REVIEW

Delivery of Adeno-Associated Virus Gene Therapy by Intravascular Limb Infusion Methods

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Recombinant adeno-associated virus (rAAV) can be delivered to the skeletal muscle of the limb (pelvic or thoracic) by means of regional intravascular delivery. This review summarizes the evolution of this technique to deliver rAAV either via the arterial blood supply or via the peripheral venous circulation. The focus of this review is on applications in large animal models, including preclinical studies. Based on this overview of past research, we aim to inform the design of preclinical and clinical studies.

INTRODUCTION

Recombinant adeno-associated virus (rAAV) targets skeletal muscle readily, and because myocytes are a nondividing cell type, therapeutic proteins can be expressed long-term, despite low levels of chromosomal vector integration.[1–5] This makes muscle a good gene therapy target for both muscle-specific disease and as a sight for production of secreted proteins.[2,6–14] While direct intramuscular (IM) vector injections are effective in targeting muscle cells, the area of spread from the injection site is limited,[11,15] meaning that only a relatively small area of myocytes can be targeted in this manner without having to administer multiple IM injections per large muscle group and the need to dose each muscle group separately. Systemic intravascular delivery can target skeletal muscle effectively, but requires large amounts of vector and may increase exposure of nontarget organs. This has led investigators to explore other means of delivering vector more widely to the muscle, while still limiting the amount of vector in the systemic circulation. This can be achieved through several different methods of infusing the limb vasculature with vector while it is isolated from systemic circulation, often through the use of a tourniquet or vessel clamps, allowing vector to access the muscle groups distal to the vessel occlusion. This technique has been adapted from procedures used to deliver regional chemotherapy, anesthesia, and antibiotics to the limb.[16–20] The goal is to either circulate the agent through the limb vasculature to allow extravasation or to increase the intravascular pressure/volume to create increased hydrodynamic pressure to expand vascular beds and perhaps increase the size and number of endothelial pores (either intracellular or intercellular).[21–23]

Regional limb delivery was initially described in the context of gene therapy as a method for delivering plasmid DNA (pDNA) constructs, rAAV, or adenovirus to myofibers via the arterial circulation using moderate to high volumes of fluid (with or without histamine and papaverine).[15,23,24] Initial work was done using high volumes of fluid to deliver luciferase expressing pDNA into the femoral artery of rats with occlusion (10 min) of both the femoral artery and vein via vascular clamps.[24] Leaving the femoral vein unclamped or delivering the pDNA over a longer time decreased luciferase expression.[24] They later demonstrated that they could express full-length dystrophin in a Duchenne muscular dystrophy mouse model using a similar procedure.[25] This method was also shown to be translatable into larger animal models, including rhesus macaques and pigs.[26,27] Interestingly the work in pigs demonstrated a decreased transgene expression in muscles with higher IM pressure during the pDNA delivery. The authors hypothesized that the increased IM pressure, due to muscle edema, may have led to the collapse of small caliber

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vessels within the muscle, decreasing extravasation of the pDNA.[27] They noted that the best pDNA expression was in muscle groups where the muscle compartment pressure did not exceed the diastolic blood pressure.

Initial work with rAAV (expressing sarcoglycan) in a hamster model of limb-girdle muscular dystrophy used a femoral artery infusion with tourniqueting of the limb and clamping of both the femoral artery and vein.[15] The rAAV dose was 7×10^{11} vector particles (VP)/animal (in 500 μl of phosphate buffered saline [PBS]) delivered after perfusion with papaverine and histamine (500 μl volume). The vector was followed by a chase of 1 ml/100 g of PBS and the limb was flushed with 3 ml of PBS to remove residual papaverine, histamine, and vector via the venous catheter.[15] This infusion procedure led to widespread sarcoglycan expression throughout the hind limb of the hamster.

Subsequent to these early studies several groups have worked to optimize muscle rAAV delivery using differing methods of regional limb infusion. In this review we will summarize many of these studies, particularly those that utilized large animal models, and detail the methods that were used (see Table 1).

ARTERIAL DELIVERY OF rAAV TO LARGE ANIMAL MODELS

The first large animal study to look at local arterial delivery of an rAAV vector involved delivery of an rAAV2 encoding the *lacZ* reporter gene or the canine factor IX (*cFIX*) gene delivered to a hemophilia B dog model.[11] This study involved the placement of an arterial catheter and simultaneous proximal thigh tourniquets with femoral artery and vein clamps. A dose of the vasodilator papaverine along with histamine, to promote vascular leakage (diluted in 2.5 ml/kg of PBS), was delivered before vector administration. The vector was diluted in 2.5 ml/kg of PBS (with histamine) followed by a 10 ml/kg bolus of PBS with a 15–20 min dwell time. Before tourniquet release, an addition flush of 15 ml/kg was administered. Following *lacZ* delivery, no transgene expression was obtained and the authors suspected an immune response to the transgene and subsequently delivered vector expressing the canine *FIX* gene, which according to their experience was fully tolerated in dogs. Following IM injection the cFIX expression was confined to the injection site; however, after limb infusion they detected widespread cFIX expression throughout the musculature of the limb supplied by the infused vessel.[11] This study demonstrated not only widespread muscle expression of cFIX in normal dogs but also clinical, long-term expression of therapeutic cFIX levels in hemophiliac dogs along with a dramatic reduction in episodes of bleeding (2 episodes in 74 total months versus an expected 34 episodes over the same time period in untreated dogs).

A second study to employ a similar methodology involved delivery of a microdystrophin gene to a muscular dystrophy mouse model using either rAAV1, rAAV6, or rAAV8 into the femoral artery.[28] Interestingly, rAAV6 and rAAV8 had good levels of transduction, but the rAAV1 transduction efficiency was very low comparatively, a sharp contrast to the transduction with rAAV1 following IM delivery. Based on the mouse results, a large animal model study was carried out using rAAV8-*eGFP* (enhanced green florescent protein) delivery to cynomolgus macaques via the femoral artery (catheter to the level of the knee) to the calf muscles.[28] Widespread eGFP expression was seen throughout the lower limb with up to 86.4% of the muscle expressing eGFP in the soleus muscle. Subsequent work by this group has shown persistent rAAV8 microdystrophin expression in the gastrocnemius of rhesus macaques following femoral artery catheterization and passage of the catheter to the level of the sural artery (tourniquets were placed proximal and distal to the gastrocnemius muscle) without the use of papaverine or histamine.[29] A detailed procedural methods chapter for this technique was published in 2011.[30] The same method was also used to deliver rAAVrh74 encoding either *MCK.GALGT2* (stimulates dystrophin and lamimin alpha2 surrogate expression) or microdystrophin to the gastrocnemius muscle of rhesus macaques.[31,32]

A study looking at rAAV6 encoding a human alkaline phosphatase (*hPLAP*) gene delivered to the canine pelvic limb via the femoral artery, comparing transgene expression with or without limb exsanguination (using a limb compression wrapping technique) before vector delivery found that expression levels were highest with prior limb exsanguination.[33] Expression levels were lower if the compression wrap was left in place for the duration of the vector dwell time versus removal before vector infusion. This study did not report the use of papaverine or histamine in association with the vector delivery.

Together, these studies indicate that femoral artery vector delivery with rAAV6, rAAV8, or rAAVrh74 with or without prior limb exaguination can result in widespread transduction of the lower limb musculature even without the use of papaverine

Table 1. *Summary of methods used in limb infusion studies delivering recombinant adeno-associated virus to large animal models*

	Femoral artery delivery				*Peripheral venous delivery*			
Study	*Arruda et al.*[11]	*Rodino-Klapac et al.*[28]	*Rodino-Klapac et al.*[29]	*Chicoine et al.*[31,32]	*Su et al.*[21]	*Toromanoff et al.*[34,35]	*Arruda et al.*[36]	*Le Guiner et al.*[38]
Species (weight)	Canine 12–22.5 kg	Cynomolgus macaque 4–5 kg	Rhesus macaque 4–8 kg	Rhesus macaque	Canine 5–11 kg	Cynomolgus macaque 3–5 kg	Canine 8.7–24 kg	Canine 8.7–24 kg
Route	Infuse, dwell, flush—ipsilateral	Infuse and dwell—ipsilateral—gastroc only	Infuse and dwell—ipsilateral	Infuse and dwell—ipsilateral—gastroc only	Hydrodynamic (ATVRX)	Hydrodynamic	Hydrodynamic (ATVRX)	Hydrodynamic
Circulated vector	No	No	No	No	No	No	No	No
Vector gene	*LacZ* and *FIX*	CMV *eGFP*	Microdystrophin	CMV *eGFP* or *MCK.GALGT2*	CMV *lacZ*	human *LEA29Y*, *cmEpo*	*cFIX*	U7snRNA-E6/E8
Dose—Vector	1.7 × 10e12–3 × 10e12 vg/kg	2 × 10e12 vg/kg	2 × 10e12 vg/kg	2 × 10e12 vg/kg	1 × 12e14 gc	5 × 10e12 vg/kg	3 × 10e12 vg/kg	1 × 10e13–5 × 10e13 vg/kg
Serotype	AAV2	AAV8	AAV8	AAVrh.74	rAAV1	rAAV 1 > rAAV8	AAV2 and AAV6	AAV8
Volume—Vector	2.5 ml/kg PBS with 10 m*M* histamine, followed by 10 ml/kg PBS	2 ml PBS—over 60 sec	2.5 ml/kg (gastroc only, tourniquet tight)	2.5 ml/kg (gastroc only, tourniquet tight)	500 ml PBS at 300 mmHg over 20 min	50 ml/kg LRS over 5 min	20 ml/kg over 3 min at 300 mmHg	12 ml/kg at 300 mmHg or 6–7 ml/kg at 10 or 35 ml/min
Volume—Pre flush	2.5 ml/kg PBS with 10 m*M* histamine	2 ml saline (pre tourniquet) 0.5 ml/kg	2.5 ml/kg (gastroc only, tourniquet snug but not tight)	2.5 ml/kg over 1 min	No	No	No	No
Volume—Post flush	15 ml/kg PBS (cimetidine and benedryl after)	2 ml PBS then tourniquet released	2.5 ml/kg with tourniquet still tight over 60 sec	2.5 ml/kg with tourniquet still tight then released	No	No	No	No
Tourniquet pressure/level	Proximal thigh (no pressure reported)	Phlebotomy tourniquet above incision	Proximal and distal to gastroc	Proximal and distal to gastroc	Groin—until femoral pulse gone	350 mmHg	Groin—until femoral pulse gone	Above elbow—310 mmHg
Limb exsanguination	No	No	No	No	No	No	No	Yes
Vessel clamping	Arterial and venous clamping	No	No	No	No	No	No	No
Arterial catheter	No size reported	3 french	3 french	3 french	No	No	No	No
Venous catheter	No	No	No	No	Saphenous, 20 gauge	Saphenous, 22 gauge	Saphenous, 14–18 gauge	Cephalic, 20 gauge
Vector dwell time	15–20 min	10 min	10 min	10 min	20 min?	15 min	15 min	15 min
Papavarine	1 mg/kg	No	No	No	No	No	No	No
Immune suppression	Cyclophosphamide	No	No	No	No	Mycophenolate and prednisone	Yes/No	No
Heparine	70 IU/kg		Systemic—50 IU/kg	Systemic—50 IU/kg	No	No	No	No
Diluent	PBS	PBS	Normal saline	PBS	PBS	LRS	PBS	LRS

rAAV, recombinant adeno-associated virus; ATVRX, afferent transvenular retrograde extravasation; PBS, phosphate buffered saline; LRS, lactated Ringer's solution.

or histamine. The safety and efficacy of this method has yet to be published in human subjects, but with its similarity to widely used limb infusion techniques applied in humans, it is likely that it will prove generally safe.

PERIPHERAL INTRAVASCULAR HYDRODYNAMIC DELIVERY OF rAAV TO LARGE ANIMAL MODELS

A method to deliver rAAV diluted in a moderate volume of fluid retrograde from a peripheral limb vein without the use of histamine or papaverine was published in rats and dogs and simplified the arterial and venous delivery methods described in the introduction for pDNA administration.[21] The group termed this method "afferent transvenular retrograde extravasation (ATVRX)." For this method in the dog, a catheter was placed in the greater saphenous vein; a tourniquet was placed at groin level and tightened until the femoral pulse was no longer palpable. The vector was then delivered in 500 ml of PBS with standard intravenous tubing and a pressure bag at a pressure of 300 mmHg.[21] The ATVRX method has the advantage of being simple to execute, which decreases anesthesia time, and does not pose the same potential risks as arterial delivery (thrombosis, emboli, etc.) and vascular modifying agents such as histamine, heparin, and papaverine. Using ATVRX, the authors were able to obtain high levels of reporter gene expression throughout the dosed limbs in both rats and dogs.[21] The level of expression was dependent on delivery pressure (<50 mmHg had lower transduction; 100 and 400 mmHg resulted in uniform transduction) but was not dependent on dwell time before tourniquet release. The authors reported no adverse clinical or histological side effects of the procedure.

The ATVRX technique was then applied in nonhuman primates (cynomolgus macaques) to compare rAAV1 versus rAAV8 transduction, biodistribution, and transgene expression.[34] There were increased numbers of vector genomes, and subsequently increased transgene expression, in the muscle following rAAV1 regional limb infusion versus rAAV8; however, the pattern of distribution was similar. Further work by the same group, also in cynomolgus macaques, demonstrated that they could get long-term muscle expression, without immunosuppression, of cynomolgus macaque erythropoietin following regional limb infusion, in contrast to IM dosing, where expression was quickly lost.[35]

This same hydrodynamic delivery method was used in dogs with hemophilia B to deliver rAAV2 expressing *cFIX* without the use of papaverine or histamine.[36] With the use of transient immune suppression (to prevent antibody formation against cFIX), they were able to demonstrate long-term cFIX expression that translated to improved clotting time and markedly fewer bleeding episodes in the treated dogs. A concurrent safety study in hemophilia B dogs further demonstrated increased expression of cFIX following limb infusion when compared to IM administration.[37] They also demonstrated immune responses to the cFIX transgene (IgG2 antibodies) and the presence of a $CD4^{+}Il\text{-}10^{+}FoxP3^{+}$ T-cell population (regulatory T-cells) that likely contributed to the sustained cFIX transgene expression. They postulated that the transient immunosuppression used may have played a role in the expansion of the regulatory T-cells that was observed.

In order to asses venous local regional delivery in a model of muscle disease as a therapeutic proof of concept, the ATVRX method was applied to the forelimb of a canine Duchenne muscular dystrophy model.[38] This study supported safety of the delivery method and a high level of muscle expression at a dose of 5×10^{13} vg/kg delivered in either 20% or 40% of the limb volume. With this dose and volume they were able to detect positive muscle fibers ranging from a mean per group of 58–76%, with a therapeutic threshold of >40%. The higher the vector dose delivered, the higher the expression they were able to detect at sights other than the injected limb (uninjected limb and diaphragm).

Two studies have looked at high-pressure transvenous limb perfusion delivery of 0.9% saline to both the pelvic and thoracic limbs of human muscular dystrophy patients.[39,40] The pelvic limb study infused saline volumes up to 20% of limb volume without adverse events beyond transient increases in muscle compartment pressures and short-term depression of limb tissue oximetry.[40] For the thoracic limb study they evaluated saline volumes up to 43% of limb volume with no adverse events up to 35% of limb volume, other than transient decreases in tissue oxygenation (returned to baseline within 5 min) and increased muscle compartment pressure (returned to baseline within 15 min) and a short-term decrease in compound muscle action potentials in a patient receiving 40% of limb volume.[39] No clinical adverse events were reported. These studies, along with the pilot data in muscular dystrophy dogs, indicate that this is a clinically feasible route and method for gene therapy in muscular dystrophy patients.

SUMMARY

Over the past decade, methods for regional limb infusion to deliver gene therapy to the skeletal muscle have shown effectiveness in increasing expression over IM delivery. The technical aspects of delivery have also improved as investigators have attempted to simplify both the arterial and venous delivery methodologies in order to move them toward clinical applicability. The true test of the usefulness of these techniques will come as they are employed in human clinical trials.

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T.R.F. is a paid consultant for Dimension Therapeutics and for Editas Medicine, and he was also the founder of AGTC but subsequently donated all stock to the Alpha-1 Foundation. A.M.G. has no competing financial interests.

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