-AAV2 antibodies. Collectively, these results demonstrate the feasibility of this approach for the treatment of HB and highlight the importance of IS to prevent immune responses to the FIX transgene product.
**Introduction**

Adeno-associated viral (AAV) vectors have demonstrated excellent safety and efficacy profiles as gene transfer tools in numerous preclinical studies \(^1\text{-}^{10}\). More recently, clinical translation of these results into humans also generated promising results \(^{11}\text{-}^{22}\). Hemophilia B represents an ideal disease model for AAV-mediated gene transfer studies; results in large and small animal models of the disease showed sustained expression of the factor IX (FIX) therapeutic transgene and correction of the disease phenotype after AAV-mediated gene transfer to muscle \(^4\text{-}^{5},^{23},^{24}\) or liver \(^6\text{-}^{7},^{10},^{25}\). Early clinical work on AAV gene transfer to muscle for hemophilia B in severely affected subjects demonstrated that this approach is feasible \(^{16},^{19}\) and led to long-term expression of the FIX transgene product \(^26\). However, we have shown that direct intramuscular administration of an AAV2 vector encoding the FIX transgene (AAV2-FIX) does not result in therapeutic levels of circulating FIX in humans at the doses tested \(^19\). Concurrently, studies in preclinical animal models of hemophilia B mice and dogs indicate that further dose-escalation of AAV-FIX vectors injected IM is associated with higher risk of development of immune responses to the transgene product, especially if large amounts (\(>1\times10^{12}\)vg) of vector are injected at a single site \(^{24},^{27},^{28}\).

One possible approach to overcoming the problem of reaching therapeutic levels of expression of the FIX transgene is to target a different tissue. Liver, for example, is an ideal target for the production of FIX, as it is the main site of synthesis of this protein. Results in experimental animal models and in severe hemophilia B subjects confirmed the dose advantage of liver vs. muscle (direct intramuscular injection) \(^6,^{20},^{29}\). In human
subjects, in particular, doses of vector delivered through the hepatic artery, comparable to those that were subtherapeutic in muscle (in the range of $10^{12}$ vg/kg) resulted in levels of circulating FIX up to 12% of normal $^{20}$. However, targeting the liver for the treatment of hemophilia presents two major obstacles. The first is the host immune system $^{30}$; experience in humans showed that the intravascular administration of an AAV2 vector through the hepatic artery results in only transient expression of the FIX transgene product, due to a capsid-specific CD8+ T cell response $^{20,31}$. While this obstacle may be overcome with the use of transient immunosuppression $^{10,30,32}$, or the use of AAV serotypes less immunogenic than AAV-2 $^{30}$, another obstacle to hepatic gene transfer is represented by the disease state of the liver. Due to the widespread use of hepatitis C virus (HCV)-contaminated plasma-derived products for replacement therapy for hemophilia prior to 1985, more than 90% of severe hemophilia patients were infected, and many now manifest variable degrees of liver disease due to HCV infection $^{33}$. The safety of administering AAV vectors to the liver in the presence of advanced liver disease has not been established. Thus, in the presence of liver disease, muscle is still a highly attractive target tissue for AAV gene transfer for hemophilia B.

We previously showed that it is possible to transduce large areas of skeletal muscle by injecting an AAV vector through the vasculature $^{34}$. This delivery method, which relies on the permeabilization of the vascular endothelium with vasoactive drugs like papaverine and histamine, resulted in circulating levels of canine FIX transgene product up to 15% in hemophilia B dogs at a dose of $3.7 \times 10^{12}$ vector genomes (vg)/kg. While a similar approach would not be amenable for clinical development, as the drugs used to
increase vascular permeability are not approved for human use, these results are at least a proof of principle that the approach is feasible and can lead to sustained expression of the FIX transgene at therapeutic levels.

A non-invasive pressurized infusion of vector-containing solution through the superficial saphenous vein without surgical or pharmacological intervention has been described. In this study, atraumatic tourniquet placement at the groin level, combined with pressurized flow of saline from a distal catheter, caused afferent flow through the valves within the major veins of the extremity but locally retrograde flow through the valveless venules, culminating in extravasation of vector across the endothelium into the interstitium followed by widespread transduction of muscle in rats and dogs.

Here we report sustained, therapeutic canine FIX transgene expression following delivery of AAV vectors through afferent transvenular retrograde extravasation (ATVRX) to skeletal muscle in sixteen severe hemophilia B dogs. Collectively these data provide the basis for future translational studies in humans with hemophilia B and liver disease.
Material and Methods

Vector production

Recombinant AAV vectors were produced by a triple transfection protocol as previously described\textsuperscript{5}, using plasmids expressing canine FIX (cFIX) under the control of the cytomegalovirus (CMV) promoter/enhancer, a second plasmid supplying adenovirus helper functions, and a third plasmid containing the AAV-2 \textit{rep} gene and the AAV-2 or AAV-6 \textit{cap} genes. Vectors were purified by repeated CsCl density gradient centrifugation.

Animals and intravascular vector delivery procedure

Sixteen adult hemophilia B dogs housed at the colony of the Department of Pathology and Laboratory Medicine at the University of North Carolina (UNC)-Chapel Hill were used in this study. These dogs exhibit severe hemophilia B, FIX levels <1%, due to a missense mutation in the \textit{F9} gene, resulting in detectable levels of mRNA transcripts but no circulating protein \textsuperscript{36}. Two normal dogs, DLAM 3 and DLAM 4, were also included in the study to test vector genomes distribution within the target and contralateral limb as well as in the liver and spleen. For the ATVRX procedure, sedation was achieved with sodium pentothal (11 mg/kg to 29 mg/kg) and anesthesia was maintained with 1% to 4% isoflurane. Pooled normal canine plasma was given to hemophilia B dogs prior to venous puncture at doses calculated to achieve at least 20% normal cFIX plasma levels.

Blood inflow to the target limb was transiently blocked by placing a tourniquet at the level of the groin, and further adjusting it until the femoral pulse was no longer detectable. An intravenous catheter (14-18 gauge) was placed under direct visualization.
into the lumen of a distal branch of the peripheral saphenous vein on the dorsum of the paw vein. At time zero, the AAV vector, diluted in 20 ml of pre-warmed 37°C sterile PBS per kg of body weight, was infused rapidly (~3 min) under elevated hydrostatic pressure, in the range of 300 mm Hg, using a sphygnomanometer placed around the bag containing the vector solution. Fifteen minutes after the pressurized vector delivery the tourniquet was released. All the animal procedures in this study were approved by the University of North Carolina at Chapel Hill’s Institutional Animal Care and Use Committee.

**Clotting assays, FIX antigen and antibodies to FIX**

Whole blood clotting time (WBCT) and activated partial thromboplastin time (aPTT) were performed as previously described 27. Canine FIX antigen concentration was determined by enzyme-linked immunosorbent assay (ELISA) using matched-pair antibodies to cFIX (Affinity Biologicals, Hamilton, ON, Canada). FIX clotting activity was determined by one-stage aPTT; plasma test samples were mixed with cFIX-deficient plasma and the aPTT values were compared with a reference standard consisting of serial dilutions of normal canine plasma mixed with canine FIX-deficient plasma. Neutralizing antibodies to canine FIX were performed by Bethesda assay as previously reported 34. Non-inhibitory antibodies to FIX were measured by an ELISA specific to dog IgG subclasses 27.
Neutralizing antibody assays to AAV-2 capsid protein

AAV2-specific neutralizing antibody titers were determined as previously described using serial dilutions of serum samples. Data are reported as the serum dilution at which a 50% inhibition of AAV transduction was measured.

Systemic and local toxicity

Hematologic and biochemical analysis of blood and serum samples for liver and kidney function tests, and muscle enzymes were performed as previously described.

Histology, immunohistochemistry, and muscle transduction

Muscle biopsies were analyzed as previously described. Briefly, serial cryosections (5µm-10µm) were stained for cFIX expression using a rabbit anti-cFIX antibody (Affinity Biologicals, Canada) at a concentration of 4µg/mL (1:500 dilution) followed by a swine anti–rabbit IgG HRP-conjugated (Dako, Denmark) as secondary antibody used at a dilution of 1:500.

To determine vector genome copy number in muscle, and other organs for which AAV-2 vectors have high tropism, tissues were collected and snap frozen in liquid nitrogen from normal dogs 3 months after ATVRX vector delivery. Determination of transgene copy number was performed by real-time quantitative PCR using a primer and probe set on the canine FIX cDNA which did not amplify the genomic cFIX gene.
Results

Transvenular delivery of AAV-cFIX to skeletal muscle of HB dogs results in long-term correction of the disease phenotype

Hemophilia B dogs from the UNC-Chapel Hill colony, with FIX activity <1% and no circulating antigen, were used in this study. In the first experiment, designed as a dose escalation study, eight hemophilic dogs received a single administration of an AAV-2 vector expressing cFIX through the saphenous vein via ATVRX (Table 1). Animals were divided into a low dose cohort, receiving a vector dose of 1x10^12 vg/kg (n=3); a mid dose cohort, receiving a vector dose of 3x10^12 vector genomes (vg)/kg (n=3); and a high dose cohort, receiving a vector dose of 8.5x10^12 vg/kg (n=2). Vector was administered under transient immunosuppression (IS) with cyclophosphamide as previously described 34. Administration of the vector-containing solution was well tolerated and no signs of persistent local or systemic toxicity were observed. Hemostasis was controlled with infusion of pooled canine plasma before the skin incision needed for catheter placement into the saphenous vein; no excessive bleeding was observed during this procedure. Rapid fluid injection (~3 min) resulted in transient leg swelling with increased limb circumference and tissue pressure. Fifteen minutes after vector infusion the tourniquet was released with no signs of cardiac or blood pressure abnormalities. Dogs recovered from the light sedation and showed no evidence of preferential use of the non-perfused limb during immediate (within 15-30 minutes post-injection) or late ambulation.

Administration of the AAV2-cFIX vector at a dose of 1x10^12 vg/kg resulted in circulating cFIX antigen levels between <1% and 3% of normal (Figure 1A). These values were in
agreement with the cFIX clotting activity, as a sustained shortening of the WBCT and aPTT was observed in all animals (Figure 1B and C). Because the cFIX levels measured in the low dose cohort were at the threshold for therapeutic efficacy for hemophilia B (1% of normal), the next dose cohort was injected with a 3-fold higher dose (3x10^{12} vg/kg). In this group, cFIX transgene antigen levels peaked at 5 to 8% of normal and at plateau ranged from 2 to 6% (Figure 1A). Detection of cFIX by ELISA was associated with a dose-dependent shortening of the WBCT and aPTT (Figure 1B and C). Long-term follow-up of the animals from the low and mid dose cohorts showed stable circulating FIX levels for periods of up to 4 years, with observation ongoing. This reflected a marked improvement in the clinical outcome, i.e. spontaneous bleeding episodes reduced by >90% compared to expected 37 bleeding rates (Table 1). No neutralizing antibodies to the cFIX transgene product were detected by Bethesda assay in any of the dogs from the low and mid dose cohorts, even after discontinuation of the immunosuppression regimen at 4 to 6 weeks post vector delivery. No antibodies to cFIX were detected, with the exception of low-titer non-neutralizing IgG2 appearing >6 months after vector administration (not shown). No elevation of the muscle enzyme creatinine phosphokinase (CPK) was detected in these animals at any time point (Figure 1D).

Percutaneous muscle biopsies from the vector-injected limb collected ~2 years post-injection from dog H48 showed local expression of cFIX protein detected by immunofluorescence (Figure 2A-D). At this time point no evidence of muscle inflammation was observed in muscle sections (Figure 2E). Complete vector distribution
within skeletal muscle of the target limb vs. the contralateral limb was evaluated in two normal dogs that received an AAV2-cFIX vector via ATVRX at a dose similar to the mid dose cohort animals. Results are summarized in Table 2; widespread, high levels of muscle transduction were detected in muscle collected from different areas of the target limb 3 months after ATVRX vector delivery. In comparison, negligible vector transduction was detectable in the contralateral (non-injected) limb and other organs with minimal dissemination to the spleen, liver and kidney (unpublished data).

**High AAV2-cFIX vector doses delivered via ATVRX result in the development of non-neutralizing anti-cFIX antibodies, even in the presence of transient immunosuppression**

ATVRX delivery of the AAV2-cFIX vector to hemophilia B dogs in the high dose cohort (8.5x10^{12} vg/kg, Table 1) did not result in the expected dose-response levels of cFIX. The first dog injected from this cohort, H24 showed circulating cFIX levels up to 6% two weeks following vector injection; this was followed by a progressive decline to ~1% of normal (Figure 3A). Despite transient IS with cyclophosphamide, the animal developed a non-neutralizing IgG2 antibody specific to cFIX (Figure 3B). Serial screening for inhibitory antibodies, performed by Bethesda assay, was persistently negative (not shown). Despite the presence of non-neutralizing antibodies to cFIX, the dog showed sustained partial shortening of WBCT and aPTT values (Figure 3C and D), suggesting that the antibody enhanced the clearance of the cFIX transgene product from the bloodstream but did not inhibit its activity. During the period of observation, H24 experienced two bleeding episodes, which were successfully treated with pooled canine
plasma (Figure 3B, arrows). Recovery and half-life of the infused canine plasma, administered at a dose sufficient to achieve 25% of normal levels, were normal when measured at day 779 post-ATVRX.

A second dog, M25, was injected in the same high dose cohort, and showed similar results. Shortly after AAV2-cFIX vector delivery, 2 weeks, M25 plasma levels of cFIX peaked at 4% of normal and then reached a plateau of ~1% of normal (Table 1 and Figure 3A). At the same time a non-neutralizing IgG2 antibody specific to cFIX was detected by ELISA (Figure 3B) and Western blot (data not shown). Similar to H24, M25 did not have any detectable Bethesda titer at any time point (not shown) and showed sustained partial correction of WBCT and aPTT values (Figure 3C and D). As was the case at lower doses, none of the high dose cohort dogs had CPK elevation (Figure 3E).

Transient immunosuppression is required to prevent immune responses to the cFIX transgene product

To test the role of transient IS with cyclophosphamide in preventing humoral responses to the cFIX transgene product after ATVRX gene transfer, two dogs were injected with the AAV2-cFIX vector via ATVRX at a dose of 3x10^{12} vg/kg with no IS (Table 1). In one of the two dogs (J03), sustained expression of cFIX antigen levels, ~2% at plateau, with improvement of the WBCT and aPTT, was observed (Figure 4A, B, and C). Only one episode of spontaneous bleeding was observed during the long-term follow up in this animal, confirming the correction of the bleeding phenotype (Table 1). Around three months after transduction J03 developed a low titer non-neutralizing anti-cFIX IgG2
antibody that did not influence the correction of the bleeding phenotype or the response to cFIX replacement (Figure 4D); no Bethesda titer was detected in this animal throughout the observation period.

A second dog (J62) from the same experimental group had a peak in cFIX transgene expression at day 14 post-ATVRX injection with subsequent return to baseline levels thereafter for a period of ~140 days (Figure 4A). This was followed by a slow continuous increment in cFIX antigen levels over time to plateau levels of ~2% of normal (Table 1 and Figure 4A). The transient loss of cFIX transgene expression was due to the formation of an inhibitory antibody that peaked at 1.5 BU at day 35, then slowly returned to titers < 1 BU (day 78 and thereafter). This dog developed an anti-cFIX IgG1 antibody with similar kinetics to the inhibitory antibody measured by Bethesda assay (Figure 4E). Whether the appearance of inhibitor in this dog was associated with local muscle inflammation is not clear, however the absence of CPK elevation or evidence for long term muscle histological changes, do not support this hypothesis. The animal also developed an IgG2 antibody response to cFIX, however this response was detected early after gene transfer (peaking at day 28) and declined slowly in titer without completely disappearing. After day 200, with the disappearance of the neutralizing antibody to cFIX, cFIX antigen levels increased and both the WBCT and the aPTT showed correction (Figure 4B and C). Infusion of pooled canine plasma to treat bleeding (the animal had 4 bleeding episodes from day 291 to day 363) resulted in increasing levels of antibody titers and decreased levels of cFIX antigen (Figure 4E). Long-term follow-up of animal J62 showed cFIX antigen levels >2% and partial correction of both WBCT and aPTT.
after clearance of the inhibitory antibody (Figure 4B and C). During this period there were no spontaneous bleeding episodes even in the absence of pooled plasma infusion. While all animals developed anti-capsid antibodies upon vector administration, no T cell responses, measured with an IFN-γ ELISpot assay, were detectable (unpublished data).

Successful expression of cFIX via transvenular delivery of AAV-6 in naïve HB dogs and HB dogs with neutralizing antibodies to AAV-2 capsid

We tested the feasibility of the readministration of AAV-2 or AAV-6 vectors encoding cFIX to hemophilia B dogs previously injected with AAV-2 vectors intramuscularly or intravenously. Vector readministration was performed via ATVRX under transient IS with cyclophosphamide as described above. Two dogs, exposed to AAV-2 vector by IM injection >5 years before the ATVRX procedure, exhibited a neutralizing antibody titer (NAB) to AAV-2 of 1:300 (dog D32, 5.7 years old) and 1:30 (dog B46, 7.6 years old). Both animals at the time of readministration had circulating cFIX levels of <1% and received an AAV2-cFIX vector at a dose of 4 x10^{12} vg/kg via ATVRX. No increase in FIX antigen or activity levels was observed after vector readministration, and bleeding episode frequency remained unchanged (Figure 5A and B; Table 3). Screening for neutralizing and non-neutralizing antibodies to cFIX was negative (not shown) and no evidence of muscle or liver damage was observed (Figure 5C and data not shown).

These findings suggest that NAB to the AAV-2 capsid prevented successful gene transfer by ATVRX of the same serotype vector, AAV-2. Dog B46 died at day 175-post ATVRX of bleeding from a necrotic lesion of the nipple first diagnosed 2 years earlier, further confirming the lack of efficacy following AAV2-cFIX readministration.
Two additional animals previously exposed to AAV-2 vectors intravascularly received 1.5×10^{12} vg/kg of an AAV6-cFIX vector via ATVRX (Table 3). The titers of NABs to the AAV-2 capsid prior to the readministration of the AAV-6 vector were 1:100 (E59, 4.6 years old) and 1:1000 (H27, 2.4 years old). While the dog with a NAB titer of 1:100 (E59) showed an increase in cFIX levels from <1% to ~5% of normal, the animal with the higher titer NAB to AAV-2 (H27, 1:1000) did not have any evidence of successful muscle transduction after AAV6 ATVRX (Figure 5D). Canine FIX expression data correlated well with WBCT in both dogs (Figure 5E) with good correction of the disease phenotype in E59 and no improvement in H27. No neutralizing antibodies to cFIX were observed in these dogs; H27 developed a low-titer non-neutralizing IgG2 antibody specific to cFIX which resulted in increased clearance of the protein without compromising the correction of the bleeding phenotype (Table 3 and Figure 5E). No muscle (Figure 5F) or liver toxicity was documented in these dogs. Thus, AAV-6 may be an attractive strategy for muscle-directed gene transfer to overcome the presence of low to medium NAB to AAV-2; however, the strategy does not seem to be effective in the presence of high-titer NAB, probably due to cross reactivity of neutralizing antibodies between alternate AAV serotypes.

Finally, the efficacy of AAV-6 in transducing muscle was further tested in two naïve HB dogs receiving 3×10^{12} vg/kg of an AAV-6 vector encoding cFIX, and transient IS with cyclophosphamide. In these dogs, cFIX levels peaked at 5-7% of normal, followed by a plateau of 4-5% of normal (Figure 6A and Table 3). The shortening of the WBCT and
the aPTT (Figure 6B and C) demonstrates that the FIX produced by the muscle was biologically active. No spontaneous bleeding was observed during the follow-up of almost one year per dog and no antibodies to cFIX were measured. Levels of cFIX transgene expression in these two naïve dogs were comparable to those obtained in animal E59, which received a similar dose of the same AAV6-cFIX vector via ATVRX in the presence of NAB to AAV2 at a titer of 1:100 (vide supra).

Discussion

Muscle represents an ideal target tissue for AAV-mediated FIX gene transfer in hemophilia B subjects with advanced liver disease due to viral hepatitis infection. Our early studies on intramuscular delivery of an AAV-2 vector encoding FIX in hemophilia B dogs and humans showed that a major obstacle that must be overcome in order to achieve therapeutic transgene expression is to effect widespread muscle transduction. In this work we show for the first time the sustained expression of the cFIX transgene product at therapeutic levels for a period of more than 4 years in a large animal model of hemophilia B. Through a simple, non-invasive procedure, which does not require the administration of vasoactive drugs 34, we were able to reach levels of cFIX transgene expression up to ten-fold higher than those measured after the intramuscular injection of the same vector at a dose of ~3x10^{12} vg/kg 5. Importantly, no acute or chronic toxicity was associated with the delivery of the vector under pressure, the tourniquet placement, or with long-term expression of cFIX from transduced muscle.
The delivery technique used in this study, ATVRX, was first described by Su and colleagues in a proof-of-concept study in which both adenovirus and AAV vectors expressing β-galactosidase were used, with up to 100% efficiency of muscle transduction in rats and dogs. More recently, the same technique was used in non-human primates in an AAV gene transfer study in which a vector encoding the secreted immunomodulatory protein LEA29Y, a mutated form of CTLA4Ig, was injected at a single dose of 5x10^{12} vg/kg. In this study, expression levels of transgene were stable for at least 34 months and 4-8-fold higher after intravascular (via ATVRX) vs. intramuscular delivery of vector. A subsequent study from the same group used the LEA29Y transgene delivered IM or intravascularly to prevent responses to the transactivator protein rtTA-M2, showing better results when the vector encoding LEA29Y was administered via ATVRX. Several other reports show successful muscle transduction with viral and non-viral vectors using intravascular routes with or without pressure delivery. For example, Qiao and colleagues showed expression of the myostatin propeptide gene in normal dogs after hydrodynamic injection of an AAV-8 vector with no apparent toxicity. However, a preliminary report from the same group showed muscle inflammation and atrophy 11 weeks after the administration of an AAV-9 vector encoding minidystrophin in neonatal Duchenne muscular dystrophy dogs. This suggests that careful evaluation of the immunogenic profile of the therapeutic transgene expressed in muscle will be required in the context of the specific disease target. This is particularly relevant for those diseases characterized by a lack of tolerance to the transgene product, like hemophilia, or by inflammation of the target organ, like muscular dystrophy. For these diseases,
immunomodulation may be necessary to prevent harmful responses to the transgene product (reviewed in 32).

The study described here represents the first ATVRX dose escalation study in which expression levels of the therapeutic transgene were followed long-term in a large animal model of disease. We showed that ATVRX administration of an AAV2 vector expressing cFIX under the control of a constitutive promoter led to a dose-dependent detection of circulating cFIX, suggesting that the muscle tissue biosynthetic machinery can produce active FIX; this is in accordance with what we have previously described 45. At high vector doses (8x10^{12} vg/kg), dose-dependence of transgene expression is lost, likely due to the development of a low-titer non-neutralizing antibody to cFIX. What makes this finding intriguing is that at high vector doses the immune response to the transgene product develops despite the use of transient immunosuppression with cyclophosphamide. However, one important observation is that, in the dogs treated at the highest vector dose with immunosuppression, the measured antibody is not neutralizing, thus the humoral response measured is somewhat benign, as it diminishes levels of cFIX without neutralizing its activity, resulting in partial correction of the disease phenotype, and preservation of the ability to respond to infused protein, albeit likely requiring higher doses.

In earlier work, we showed that intravascular delivery of AAV vectors to skeletal muscle of hemophilia B dogs with co-administration of vasoactive drugs requires transient immunosuppression with cyclophosphamide to prevent the formation of neutralizing
antibodies to the cFIX transgene \(^{34}\). In the absence of immunosuppression, vector administration through this procedure resulted in high titer inhibitory antibodies to AAV (up to ~30 BU). Here, two hemophilia B dogs received an AAV2-cFIX vector via ATVRX with no immunomodulation; one dog expressed the transgene at levels comparable to the immunosuppressed dogs which received the same dose, while the other developed a transient inhibitory antibody to the cFIX transgene peaking at 1.5 BU. This dog developed a predominantly Th1 response to the cFIX antigen, with an increase in IgG1 antibodies coinciding with the detection of inhibitor; after that, a non-neutralizing antibody response (Th2-driven, with production of anti-cFIX IgG2) became predominant with detectable cFIX in the circulation and correction of the disease phenotype. Although this would argue for a lower immunogenicity of ATVRX compared to the IM \(^{38,46}\) and intravascular routes \(^{34}\), in terms of safe translation of this approach to the clinics IS seems to be a requirement in the context of muscle directed gene transfer. This is particularly true for loss-of-function diseases, in which absence of a gene product prevents thymic deletion of autoreactive T cell clones. The use of immunosuppression in the context of AAV-mediated muscle gene transfer has already been safely tested in other disease models \(^{47,48}\) including human subjects undergoing AAV1 intramuscular gene transfer for lipoprotein lipase deficiency \(^{49,50}\) and AAV6 gene transfer in dog model of Duchenne muscular dystrophy \(^{47}\). While the purpose of IS has been mostly to explore to prevent capsid-specific T cell responses there are examples transient immunosuppression coupled with AAV delivery to skeletal muscle the approach is feasible and safe. When designing muscle-targeted AAV gene transfer strategies, the risk of immune responses to the vector transgene product should be evaluated on a case-by-case basis, taking into account the
role of danger signals, such as pre-existing inflammation in the muscle, and the level of
tolerance to the transgene at the time of gene transfer. Finally, the risk of incurring
harmful T cell responses to the gene transfer vector itself should also be taken into
consideration when an intravascular route of administration is chosen, as systemic
exposure upon tourniquet release may result in enhanced immunogenicity in the target
tissue or other organs. For example, there are modest levels vector biodistribution to the
liver after ATVRX in dogs, presumably due to systemic exposure after the tourniquet is
released (unpublished data).

Results with the ATVRX delivery of an AAV6-cFIX vector presented here have multiple
implications. First, in the presence of anti-AAV-2 NAB titers of 1:100, which can be
considered high, efficient muscle transduction can be achieved. This is an important
result, as antibodies to AAV serotype 2 are highly prevalent in the general population and
switching serotypes is a feasible approach to vector readministration. Second, using AAV
serotypes that more efficiently transduce muscle, combined with ATVRX, closes the gap
in terms of levels of transgene expression in muscle- vs. liver- directed AAV gene
transfer. Third, an earlier objection to muscle-directed gene transfer, that it would likely
require transient immunosuppression in the setting of a genetic disease in which the host
may not be tolerant to the transgene product, may also be lessened if it becomes clear that
AAV-mediated gene transfer to liver will also require short-term immunosuppression

These considerations bolster the case for continued development of ATVRX as a
delivery method for AAV-mediated gene transfer in the setting of hemophilia.
In summary, our results showed that the intravascular delivery of an AAV vector to muscle results in sustained expression of the FIX transgene product at therapeutic levels. This procedure has proven to be safe and effective in correcting the disease phenotype in a large animal model of hemophilia B, thus laying the foundation for future clinical development of this novel therapeutic approach in humans with advanced liver disease.
Acknowledgments

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Author contributions

V.R.A. directed designed and execution of the experiments and drafted the manuscript. H.H.S provided insights on the surgical protocol design and execution. V.A.H., G.B., S.B., P.F., and Y.C. participated in the animals’ laboratory evaluations and follow up. S.Z. and J.F.W. provided the AAV vectors used in the study. L.B.C., H.J., and G.F.P. provided assistance in experimental design. T.C.N. and D.A.B. performed the vector administrations, surgical procedures, provided care to the animals, and assisted with experimental design and interpretation. F.M. provided insights protocol design and drafted the manuscript. K.A.H. directed experimental design, conducted data analysis and interpretation, and drafted the manuscript.

Conflict of interest statement

V.R.A., J.F.W., F.M. and K.A.H. are consultants for companies that are developing AAV-based therapeutics not in the field of hemophilia and hold patents related to AAV gene therapy. All other authors declare no competing financial interests.
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Table 1: Summary of results in naïve hemophilia B dogs following ATVRX delivery of an AAV2-cFIX vector

<table>
<thead>
<tr>
<th>Group/ Dog ID</th>
<th>Age at time of injection (Months)</th>
<th>Sex</th>
<th>Weight (Kg)</th>
<th>Circulating cFIX (ng/ml)*</th>
<th>Inhibitor (Bethesda Unit)</th>
<th>Bleeding episodes (expected†)</th>
<th>Follow-up (months)</th>
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<tr>
<td>I05</td>
<td>9</td>
<td>M</td>
<td>17.5</td>
<td>141 ± 18</td>
<td>No</td>
<td>1 (27)</td>
<td>59</td>
</tr>
<tr>
<td>J04</td>
<td>4.5</td>
<td>M</td>
<td>12.4</td>
<td>27 ± 4</td>
<td>No</td>
<td>1 (19)</td>
<td>42</td>
</tr>
<tr>
<td>Mid Dose (3 x10^{12} vg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H48</td>
<td>6.5</td>
<td>M</td>
<td>15</td>
<td>275 ± 75</td>
<td>No</td>
<td>0 (20)</td>
<td>44</td>
</tr>
<tr>
<td>H34</td>
<td>11</td>
<td>F</td>
<td>15</td>
<td>76 ± 14</td>
<td>No</td>
<td>1 (29)</td>
<td>63</td>
</tr>
<tr>
<td>I07</td>
<td>8</td>
<td>F</td>
<td>16</td>
<td>125 ± 39</td>
<td>No</td>
<td>0 (22)</td>
<td>47</td>
</tr>
<tr>
<td>High Dose (8.5 x10^{12} vg/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H24</td>
<td>9</td>
<td>M</td>
<td>24</td>
<td>20 ± 3</td>
<td>No</td>
<td>3 (29)</td>
<td>64</td>
</tr>
<tr>
<td>M25</td>
<td>3.5</td>
<td>F</td>
<td>8.7</td>
<td>23 ± 3</td>
<td>No</td>
<td>1 (6)</td>
<td>12</td>
</tr>
<tr>
<td>Mid Dose: No Immunosuppression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J03</td>
<td>11</td>
<td>M</td>
<td>17.5</td>
<td>81 ± 13</td>
<td>No</td>
<td>1 (20)</td>
<td>44</td>
</tr>
<tr>
<td>J62</td>
<td>6</td>
<td>M</td>
<td>14</td>
<td>120**</td>
<td>Transient (1.5 BU)</td>
<td>6*** (5)</td>
<td>40</td>
</tr>
</tbody>
</table>

*Average ± standard deviation of plateau levels >3 months; ** Measured at day >600 after resolution of inhibitor;
*** Occurred from day 15 to 363, while inhibitor persisted; † Based on an average of ~5 bleeding episodes/year for untreated dogs. 51
Table 2. Determination of vector genome copy number after ATVRX delivery of an AAV2-cFIX vector.

<table>
<thead>
<tr>
<th>Biopsy site</th>
<th>DLAM 3 (vg/diploid genome)</th>
<th>DLAM 4 (vg/diploid genome)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target limb 1</td>
<td>0.163 ± 0.111</td>
<td>0.09*</td>
</tr>
<tr>
<td>Target limb 2</td>
<td>0.091 ± 0.012</td>
<td>0.186 ± 0.251</td>
</tr>
<tr>
<td>Target limb 3</td>
<td>14.018 ± 1.479</td>
<td>9.146 ± 1.587</td>
</tr>
<tr>
<td>Target limb 4</td>
<td>6.058 ± 0.591</td>
<td>0.505 ± 0.057</td>
</tr>
<tr>
<td>Target limb 5</td>
<td>0.019 ± 0.005</td>
<td>5.358 ± 0.592</td>
</tr>
<tr>
<td>Contralateral limb 1</td>
<td>0.004 ± 0.001</td>
<td>-</td>
</tr>
<tr>
<td>Contralateral limb 2</td>
<td>0.014 ± 0.015</td>
<td>0.094 ± 0.108</td>
</tr>
<tr>
<td>Contralateral limb 3</td>
<td>0.004 ± 0.003</td>
<td>-</td>
</tr>
<tr>
<td>Contralateral limb 4</td>
<td>0.004 ± 0.001</td>
<td>-</td>
</tr>
<tr>
<td>Contralateral limb 5</td>
<td>0.004 ± 0.004</td>
<td>0.012 ± 0.003</td>
</tr>
</tbody>
</table>

Results are expressed as average ± standard deviation. All the experiment where repeated twice using 500-1000 ng of genomic DNA per reaction. * single experiment with 344 ng of genomic DNA in the reaction. 1-5
Table 3: Summary of results following delivery of an AAV6-cF.IX vector via ATVRX in hemophilia B dogs naïve or previously exposure to an AAV-2 vector

<table>
<thead>
<tr>
<th>Group/ Dog ID</th>
<th>Age at time of injection</th>
<th>Sex</th>
<th>Weight (Kg)</th>
<th>Serotype</th>
<th>NAB to AAV-2 capsid*</th>
<th>Circulating cF.IX** (ng/ml)</th>
<th>Inhibitor to cF.IX</th>
<th>Bleeding episodes (expected†)</th>
<th>Follow-up (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HB dogs previously exposed to AAV-2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E59</td>
<td>4.6 years</td>
<td>F</td>
<td>21</td>
<td>AAV-6</td>
<td>1:100</td>
<td>259 ± 50</td>
<td>No</td>
<td>1§ (21)</td>
<td>46</td>
</tr>
<tr>
<td>H27</td>
<td>2.4 years</td>
<td>F</td>
<td>20</td>
<td>AAA-6</td>
<td>1:1000</td>
<td>89 ± 47</td>
<td>No</td>
<td>0 (19)</td>
<td>42</td>
</tr>
<tr>
<td>D32</td>
<td>5.7 years</td>
<td>M</td>
<td>18</td>
<td>AAV-2</td>
<td>1:300</td>
<td>&lt; 10</td>
<td>No</td>
<td>8 (10)</td>
<td>22</td>
</tr>
<tr>
<td>B46</td>
<td>7.6 years</td>
<td>M</td>
<td>24</td>
<td>AAV-2</td>
<td>1:30</td>
<td>&lt;10</td>
<td>No</td>
<td>4 (3)</td>
<td>6</td>
</tr>
<tr>
<td><strong>Naïve HB Dogs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13</td>
<td>5 months</td>
<td>F</td>
<td>13</td>
<td>AAV-6</td>
<td>n.d.</td>
<td>259 ± 37</td>
<td>No</td>
<td>0 (6)</td>
<td>12</td>
</tr>
<tr>
<td>M20</td>
<td>4 months</td>
<td>M</td>
<td>8</td>
<td>AAV-6</td>
<td>n.d.</td>
<td>213 ± 25</td>
<td>No</td>
<td>0 (6)</td>
<td>12</td>
</tr>
</tbody>
</table>

NAB, Neutralizing antibody titer; n.d., Not determined;
* Measured at the time of vector readministration by ATVRX, values expressed as reciprocal dilutions; ** Average±standard deviation of plateau levels >3 months; † Based on an average of ~5 bleeding episodes/year for untreated dogs 51.
§ Two severe bleeding episodes and 3 post-partum bleeding episodes despite prophylactic plasma transfusion prior to readministration.
Figure legends

Figure 1. Time course of circulating canine FIX (cFIX) and coagulation activity in naïve hemophilia B dogs following ATVRX vector delivery. A. Canine FIX antigen levels measured by ELISA; B. Activated partial thromboplastin time (aPTT); s, seconds; C. Whole blood clotting time (WBCT); m, minutes. D. Creatinine phosphokinase (CPK) levels. Each line represents an individual dog; in red are indicated dogs from the low dose cohort (1x10^{12} vg/kg), in black dogs from the mid dose cohort (3x10^{12} vg/kg). Shaded gray areas represent indicate range of values for hemostatically normal animals. x axis, time in days.

Figure 2. Canine FIX staining and histology of muscle biopsies from dog H48 (3x10^{12} vg/kg) 2 years after ATVRX delivery of an AAV2-cFIX vector. Left panel, sites of muscle biopsy sampling, AAV injected target limb: A, semimembranosus; B, vastus lateralis; C, semitendinosus; A, B, and C. cFIX immunostaining of muscle biopsies collected at the sites indicated; D. cFIX immunostaining of muscle biopsies from the contralateral, non-injected, limb; E. Hematotoxylin and eosin staining of a the muscle biopsy collected from the target leg. Images are taken at a 100X magnification.

Figure 3. Transient non-neutralizing anti-cFIX antibodies develop at high doses of AAV2- cFIX vector delivered via ATVRX. A. Canine FIX transgene antigen levels measured by ELISA; B. Anti-canine FIX IgG1 and IgG2 subclass antibody titer measured by ELISA; C and D. Blood coagulation activity measured by aPTT (s,
seconds) and WBCT (m, minutes); E. Creatinine phosphokinase (CPK) levels. Shaded gray areas indicate the range of values for hemostatically normal animals. Vertical arrows represent normal pooled plasma administrations. x axis, time in days.

**Figure 4. Delivery of AAV2 -cFIX via ATVRX with no IS.** A. Canine FIX antigen levels measured by ELISA; B and C. Coagulation parameters measured by aPTT (s, seconds) and WBCT (m, minutes); D and E. Anti-canine FIX IgG1 and IgG2 subclass antibody titer measured by ELISA in J03 (D) and J62 (E); *, Peak neutralizing antibody titer (1.5 BU at day 35 post-ATVRX). Shaded gray areas represent indicate range of values for hemostatically normal animals. Vertical arrows represent pooled normal plasma administrations. x axis, time in days.

**Figure 5. Readministration of AAV vectors by ATVRX in HB dogs previously exposed to AAV-2 vectors.** A and B. Canine FIX levels measured by ELISA and coagulation activity (WBCT in minutes, m) in dogs D32 (red) and B46 (black) upon readministration of an AAV2-cFIX vector via ATVRX. D and E. Canine FIX levels measured by ELISA and coagulation activity (WBCT in minutes, m) in dogs E59 (red) and H27 (black) upon readministration of an AAV6-cFIX vector via ATVRX. C and F. Creatinine phosphokinase (CPK) levels. x axis, time in days.

**Figure 6. Time course of canine FIX expression and coagulation activity in naïve hemophilia B dogs following ATRVX delivery of an AAV6-cFIX vector.** A. Canine FIX antigen levels measured by ELISA; B. Whole blood clotting time (WBCT); m,
minutes; C. Activated partial thromboplastin time (aPTT); s, seconds. x axis, time in days.
Figure 1

A 1 x 10^{12} vg/kg

3 x 10^{12} vg/kg

I05
I04
J04
H34
H48
I07

B Untreated hemophilia

C Untreated hemophilia

D Upper limit of normal

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Figure 2
Peripheral transvenular delivery of adeno-associated viral vectors to skeletal muscle as a novel therapy for hemophilia B