HYDRODYNAMIC GENE TRANSFER OF ALPHA-GALACTOSIDASE (GLA) IN THE GLA KNOCKOUT MOUSE PARTIALLY REVERSES BIOCHEMICAL DEFICITS

by

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Hydrodynamic gene transfer of alpha-galactosidase (GLA) in the GLA knockout mouse partially reverses biochemical deficits

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In humans, deficiency of the GLA enzyme is an inheritable, X-linked recessive disorder (Fabry disease) that results in accumulation of glycosphingolipid globotriaosylceramide (Gb3) within blood vessels, various tissues, and organs leading to severe pain, renal failure, cardiovascular disease and resultant premature mortality. Enzyme replacement therapy (ERT) is the only approved pharmacological treatment and is prohibitively expensive. Gene therapy may be a viable alternative to ERT. This study investigates the use of hydrodynamic gene delivery (HGD) of the GLA gene to GLA knockout mice, the Fabry disease animal model. The objective of this study is to evaluate the safety, efficacy and therapeutic outcome of administering GLA-containing plasmids via HGD to GLA knockout mice. The human GLA gene cDNA sequence was successfully inserted into the pAAV-MCS vector at the EcoR1 and Xho1 cloning sites resulting in pAAV-GLA plasmids and into the pLIVE vector at BamH1 and Xho1 sites resulting in pLIVE-GLA. It was determined that an 8% (of body weight) injection volume significantly increased GLA activity when compared to 6% ($p<0.05$) and injection volumes greater than 8% resulted in mortality. DNA doses of 5 to 15 μg/ml produced higher serum levels of GLA in the GLA knockout mice when compared to wild-type (WT) mice. The DNA dose used in this study was 15 μg/ml with an injection volume of 8%. Results show that pAAV-GLA generated greater activity (7600 %) in serum when compared to pLIVE-GLA (2500 %). With the pAAV-GLA plasmid, serum activity was maintained for two days. However, for pLIVE-GLA, at 18 days post-injection, GLA activity in liver and heart tissues in GLA knockout mice was significantly ($p<0.05$) increased when compared to WT. These levels were sustained for up to 66 days after the treatment when 3 treatments were administered, each 2 weeks apart. These data demonstrate that HGD of pLIVE-GLA safely and effectively corrected GLA deficiency in GLA knockout mice. Future studies should address Gb3 accumulation relative to GLA expression using the same model.

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1.0 INTRODUCTION

Lysosomal storage diseases (LSDs) comprise a group of at least 50 distinct genetic diseases, each disorder resulting from a deficiency of a particular lysosomal protein or from non-lysosomal activities necessary for protein maturation.[1] First described by William Anderson and Johannes Fabry in 1898, Fabry disease is the second most common LSD, after Gaucher disease.[1] Fabry disease is an inheritable, X-linked gene mutation that results in a deficiency of α-galactosidase A (GLA) leading to excessive deposition of globotriaosylceramide (Gb3) lipids.[2] Gb3 lipids accumulate in various tissues, particularly in kidney, heart, brain and liver due to the inborn error of GLA metabolism.[3]

GLA gene is mapped to the X chromosome (Xq22) from base pair 100,652,778 to base pair 100,663,000.[4] It is a 12 kb long gene containing seven exons with introns from 200 bp to 3.7 kb.[5] The full-length cDNA contains 1290 bp coding sequence and encodes the 398 residues of the mature enzyme subunits forming the functional homodimer.[6] Fabry disease affects mainly hemizygous males, homozygous females, and sometimes the heterozygous females who are also carriers of the mutation. The Gb3 accumulation mechanism that causes symptoms of Fabry disease such as pain, renal failure, dermatitis, hypertension, cardiomyopathy and keratopathy is still unknown. In some cases, men with Fabry disease are known to develop proteinuria and resultant renal failure. The symptoms are often exhibited in childhood and adolescence and range from mild to life-threatening, making diagnosis difficult. Nearly all men who inherit the genetic

mutation will develop symptoms and the complications that accompany Fabry disease. Women with the mutation often exhibit milder or no symptoms at all.

Fabry disease has been successfully treated with enzyme replacement therapy (ERT) by administering synthetic GLA enzyme intravenously to patients. GLA enzyme enters the diseased cells via the mannose-6-phosphare (M6P) receptor system, and traffics intracellularly to the lysosomes.[7-8] GLA is therefore taken up and catabolizes Gb3 lipids in fibroblasts in Fabry patients.[9] The major barrier for using ERT is high cost of GLA replacement therapy and the lack of large-scale production of GLA enzyme.

Gene therapy-based enzyme replacement therapy has been suggested as an alternative to exogenous GLA enzyme replacement therapy for Fabry disease. Gene therapy works by expressing the GLA enzyme endogenously in a target organ, rather than the direct infusion of GLA protein.[8] Recent studies indicated that gene therapy can play an important role in terms of partial or full correction of Fabry disease.[5] Several studies have been done to investigate the usefulness of using viral gene therapy in GLA knockout mice (the Fabry disease model) such as using the adeno-associated virus (AAV) vector. However, there are limitations in terms of the size of the transgene that recombinant viruses can carry, influencing the production of viral vectors.

In this study, we proposed using hydrodynamic gene delivery (HGD) as a non-viral gene delivery method and as an alternative approach to GLA enzyme therapy. HGD has been reported as a simple and efficient non-viral gene delivery method that can be used as an effective tool for studying gene function and regulation through gene transfer, as well as for expressing proteins in

animals.[10] HGD is performed by rapid intravascular injection of DNA solution into the tail vein of mice to increase the permeability of the plasma membrane, primarily in the liver.

This study tested the feasibility of using the HGD method to deliver the GLA gene to GLA-knockout mice. The goals of the study were: 1) to insert the human GLA gene cDNA sequence into the cloning sites of two vectors, p-AAV and p-LIVE, 2) to compare the pattern of GLA expression in GLA-knockout mice using the two vectors, 3) and to evaluate the therapeutic outcomes in GLA-knockout mice using HGD of GLA- containing plasmids.

2.0 MATERIALS AND METHODS

2.1 PLASMID VECTORS

A cDNA sequence corresponding to the human GLA mRNA (GI:125661058, kindly provided by Dr. Michael J Passineau (Allegheny-Singer Research Institute, West Penn Allegheny Health System) was inserted into the multiple cloning site of the AAV-MCS vector (Stratagene, La Jolla, CA) at the EcoR1 and Xho1 sites to make pAAV-GLA plasmids downstream of a Cytomegalovius (CMV) promoter and confirmed with direct sequencing to create pAAV-GLA. Same cDNA sequence was inserted into pLIVE vector (Mirus, Madison, WI), at BamH1 and Xho1 sites to make pLIVE-GLA downstream of an albumin promoter. Plasmid purity and GLA insertion were confirmed by restriction enzyme digestion by the size of DNA fragments resulted from restriction enzyme (Figure 1). Vectors suspended in phosphate-buffered saline (PBS) at the concentrations indicated in (Figure 1) prior to hydrodynamic gene transfer.

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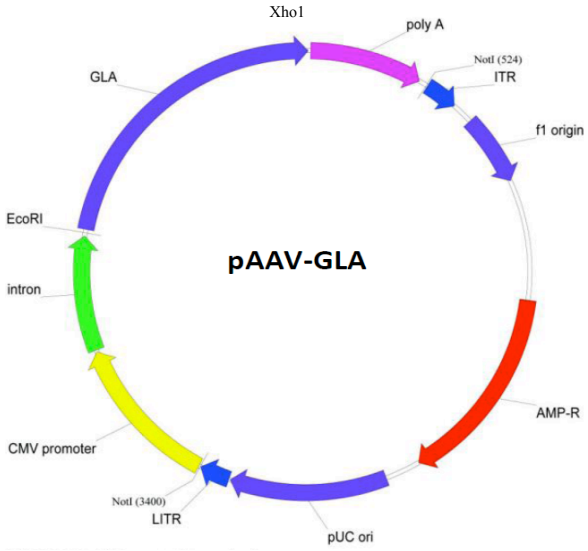
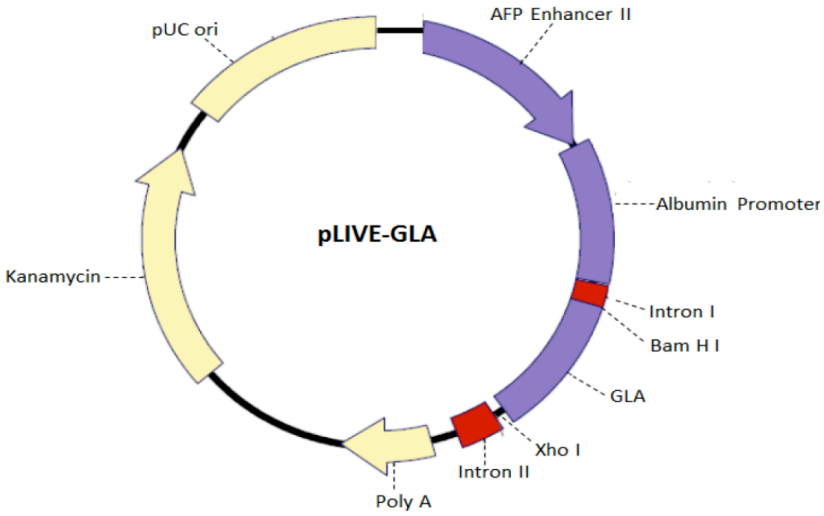
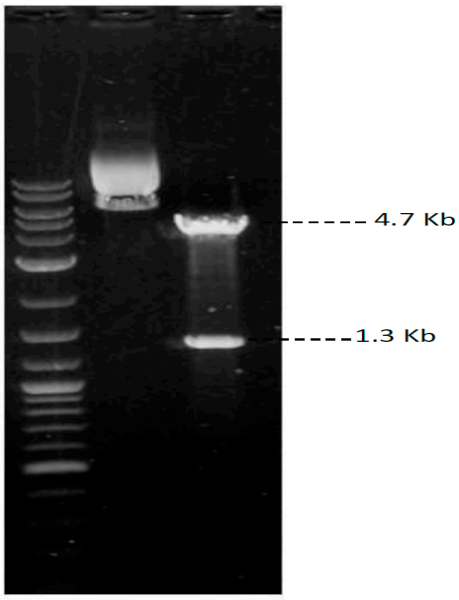
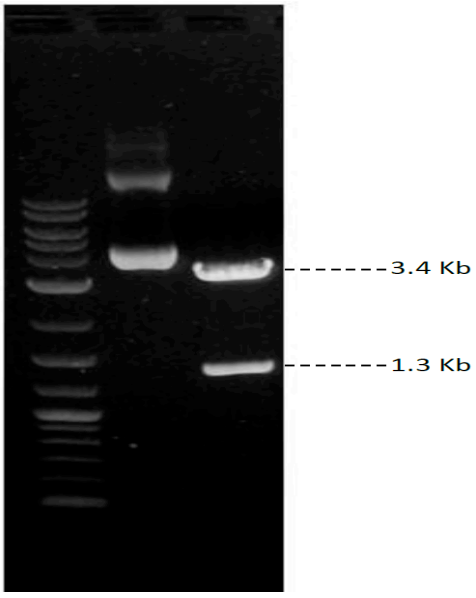






Marker Plasmid EcoR1/Xho1



Marker Plasmid BamH1/Xho1

Figure 1. Cloning and Preparation of GLA Plasmid

2.2 ANIMALS

Fabry model mice were bred by Dr. Michael J Passineau (Allegheny-Singer Research Institute, West Penn Allegheny Health System). All animal experiments were performed according to the protocol of the Institutional Animal Care and Use Committee of Allegheny-Singer Research Institute. Fabry mouse model has the GLA gene disrupted by replacement of a portion of exon 3 and intron 4 with a neomycin resistance sequence (Ohshima et al., 1997). Mice were PCR genotyped using primers recognizing the portion of exon 3 containing the insertion/deletion, with the wild-type allele producing an amplicon of 295 base pairs and the disrupted allele producing a 202-base pair amplicon. All animals were individually genotyped by tail biopsy before entering into experimental groups. All experiments were performed on male mice. Fabry mice were injected hydrodynamically by Dr. Dexi Liu into the tail vein. Blood samples were collected from the tail of Fabry mice hours and days after the injection. Tissues were also collected 18, 38, and 66 days after the hydrodynamic injection.

2.3 HYDRODYNAMIC GENE TRANSFER

A 27.5 gauge needle was inserted hydrodynamically into the tail vein of Fabry miice by Dr. Dexi Liu (Liu et al., 1999). A fluid bolus of PBS/vector corresponding to 6%, 8%, or 10% of the animal's body weight was injected in less than 5 seconds.

2.4 BLOOD AND TISSUE COLLECTION

Blood was collected in a microcentrifuge tube by orbital bleeding at the timepoints indicated. 20μl of 15% EDTA was added and the sample placed on ice. Blood was centrifuged at 14,000 pm for 15 minutes at 4C, and the serum was removed and placed in a fresh microcentrifuge tube and frozen at -80C. For frequent blood collections, blood was collected from the mice tail or alternating eyes, with at least 48 hours elapsing between bleedings. Tissues were collected by administering a lethal dose of ketamine to the animal and quickly dissected, washed in ice-cold PBS and homogenized in ice-cold T-PER Buffer (Thermo Scientific) using a Fast Prep-24 (MP Biomedical) and a garnet/ceramic lysing matrix (MP Biomedical Lysing Matrix A). Following homogenization, the samples were centrifuged at 1000g for 10 minutes at 4C. The supernatant was collected and protein concentration determined using the Bio-Rad Protein Assay (Bio-Rad Life Science Research Products).

2.5 GLA ENZYME ACTIVITY ASSAYS

Our GLA enzyme assay is adapted from principles used in clinical diagnosis of Fabry disease in humans(Beutler and Kuhl, 1972; Desnick et al., 1973; Kint, 1970). 50μl of sample (serum or tissue) was added to 400μl of 0.2 M sodium citrate buffer at pH 4.0, 100 μl n-acetyl-d-galactosamine (Sigma) and 20 μl of 20 mM 4-methylumbelliferyl α-galactoside (Sigma) and incubated at 37C for one hour. After one hour, 1 ml of high pH stop buffer (0.2 M

glycine/NaOH) was added, and sample fluorescence was determined using a Versa Fluor spectrofluorometer (Bio-Rad) with 350/50 excitation and 460/10 emission filters. Relative GLA activity is calculated as EM (460)/μg of tissue or EM460 and expressed as % wild-type activity using the formula: (EM460 (Sample) – EM460 (Knockout))/(EM460 (Wild Type) – EM460 (Knockout)). In this formula, EM460 has been corrected for protein concentration, and EM460 for wild-type and knockout tissues are an average of at least 5 samples, which in our hands have less than 5% sample-to-sample variability.

3.0 RESULTS

3.1 HGD MAGNITUDE OPTIMIZED AT 8% INJECTION VOLUME

The level of GLA activity was detected by measuring GLA activity after the hydrodynamic injection. Four groups of Fabry mice were injected with pAAV-GLA (20 μg/ml) equivalent to 6, 8, 9, 10 % of body weight and blood samples were collected at 2, 4, 8, 24, 36, 48, 72, and 96 hours post hydrodynamic injection. Fabry mice that were injected with 9 and 10% of body weight died right after the injection or few hours after the injection suggesting that mice cannot tolerate the high injection volume. Studies have shown that the standard procedure involves a tail vein injection in 5–7 seconds of physiological solution, equivalent to 8–10% of body weight.[11] Due to the mortality rate, GLA was then measured for the 6 and 8% groups at 8 hours after the hydrodynamic injection because 8 hours in both groups have the highest peak of the GLA expression. First group was injected with 6% body weight and showed high activity of GLA than what it was detected in both wild type and knockout. Second group was injected with 8% body weight and showed GLA level significantly higher than the first group ($p<0.05$) and the mice still look healthy. Therefore, 8% injection volume is an appropriate injection volume in order to generate the highest peak of GLA expression and to also maintain the GLA activity in the tissues. (Figure2).

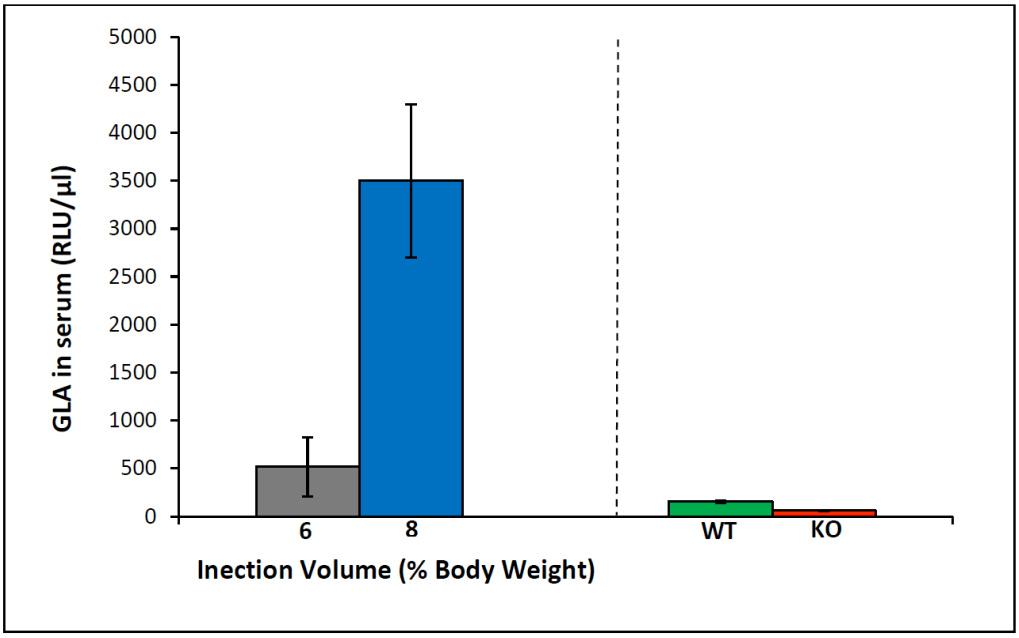


Figure 2. Optimization of Injection Volume. Fabry mice were injected with pAAV-GLA (20 µg/ml) in different volume equals to either 6% or 8% body weight. Serum GLA level was measured 8 hours post hydrodynamic injection. Values are the mean ± SD (*n*=5).
RLU; relative light units.

3.2 HGD MAXIMIZES DNA DOSE AT 5 µG/ML

The DNA dose was also optimized by injecting the Fabry mice with pAAV-GLA plasmids at different concentration with 8% body weight injection volume. Serum GLA activity showed that approximately from 5 to 15 µg/ml is able to show higher GLA level at 8 hours post the

hydrodynamic injection indicating that the DNA dose has to be from 5 to 15 µg/ml in order to maintain the highest GLA expression (Figure 3).

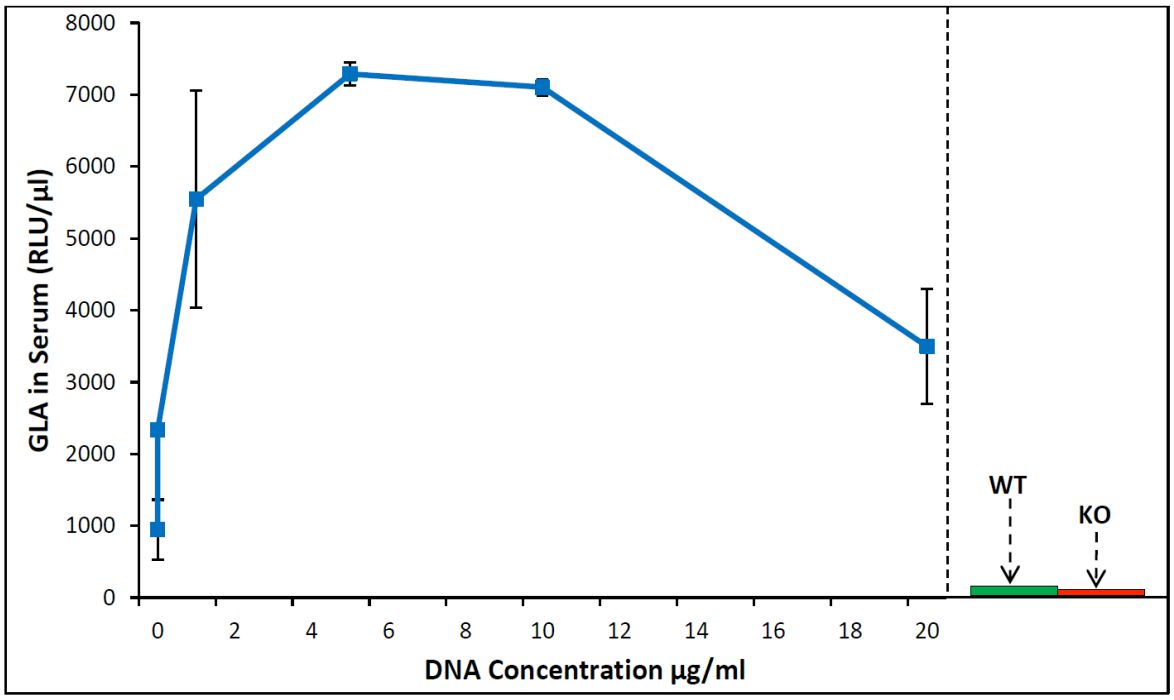


Figure 3. Optimization of DNA Dose. Fabry mice were hydrodynamically injected with pAAV-GLA plasmids at different concentration in volume equal to 8% body weight. GLA activity in serum was determined 8 hours post hydrodynamic gene delivery. Values are expressed as the mean ± SD (*n*=5).

3.3 PHARMACODYNAMICS OF GLA EXPRESSION INFLUENCED BY THE TIME AND THE TYPE OF THE VECTOR

Two groups of Fabry mice were injected hydrodynamically and the blood was collected from the tail of the mice at different time. Serum was obtained. The first group was injected with pAAV-GLA and serum was collected from day 1 to day 5. High level of GLA was detected at the first day and it declined quickly from the second day after the injection (Figure 4). Second group of Fabry mice were also injected hydrodynamically with pLIVE-GLA and the serum was collected at 2, 4, 8, 24, 48, 96 hours until 66 days post injection. The GLA expression was sustained for more than two months with repeating the injection and without causing mortality in our mice. Mice were sacrificed whenever the GLA expression in the serum decreased (18, 38, and 66 days post injection) and tissues samples (liver, kidney, heart, lung, spleen, brain and leg muscle) were collected (Figure 4).

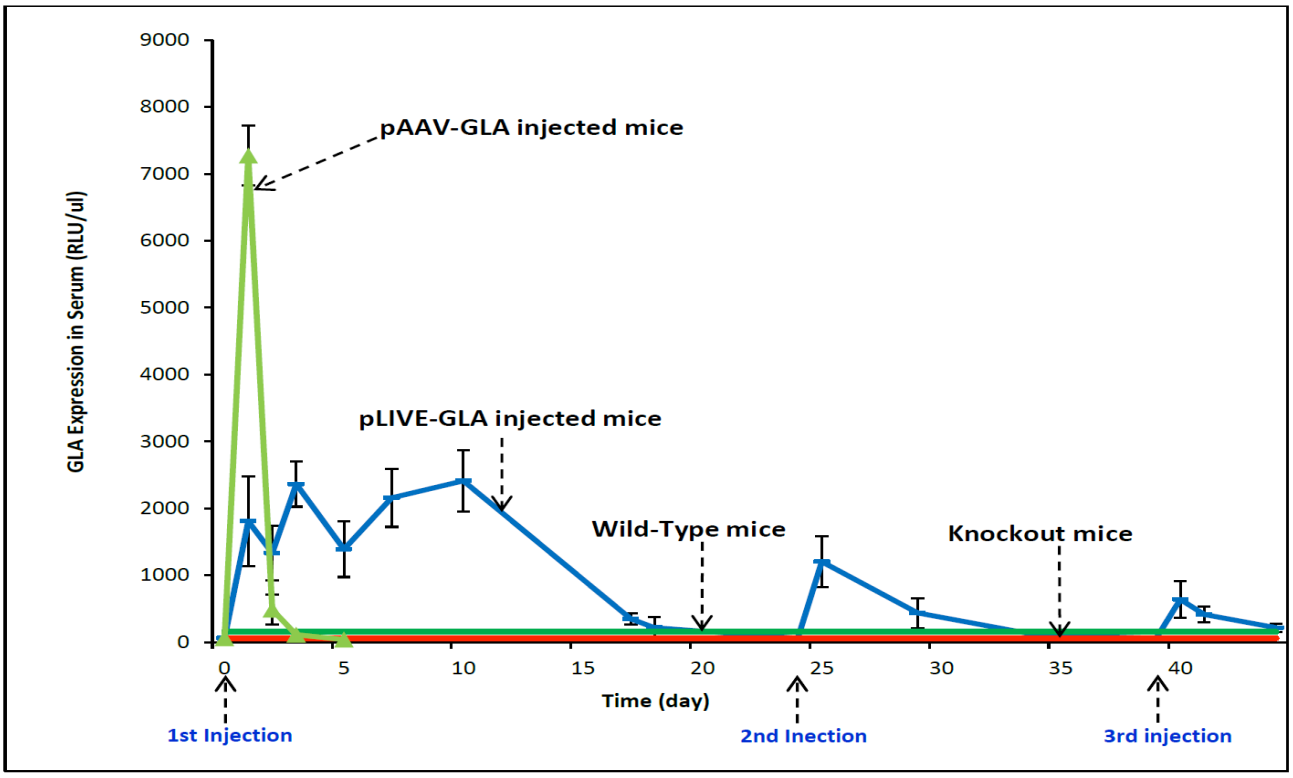


Figure 4. Plasmid Dependent GLA Expression in Fabry Mice. Fabry mice were hydrodynamically injected with either pAAV-GLA or pLIVE-GLA at concentration of 15 µg/ml and in a volume of 8% body weight. Serum GLA level was determined at different time and repeated injection was performed in animals injected with pLIVE-GLA plasmids. Values are the mean ± SD (*n*=5).

3.4 HYDRODYNAMIC GENE THERAPY OF GLA CORRECTS THE ORGAN BIOCHEMICAL DEFICITS

GLA activity was measured at different time points. Our findings demonstrated that GLA was taken up by the systemic organs (Table 1). Relative GLA levels in various organs are also presented in the (Figure 5). After the HGD, GLA activity was (20, 20, 40, 10, 70% of the normal GLA level) in the kidney, lung, spleen, brain, and leg muscle respectively. At 18 days post injection, GLA activity was significantly ($p<0.05$) increased in liver and heart. However, GLA level was ≈ 20 % in kidney and lung, 40% in spleen, 10% in brain and 70% in leg muscle of the normal GLA level. These levels were sustained up to 66 days after the treatment with a significant increasing in the liver, quite improving in the kidney and decreasing by 50% in the heart.

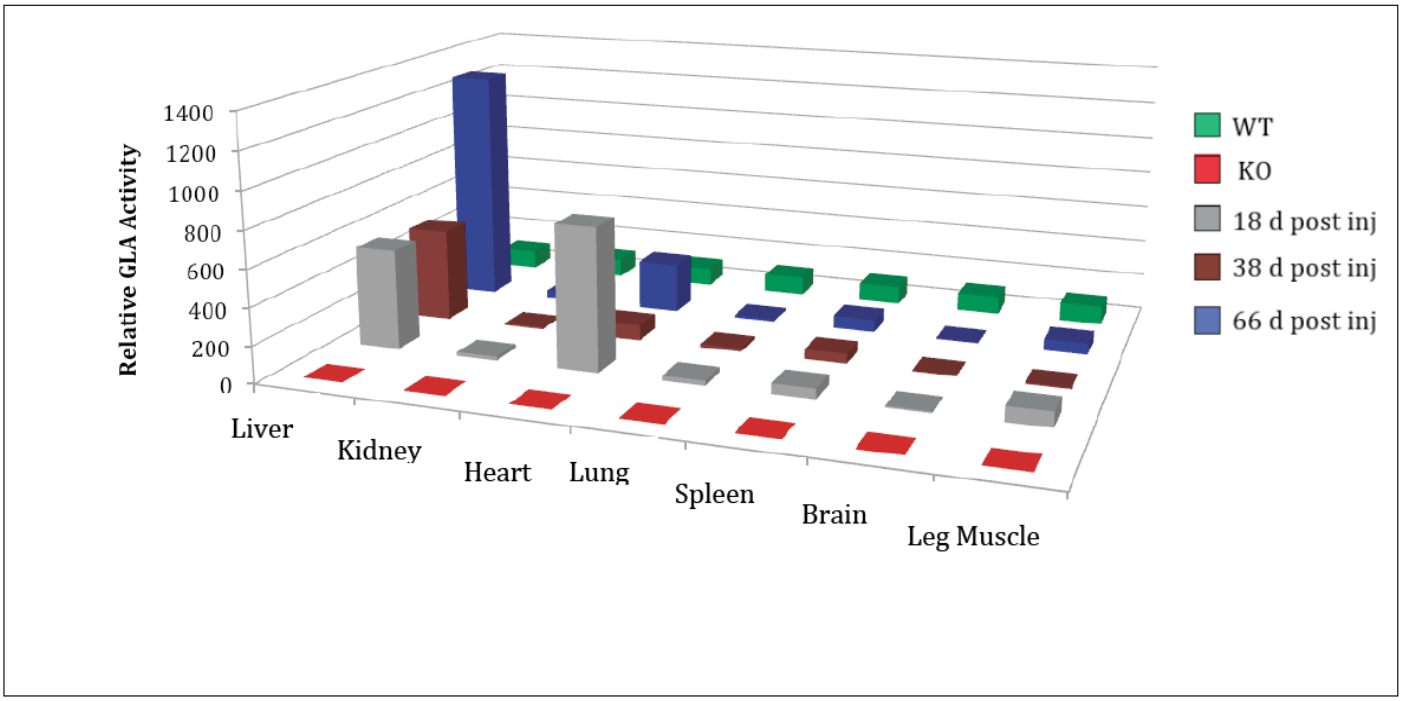
Table 1. GLA Activities/ µg Protein in tissues from untreated and treated Fabry mice, and wild-type mice

Organs	Wild-Type	Knockout	18 days	38 days	66 days
Liver	10.7±2.0	0.98±0.001	22.2±3.2	20.8±2.9	47.1±1.4
Kidney	6.4±0.7	0.93±0.01	1.5±1.7	1.23±1.7	2.1±0.9
Heart	3.1±0.5	1.02±0.0	5.5±0.5	1.503±0.6	2.6±0.2
Lung	14±0.16	1.3±0.01	3.1±4.2	2.5±4.2	2.2±2.2
Spleen	23.3±4.0	1.6±0.08	8.9±7.3	8.7±6.6	10.7±4.6
Brain	13.9±0.1	1.0±0.03	1.7±4.3	1.424±4.4	1.35±2.6
Leg Muscle	3.6±0.4	1.1±0.1	2.4±0.8	1.22±0.8	2.1±0.6

Organs were obtained from treated mice sacrificed at 18, 38 and 66 post pLIVE-GLA injection.

Figure 5. Relative GLA Levels in Organs of Treated Fabry Mice

$$\textbf{Relative GLA Activity} = \frac{\text{EM (460) Treated} - \text{EM (460) KO}}{\text{EM (460) WT} - \text{EM (460) KO}} * 100$$



GLA levels of different organs of wild-type (WT) and knockout (KO) Fabry mice, and treated mice at 18, 38 and 66 days post-hydrodynamic injection.

4.0 DISCUSSION

Based on our findings, hydrodynamic gene transfer is a reasonable approach for delivering GLA gene mediated enzyme and treating Fabry disease. Our study demonstrated that hydrodynamic gene delivery can be repeated in the same group of animals. We have cloned and prepared two GLA plasmids and we found that the GLA expression was mainly based on the type and of the vector that was utilized. Wild-type mice exhibit higher GLA expression than the GLA deficient mice suggesting the successful optimization of the hydrodynamic GLA gene transfer and the successful disruption of the GLA gene. Our results also suggest that the appropriate DNA dose is between 5 to 15 μg/ml in order to optimize and reach the significant level of GLA greater than the normal mice.

Some studies showed that GLA as an enzyme replacement therapy for Fabry patients can be given every 2 weeks suggesting that this period can be adequate in terms of preventing the Gb3 accumulation.[7] However, cost issue and difficulty in the enzyme production remained as limitations for ERT. Gene therapy approach has a strong potential for the treatment of metabolic disorders.[12-13] But this approach is still facing some obstacles in obtaining a sufficient gene expression in treated organs.[14] Our findings demonstrated that maintaining GLA expression in the serum and tissues for long period of time (~two months) can be accomplished by using pLIVE-GLA plasmid vector. Taking together, pAAV-GLA is more effective in generating peak level of GLA expression but pLIVE-GLA is more effective in sustained GLA expression.

These results suggest the possibility of using other vectors such as “minicircle” vectors that have been recently known to mediate gene expression. Our findings showed that hydrodynamic gene therapy of Fabry knockout mice results in GLA expression that is higher than is needed for the therapeutic level suggesting that the feasibility of using or applying another gene delivery approach such as HGD in large animals. Clinical study indicated that only 1–5% of normal enzyme activity is required to prevent LSDs suggesting that we successfully reached the GLA therapeutic level in the diseased organs. Gb3 analysis is also an interest parameter to determine and confirm the therapeutic outcomes. It is expected that tissues that have high GLA activity will exhibit much more reduction in the Gb3 Future studies can be done to investigate and measure the Gb3 lipid accumulation in target organs.

5.0 SUMMARY AND CONCLUSION

In summary, our study demonstrated that hydrodynamic gene delivery is a safe and very effective approach for GLA long-term systemic expression. It is also a promising approach in terms of treatment for Fabry disease. We were able to treat and correct the GLA deficiency in the serum, liver, heart and other tissues of Fabry mice. Further work should address the Gb3 analysis that can reflect the successful therapy for Fabry disease.

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