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THERAPY

Prednisolone Does Not Regulate Factor VIII Expression in Mice Receiving AAV5-hFVIII-SQ: Valoctocogene Roxaparvovec

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AAV5-hFVIII-SQ (valoctocogene roxaparvovec) is an adeno-associated virus (AAV)-mediated gene therapy vector containing a B-domain-deleted human factor VIII (hFVIII-SQ) transgene. In a phase 1/2 clinical study of AAV5-hFVIII-SQ for severe hemophilia A (FVIII < 1 IU/dL), participants received prednisolone to mitigate potential immune-mediated reactions to the gene therapy and demonstrated concomitant elevations in plasma FVIII levels, following a single administration of AAV5-hFVIII-SQ. To assess whether prednisolone is capable of directly modulating transgene expression or levels of circulating hepatic enzymes, C57BL/6 mice were given intravenous vehicle, 2×10^{13} vector genomes (vg)/kg AAV5-hFVIII-SQ, or 6×10^{13} vg/kg AAV5-hFVIII-SQ, followed by either daily oral prednisolone or water. Mice were euthanized 4 or 13 weeks after vector administration. Hepatic hFVIII-SQ DNA, RNA, and protein (immunostaining), plasma hFVIII-SQ protein and FVIII activity, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured. Liver hFVIII-SQ DNA, RNA, and plasma hFVIII-SQ protein and activity increased in a dose-dependent manner, with or without prednisolone. In summary, chronic prednisolone treatment in mice treated with AAV5-hFVIII-SQ did not modulate levels of liver hFVIII-SQ DNA, RNA, or the percentage and distribution of hFVIII-SQ-positive hepatocytes, nor did it regulate levels of plasma hFVIII-SQ protein or activity, or affect levels of plasma AST or ALT.

INTRODUCTION

Hemophilia A is an X-linked bleeding disorder arising from mutations in the gene encoding coagulation factor VIII (FVIII), an essential blood-clotting protein. The condition affects 1 in 4,000 male live births globally, with individuals presenting with a mild, moderate, or severe phenotype.[1] Individuals with the severe phenotype (<1% of normal FVIII activity) are at risk of uncontrolled spontaneous or traumatic bleeding into the joints and soft tissue, which may result in debilitating musculoskeletal damage.[2]

The current standard of care for severe hemophilia A is lifelong acute or prophylactic treatment with intravenous recombinant or plasma-derived FVIII. However, frequent infusions make adherence a challenge

and can have a negative impact on patient quality of life.[3,4] Moreover, multiple studies have reported that the use of recombinant or plasma-derived FVIII carries an increased risk of FVIII neutralizing antibody development, a serious complication estimated to affect up to 30% of people with hemophilia A and reducing the efficacy of FVIII replacement therapy.[3,5,6] Gene therapy may have the potential to provide a long-term approach to hemophilia A management, by reducing the need for frequent infusions, reducing the number of breakthrough bleeding events, and improving patient quality of life.[7]

AAV5-hFVIII-SQ (valoctocogene roxaparvovec) is a gene therapy in development for people living with severe hemophilia A. It comprises a replication-incompetent adeno-associated virus 5 (AAV5) vector containing a B-domain-deleted human FVIII gene (hFVIII-SQ) driven by a liver-specific promoter.[8] In a phase 1/2 study of individuals with severe hemophilia A, participants who received a single intravenous infusion of AAV5-hFVIII-SQ at a dose of 6×10^{13} vector genomes (vg)/kg showed sustained increases in FVIII levels, decreases in annualized bleeding rate, and decreases in reliance on exogenous FVIII replacement therapies at 1 year post-administration.[7]

In a study of AAV2 gene therapy for hemophilia B, participants were not given glucocorticoids, and a loss of transgene expression was observed in association with elevated alanine aminotransferase (ALT) levels.[9] A subsequent study of AAV8 gene therapy for hemophilia B showed that transient increases in serum transaminase levels resolved following prednisolone therapy, and long-term transgene expression was achieved.[10] Similar findings of increased transaminase levels have been observed with other AAV-delivered gene therapies.[11,12]

Transient, non-serious ALT elevations were the most commonly observed adverse events in the phase 1/2 study of AAV5-hFVIII-SQ for hemophilia A.[7] These ALT elevations were not associated with
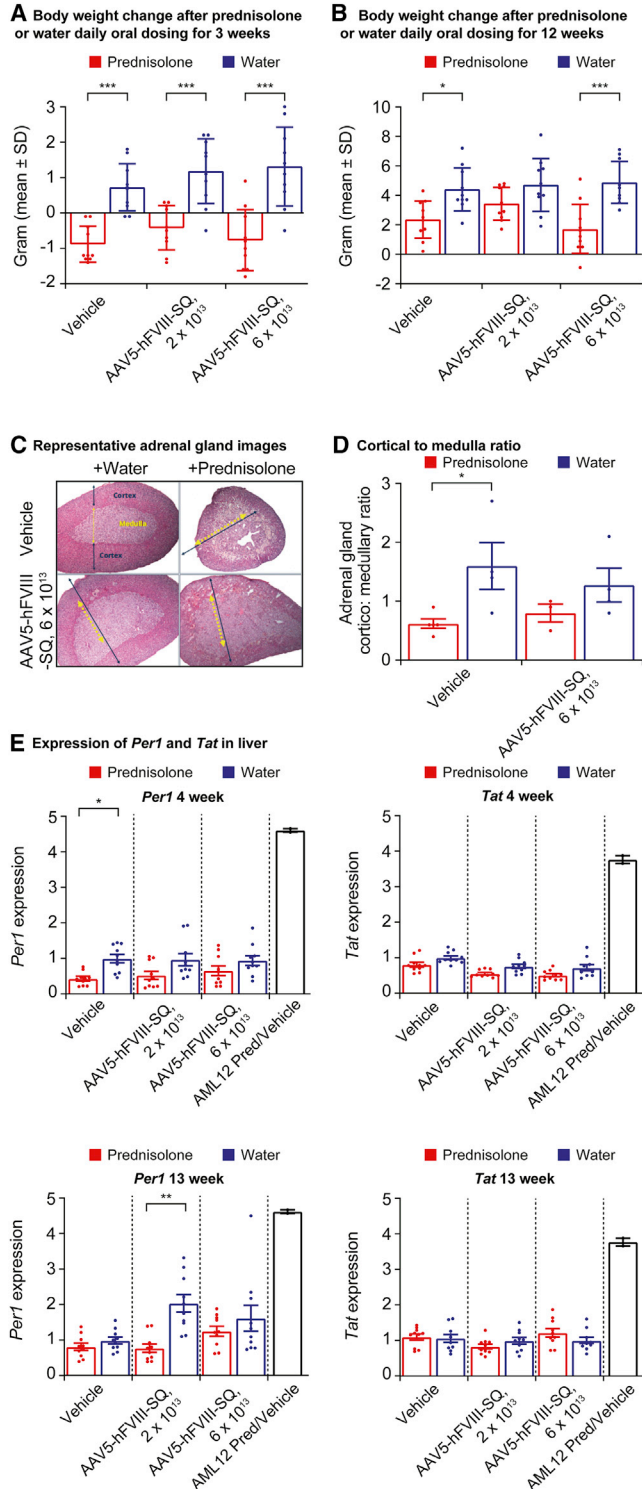
Figure 1. Demonstration of Prednisolone Exposure

(A and B) Mouse body weight change from baseline in the (A) 4-week and (B) 13-week cohorts. (C–E) Representative adrenal gland images (C), cortical-to-medulla ratio (D), and liver *Per1* and *Tat* expression (E) in water and prednisolone treatment groups in the 4- and 13-week cohorts, following a single tail vein administration of vehicle or AAV5-

hFVIII-SQ at 2×10^{13} or 6×10^{13} vg/kg, with or without daily prednisolone treatment for 3 or 12 weeks. AML12 is the cell line "active control." Results are mean ± SD (D) or SEM (n = 10 per group). AAV5-hFVIII-SQ, valoctocogene roxaparvovec; Pred, prednisolone. *p < 0.05. ***p < 0.001. When not indicated, comparisons for prednisolone- versus water-treated groups were non-significant.

any clinical sequelae or liver dysfunction, but it was speculated that increased ALT could correlate with FVIII expression decline, as has been seen in other gene therapy studies for hemophilia.[10] To minimize the potential for immune-mediated reactions and associated decreases in hFVIII-SQ expression levels, prednisolone was administered to patients either prophylactically or on demand.[7] However, participants receiving prednisolone also showed a concomitant increase in FVIII activity after AAV5-hFVIII-SQ infusion,[7] raising the question of whether prednisolone may directly regulate transgene expression. Thus, the preclinical study reported herein aimed to elucidate whether chronic prednisolone administration following treatment with AAV5-hFVIII-SQ affects transgene expression. Additionally, this study aimed to determine whether wild-type mice could be used to model transient ALT elevations seen in humans treated with AAV-mediated gene therapy.

RESULTS

All animals (male C57BL/6J mice, aged 8 weeks, n = 120) were observed to be in good health throughout the study period, with animals receiving scheduled daily treatment with either prednisolone (2 mg/kg) or water (control) by oral gavage initiated 1 week after AAV5-hFVIII-SQ or vehicle administration (six groups of 10 per group for 4-week and 13-week cohorts).

Efficacy of Prednisolone

Biological activity of prednisolone was confirmed by its effects on body weight, adrenal cortical atrophy, and expression of the steroid-responsive genes *Per1* and *Tat* in liver tissue.

In the 4-week cohort, animals in all groups given water experienced weight gain (mean starting weight [SD], 26.16 [1.46] g; mean final weight [SD], 27.23 [1.70] g; mean change in weight [SD] at 4 weeks of age, 1.07 [0.93] g), while animals in all groups given prednisolone experienced weight loss (mean starting weight [SD], 26.82 [1.65] g; mean final weight [SD], 26.13 [1.70] g; mean change in weight [SD], 0.69 [0.70] g; p < 0.0001). However, in the 13-week cohort, animals in both water and prednisolone groups gained weight, although mean weight gain was greater in water-treated animals (mean starting weight [SD], 25.40 [1.29] g; mean final weight [SD], 30.06 [2.63] g; mean change in weight [SD], 4.66 [1.56] g) than in prednisolone-treated animals (mean starting weight [SD], 26.02 [1.29] g; mean final weight [SD], 28.51 [2.12] g; mean change in weight [SD], 2.49 [1.55] g; p < 0.0001).

For the 4-week cohort, this trend in weight change for water versus prednisolone-treated groups was consistent across vehicle and AAV5-hFVIII-SQ dose groups (each group p < 0.001; Figure 1A). For the 13-week cohort, the difference in weight gain between the
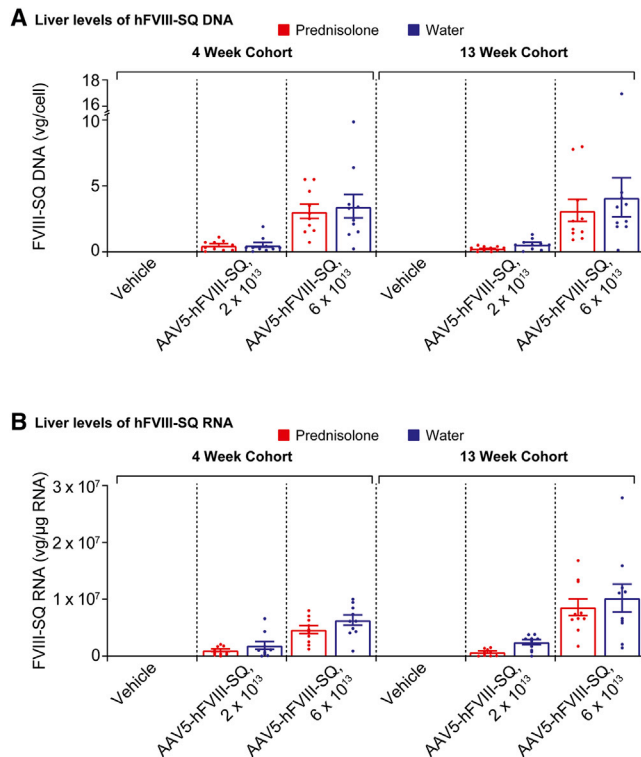
Figure 2. Evaluation of Levels of hFVIII-SQ DNA and RNA in the Liver

(A and B) Levels of liver hFVIII-SQ DNA (A) and RNA (B) in water-treated (blue bars) and prednisolone-treated (red bars) cohorts 4 or 13 weeks following a single tail vein administration of vehicle or AAV5-hFVIII-SQ at 2×10^{13} or 6×10^{13} vg/kg, with or without daily prednisolone treatment for 3 or 12 weeks. AAV5-hFVIII-SQ, valoctocogene roxaparvovec; Pred, prednisolone. Results are mean ± SEM (n = 10 per group). All comparisons for prednisolone- versus water-treated cohorts at equivalent dose level of AAV5-hFVIII-SQ were non-significant.

water and prednisolone groups was significant for the 6×10^{13} vg/kg AAV5-hFVIII-SQ dose group (p < 0.001) and for the vehicle group (p < 0.05) (Figure 1B).

Representative images of the adrenal gland from vehicle and 6×10^{13} vg/kg dose groups treated with either water or prednisolone from the 13-week cohort are shown in Figure 1C. Prednisolone-treated animals had a cortical-to-medullary (C:M) ratio less than 1 (mean [SD] C:M ratio of 0.6 [0.1] for vehicle and 0.8 [0.1] for 6×10^{13} vg/kg), indicative of adrenal atrophy. In water-treated animals, mean (SD) C:M ratios were 1.6 (0.4) for vehicle-treated and 1.2 (0.3) for 6×10^{13} vg/kg-treated animals, with the difference between the water-dosed and prednisolone-dosed groups reaching statistical significance in mice receiving vehicle (p < 0.05; Figure 1D). The changes in C:M ratio with prednisolone treatment were indicative of adrenal cortical atrophy due to chronic glucocorticoid treatment.

At 4 weeks, administration of prednisolone was associated with a trend toward suppressed expression of *Per1*, irrespective of vector dose. However, by 13 weeks, this trend had largely disappeared, sug-

gesting that the animals had adjusted in response to prolonged treatment. No significant changes were evident for *Tat* at either 4 or 13 weeks (Figure 1E).

Changes in body weight and reduced expression of the steroid-responsive glucocorticoid receptor (GR) target genes *Per1* and *Tat* in hepatic tissue in this study are consistent with effects shown with chronic glucocorticoid exposure for up to 1 week in the same mouse model.[13]

Collectively, assessments of prednisolone effects on body weight, adrenal cortical atrophy, and expression of the steroid-responsive genes *Per1* and *Tat* in liver tissue confirm that the prednisolone dosage used in our study was appropriate for exploring the impact of glucocorticoid exposure on AAV5-hFVIII-SQ transgene expression in the wild-type C57BL/6J mouse.

Hepatic hFVIII-SQ DNA and RNA

A dose-dependent increase in both hFVIII-SQ DNA and RNA was seen in the liver of both water- and prednisolone-treated mice at both 4 and 13 weeks following 2×10^{13} and 6×10^{13} vg/kg AAV5-hFVIII-SQ administration (Figure 2). Notably, treatment with prednisolone for 3 and 12 weeks did not significantly modulate the levels of hFVIII-SQ DNA or RNA in liver, compared with water treatment, at either AAV5-hFVIII-SQ dose level. No hFVIII-SQ DNA and RNA was detectable in mice that did not receive AAV5-hFVIII-SQ.

Liver hFVIII-SQ RNA also did not change significantly between 4 and 13 weeks for animals dosed at 2×10^{13} vg/kg; an increase in RNA was seen at the higher dose of AAV5-hFVIII-SQ between 4 and 13 weeks, with or without prednisolone treatment. These findings support the observations that valoctocogene roxaparvovec-mediated hFVIII-SQ production reaches steady state by 12 weeks post-dosing.[8]

hFVIII-SQ Protein in Liver and Plasma

To investigate whether chronic prednisolone treatment could impact hFVIII-SQ expression post-transcriptionally, plasma hFVIII-SQ protein was measured and patterns of hepatic immunostaining were recorded. Dose-dependent increases in plasma hFVIII-SQ protein level were observed at 4 and 13 weeks (Figures 3A and 3B) following administration of AAV5-hFVIII-SQ. Moreover, when compared with water, prednisolone did not modulate hFVIII-SQ protein levels in plasma at either vector dose (Figures 3A and 3B).

The hFVIII-SQ protein immunostaining pattern was assessed in the 13-week cohort for the dose group receiving 6×10^{13} vg/kg AAV5-hFVIII-SQ and was similar regardless of whether prednisolone or water was administered for 12 weeks (Figure 3C). Specifically, prednisolone treatment for 12 weeks did not affect the proportion of hepatocytes that were immunopositive for hFVIII-SQ (mean ± SEM: prednisolone group, 12.8 ± 1.5%; water group, 11.5 ± 1.2%), and this immunopositivity was mainly observed surrounding central veins (Figure 3D).

A Plasma hFVIII-SQ protein

B Plasma FVIII activity

C Immunostaining of hFVIII-SQ in the 13 week cohort

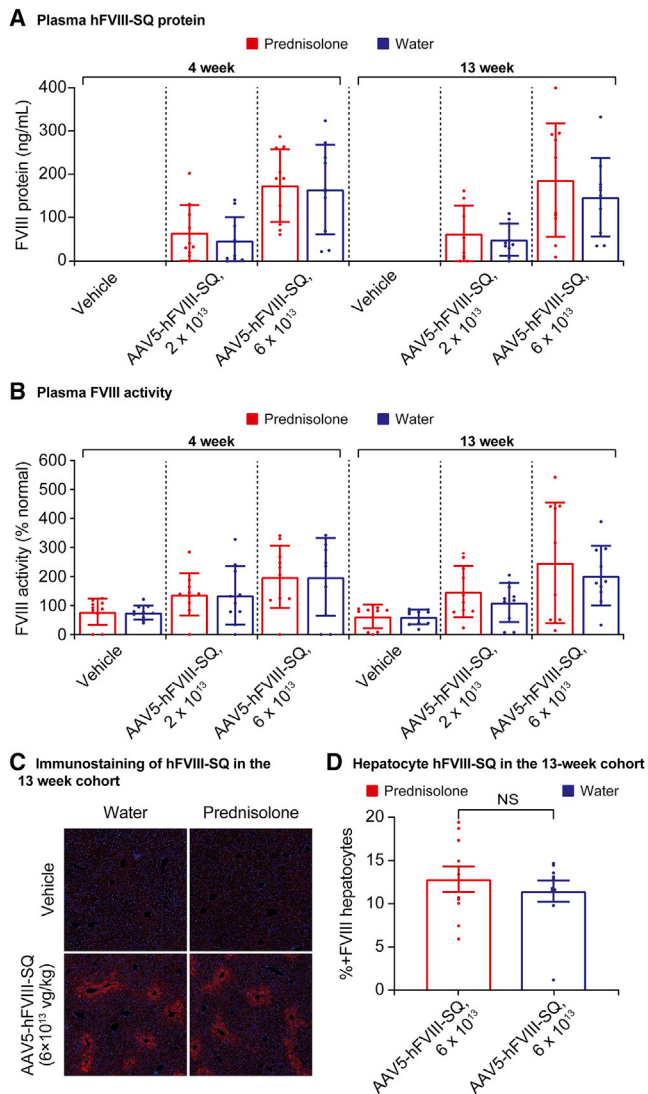## D Hepatocyte hFVIII-SQ in the 13-week cohort

Figure 3. hFVIII-SQ Protein and FVIII Activity Levels in Liver and Plasma

(A and B) Plasma levels of hFVIII-SQ protein (A) and FVIII activity (B) in water- or prednisolone-treated cohorts 4 and 13 weeks following a single tail vein administration of vehicle or AAV5-hFVIII-SQ at 2×10^{13} or 6×10^{13} vg/kg. (C and D) Liver hFVIII-SQ protein immunostaining (C) and quantitative analysis (D) of percentage of hepatocytes with detectable hFVIII-SQ protein staining in a water- or prednisolone-treated 13-week cohort following a single tail vein administration of vehicle or 6×10^{13} vg/kg AAV5-hFVIII-SQ. Results are mean ± SD (n = 10) for (A) and (B). Results are mean ± SEM (n = 10 per group), except vehicle (Veh) groups: Veh+Pred (n = 9), Veh+Water (n = 7) for (D). AAV5-hFVIII-SQ, valoctocogene roxaparvovec; NS, not significant; Pred, prednisolone. All comparisons for prednisolone- versus water-treated cohorts at equivalent dose levels of AAV5-hFVIII-SQ were non-significant.

Assessment of Liver Injury

There were no significant increases in mean plasma levels of aspartate aminotransferase (AST) or ALT in response to treatment with AAV5-hFVIII-SQ at either vector dose or in response to prednisolone (Figure 4). However, several mice treated with prednisolone appeared to show non-significant increases in levels of ALT

and/or AST, regardless of whether they received vehicle or AAV5-hFVIII-SQ treatment. One animal treated with vehicle+prednislone showed an ALT level above the upper limit of normal (ULN; normal range, 18–94 U/L), but no animal had an AST level above ULN (normal range, 32–122 U/L).[14] Mice that showed increased ALT levels also tended to have higher AST levels. In general, greater inter-animal variability was observed in animals treated with prednisolone compared with water.

DISCUSSION

In a phase 1/2 study of AAV5-hFVIII-SQ for severe hemophilia A, adult male participants received prednisolone to manage ALT elevations, which were the most commonly observed adverse event in this study and have also been commonly reported in other AAV gene therapy trials.[9–12] In one patient receiving 6×10^{13} vg/kg AAV5-hFVIII-SQ, mildly increased ALT was accompanied by a decline in FVIII activity, calling into question a potential relationship between ALT and FVIII levels, as well as the role of prednisolone in potentially influencing the latter. Furthermore, FVIII activity appeared to increase with time in patients who received prednisolone; as AAV5-mediated delivery of the FVIII transgene reportedly takes longer to reach a steady state than other serotypes, this might simply mirror FVIII levels having not yet reached steady state following administration of AAV5-hFVIII-SQ.[7] The preclinical research described herein uses the same investigational vector at two of the same doses explored in the phase 1/2 study to investigate whether chronic glucocorticoid treatment directly affects hFVIII-SQ expression following gene transfer with AAV5-hFVIII-SQ.

Following AAV5-hFVIII-SQ administration in mice, dose-dependent levels of hFVIII-SQ DNA were detected in hepatocytes. There was no evidence that daily administration of prednisolone for up to 13 weeks had an impact on levels of hFVIII-SQ DNA, RNA, or protein expression in the liver, that suggesting prednisolone treatment does not modulate DNA retention or regulate transcription of hFVIII-SQ DNA in hepatocytes via the promoter. Immunohistochemistry showed that hFVIII-SQ protein was localized to hepatocytes, mainly those surrounding central veins, which is consistent with the distribution pattern seen in a hemophilia mouse model following administration of AAV5-hFVIII-SQ.[8] In this study, distribution was unaffected by chronic prednisolone administration. Moreover, neither the levels of plasma hFVIII-SQ nor plasma FVIII activity was impacted by the prednisolone regimen, suggesting that glucocorticoid treatment did not affect hFVIII-SQ secretion from the liver. The present study in mice showed that hFVIII-SQ protein levels increased between 4 weeks and 13 weeks, regardless of prednisolone treatment. This finding is consistent with recent observations in mouse models[8] and humans,[7] and it suggests that the relationship between observed increases in FVIII activity and prednisolone administration is a temporal association and not direct or causal.

In clinical trials of AAV-based vectors in hemophilia B, glucocorticoids were administered to protect against immune-mediated hepatocyte injury, which could also decrease or prevent transgene
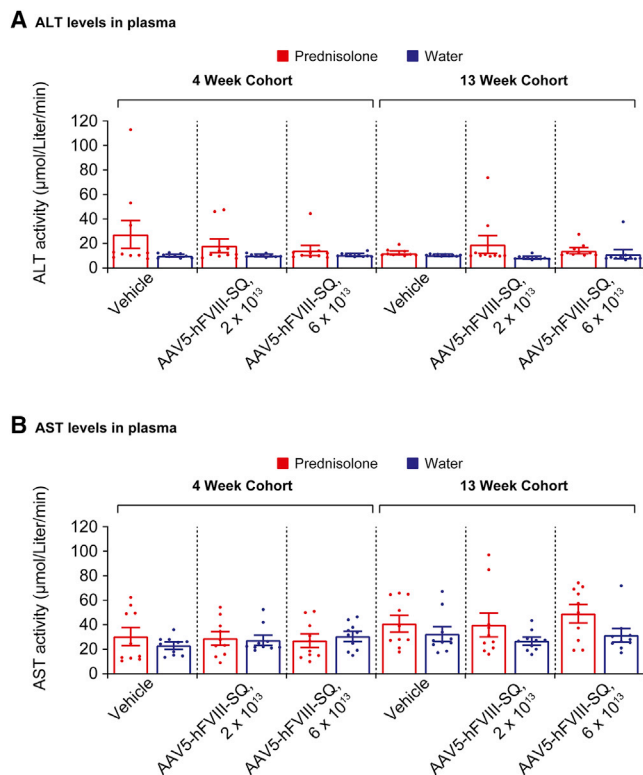
A ALT levels in plasma

B AST levels in plasma

Figure 4. Evaluation of Liver Enzymes in Plasma

(A and B) Plasma levels of (A) ALT and (B) AST in water- and prednisolone-treated cohorts 4 or 13 weeks following a single tail vein administration of vehicle or AAV5-hFVIII-SQ at 2×10^{13} or 6×10^{13} vg/kg, with or without daily prednisolone treatment for 2 or 12 weeks. Results are mean ± SD (n = 10 per group). AAV5-hFVIII-SQ, valoctocogene roxaparvovec; ALT, alanine transaminase; AST, aspartate transaminase; Pred, prednisolone.

expression.[9,10,12,15] Similarly, glucocorticoids were used to manage transient ALT elevations in the phase 1/2 study of AAV5-hFVIII-SQ.[7] Therefore, the present study also explored whether the transient elevations in liver enzymes observed in some patients following AAV5-hFVIII-SQ administration in the phase 1/2 study could be modeled in wild-type, as opposed to immune-compromised, mice. However, no significant elevations of ALT or AST were observed in mice following treatment with AAV5-hFVIII-SQ at either vector dose, regardless of whether or not they were also given prednisolone.

Mild, asymptomatic increases in ALT have been observed following clinical administration of AAV gene therapy for hemophilia A,[7] hemophilia B,[9–12] and spinal muscular atrophy.[16] In all cases, elevation of ALT levels were resolved with prednisolone treatment. The impact of this remains unclear, although during AAV clinical trials for hemophilia B, a dose-dependent elevation of serum ALT was associated with decreased factor IX levels.[10,15] Moreover, two patients who experienced ALT elevations following hemophilia B gene therapy had also shown a capsid-directed immune response that was managed with

corticosteroid treatment.[11] To date, only one patient receiving AAV5-hFVIII-SQ at 6×10^{13} vg/kg has had an increase in ALT accompanied by a decline in FVIII activity, with no clear indication of a consistent relationship or temporal correlation.[7]

Despite murine strains being frequently used to model human disease and to prove therapeutic concepts, limitations exist when it comes to translating results into the clinical setting.[17] However, pre-clinical findings can guide the design and implementation of future clinical studies. Follow-up studies should also investigate the potential effect of glucocorticoids on vector transduction, which was likely to have already occurred at the point in time when prednisolone was administered in this study. ALT and AST elevation patterns in preclinical studies have not mirrored those seen in the clinical setting, with very few elevations observed in mice (up to 2×10^{14} vg/kg) and monkeys (up to 6×10^{13} vg/kg) who received AAV5-hFVIII-SQ. Thus, although clinical trials with AAV5-hFVIII-SQ showed transient elevation of liver enzymes, these preclinical species do not appear to model an ALT rise following AAV5-hFVIII-SQ treatment. We cannot rule out whether higher doses might induce a transient rise in hepatic enzymes in these species. We did observe sporadic elevation of ALT/AST in a few mice, presumably associated with prednisolone treatment.[18]

With the number of clinical gene therapy trials increasing rapidly and the concomitant administration of glucocorticoids becoming standard practice, a thorough understanding of the effects of prednisolone on transgene expression is critical. This study finds that chronic prednisolone treatment initiated 1 week following AAV5-hFVIII-SQ administration in immune-competent mice did not modulate hepatic AAV5-hFVIII-SQ DNA retention, transcription, or hFVIII-SQ protein expression and distribution, nor did it elevate levels of plasma hFVIII-SQ protein or liver enzymes.

MATERIALS AND METHODS

Mouse Studies

All *in vivo* mouse experimentation was performed in accordance with institutional guidelines under protocols approved by the Institutional Animal Care and Use Committee of the Buck Institute (Novato, CA, USA). In the current study, C57BL/6J mice (Jackson Laboratory, Western Sacramento, CA, USA) were used mainly because daily oral gavage required to administer prednisolone could cause injury of the esophagus and internal bleeding, more likely leading to death of hemophilia A (HA) mice than C57BL/6J mice. Previous studies indicated that hFVIII-SQ expression in HA and C57BL/6J mice is comparable.[8]

Treatment Schedule

Male C57BL/6J mice (n = 120) aged 8 weeks were distributed into two cohorts, according to the time they were to be euthanized (either at 4 weeks or 13 weeks), with six groups per cohort (n = 10 per group). Two groups in each cohort received either vehicle or one of two doses of valoctocogene roxaparvovec (AAV5-hFVIII-SQ; 2×10^{13} vg/kg or 6×10^{13} vg/kg) by single intravenous bolus injection via the tail vein. One of the two groups in each dosing group received daily treatment

Table 1. Dose Schedule

	4-Week Cohort (n = 60)		13-Week Cohort (n = 60)	
Group	Week 0	Weeks 1–4[a]	Week 0	Weeks 1–13[a]
Group 1 (n = 10)	vehicle	prednisolone	vehicle	prednisolone
Group 2 (n = 10)	AAV5-hFVIII-SQ, 2×10^{13} vg/kg	prednisolone	AAV5-hFVIII-SQ, 2×10^{13} vg/kg	prednisolone
Group 3 (n = 10)	AAV5-hFVIII-SQ, 6×10^{13} vg/kg	prednisolone	AAV5-hFVIII-SQ, 6×10^{13} vg/kg	prednisolone
Group 4 (n = 10)	vehicle	water	vehicle	water
Group 5 (n = 10)	AAV5-hFVIII-SQ, 2×10^{13} vg/kg	water	AAV5-hFVIII-SQ, 2×10^{13} vg/kg	water
Group 6 (n = 10)	AAV5-hFVIII-SQ, 6×10^{13} vg/kg	water	AAV5-hFVIII-SQ, 6×10^{13} vg/kg	water

[a]Prednisolone was administered orally at a dose of 2 mg/kg daily, starting 1 week after vehicle or AAV5-hFVIII-SQ (valoctocogene roxaparvovec) and continued for 3 weeks in total in the 4-week cohort and for 12 weeks in total in the 13-week cohort.

with prednisolone at 2 mg/kg, and the other served as a control and received water by oral gavage initiated 1 week after vehicle or AAV5-hFVIII-SQ (Table 1). Mice were euthanized either 3 weeks after initiating water/prednisolone (4-week cohort) or 12 weeks after initiating water/prednisolone (13-week cohort).

Efficacy of Prednisolone

Efficacy of prednisolone treatment was assessed by change in body weight from baseline to termination, as well as histopathological examination of the adrenal gland for evidence of cortical atrophy. Additionally, determination of expression of the steroid-responsive genes period circadian protein (*Per1*) and tyrosine amino transferase (*Tat*) was also performed; the expression of these GR marker genes is characteristically reduced by prednisolone.[19]

Histopathological Examination of the Adrenal Gland

Histopathological analysis was performed on adrenal glands from mice in the 13-week cohort that received vehicle with/without prednisolone or high-dose AAV5-hFVIII-SQ (6×10^{13} vg/kg) with/without prednisolone (n = 40; four groups of 10). Adrenal glands were harvested, formalin-fixed/paraffin-embedded (FFPE), and stained with H&E. The presence of a full-thickness adrenal gland was required for inclusion; 16 samples met this criterion. All stained tissues were scanned on a Zeiss Axio Scan.Z1 using a Plan-Apochromat 20×/0.8 objective equipped with a Hamamatsu Orca Flash camera. A μm bar set by Zeiss Axio Scan.Z1 image analysis software was used as a calibration measurement. Three width measurements were recorded for each adrenal gland, comprising the upper cortex, medulla, and lower cortex, which were used to calculate the C:M ratio. A C:M ratio of <1 indicated adrenal gland atrophy.

Expression of *Per1* and *Tat*

Expression levels of steroid-responsive genes *Per1* and *Tat* were monitored as molecular biomarkers of prednisolone activity determined in mouse liver samples using a Droplet Digital PCR (ddPCR) assay with a QX200 ddPCR system (Bio-Rad, Hercules, CA, USA). Each ddPCR assay reaction (25 μL) contained 1× ddPCR Supermix for Probes (no deoxyuridine triphosphate [dUTP]) (Bio-Rad, Hercules, CA, USA), 1× gene-specific PrimePCR assay of either

mTat (FAM) or mPer1 (FAM) and control primers/probe, mRplp0 (HEX), and diluted cDNA (1:30 dilution). Droplets were generated from the reaction mix and QX200 droplet generation oil for probes (Bio-Rad) using a QX200 droplet generator (Bio-Rad). After droplet generation, droplets were transferred to 96-well PCR plates, which were subsequently sealed using a PX1 PCR plate sealer (Bio-Rad). PCR was performed in a C1000 Touch thermal cycler (Bio-Rad) with the following cycling conditions: once at 95°C for 10 min, 40 times at 95°C for 30 s/58°C for 1 min, and once at 98°C for 10 min/4°C for hold with a 2°C/s ramp rate. Following PCR, the samples were analyzed using a QX200 droplet reader (Bio-Rad). Target mRNA transcript concentrations (copies/μL) were calculated using Poisson statistics in QuantaSoft software (Bio-Rad), and values were normalized to *mRplp0* levels. All *mTat* (assay ID dMmuCPE5094458), *mPer1* (assay ID dMmuCPE5094220), and *mRplp0* (assay ID dMmuCPE5195429) primers/probe sets for the ddPCR assay were from Bio-Rad.

Hepatic hFVIII-SQ DNA and RNA and Protein Expression

Expression levels of hFVIII-SQ DNA (qPCR) and hFVIII-SQ RNA (reverse transcription followed by qPCR) were assessed in a liver fragment of ∼25 mg from all mice in the 4-week and 13-week cohorts. Liver fragments were homogenized separately in 2 mL of lysing matrix D tubes (MP Biomedicals, OH, USA) containing 600 μL of RLT buffer (QIAGEN, Hulsterweg, the Netherlands) with 1% β-mercaptoethanol using the FastPrep-24 instrument (MP Biomedicals) with the setting 6.0 m/s for 40 s at room temperature. Total RNA and genomic DNA in liver homogenate were extracted from the same homogenate using the QIAGEN DNA/RNA/Protein AllPrep kit following the manufacturer's instructions for manual or QIAcube extraction. Genomic DNA was eluted in 50–100 μL of QIAGEN elution buffer (EB), and RNA was eluted in 100 μL of nuclease-free water (QIAGEN). The concentration of the extracted RNA and DNA was measured using Nano-Drop 2000 (Thermo Scientific, Cheshire, UK) using 2 μL of samples. Each extracted DNA sample was diluted to 10 ng/μL, and each RNA sample was diluted to 200 ng/μL.

Hepatic expression and distribution of hFVIII-SQ protein was measured by immunohistochemistry in the 13-week cohort only in four groups, including those that received vehicle with prednisolone,

vehicle without prednisolone, AAV5-hFVIII-SQ at 6×10^{13} vg/kg with prednisolone, and AAV5-hFVIII-SQ at 6×10^{13} vg/kg without prednisolone (n = 10 per group; 40 in total). FFPE livers were sectioned at 5-μm thickness on SuperFrost Plus slides. Slides were deparaffinized and rehydrated in a series of decreasing graded ethanols. Antigen retrieval solution (Ventana Discovery, Tucson, AZ, USA) was used to retrieve antigen at 95°C. Sections were blocked in 2% normal donkey serum (NDS), 0.1% BSA, and 0.3% Triton X-100 in $1\times$ Tris-buffered saline (TBS) for 45 min at room temperature. Anti-FVIII antibody Abcam (Cambridge, MA, USA, catalog no. ab139391) was diluted 1:1,000 in Ventana reaction buffer (Ventana Medical Systems, Tuscon, AZ, USA; catalog no. 950-300) and slides were incubated overnight at 4°C. Slides were washed in $1\times$ TBS. Donkey anti-sheep 555 antibody (Life Technologies, Waltham, MA, USA, catalog no. A21436) was diluted 1:500 in Ventana reaction buffer and sections were incubated for 1 h at room temperature. Slides were washed in $1\times$ TBS, counterstained with DAPI, and mounted with Fluoromount G. Slides were imaged on a Zeiss Axio Scan.Z1 using a Plan-Apochromat $20\times/0.8$ objective equipped with a Hamamatsu Orca Flash camera. One whole section of liver was acquired per animal, and two regions were randomly selected for export and image analysis. Total FVIII-positive hepatocytes were counted with custom macros using Volocity version 6.3 software (PerkinElmer, Waltham, MA, USA).

Plasma hFVIII-SQ Protein Levels and FVIII Activity

Blood samples were collected at termination for evaluation of plasma hFVIII-SQ protein levels using a sandwich ELISA, and plasma FVIII activity was assessed using a chromogenic FXa-activating assay. The ELISA utilized human-specific anti-FVIII capture (GMA-8023, Green Mountain Antibodies, Burlington, VT, USA) and detection (F8C-EIA, Affinity Biologicals, Ancaster, ON, Canada) using antibody pairs to specifically measure human FVIII rather than endogenous mouse FVIII in high-binding black polypropylene plates coated with 4 μg/mL anti-FVIII (domain A2) antibodies. Samples were diluted 1:10 in a diluent buffer comprising 6% BSA in $1\times$ TBS with Tween 20 (TBST) and incubated for ~2 h at ambient temperature. Human FVIII was detected by the addition of sheep anti-FVIII antibodies conjugated to horseradish peroxidase (HRP) and incubated at ambient temperature for 1 h. After the final wash, QuantaBlu substrate solution was used for detection. The relative fluorescent units detected on a FlexStation 3 instrument (Molecular Devices, San Jose, CA, USA) were proportional to the levels of hFVIII-SQ protein in the samples, and the concentrations were extrapolated from an 11-point standard curve prepared by spiking in clinical grade recombinant B-domain-deleted human FVIII (Xyntha; Pfizer, manufactured by Wyeth Pharmaceuticals, Philadelphia, PA, USA) in human FVIII-deficient plasma (George King Bio-Medical, Overland Park, KS, USA) with the range of 0.684–700 ng/mL. The quantitative range for this assay was determined to be 2.73–700 ng/mL Xyntha (FVIII-SQ) in neat mouse plasma.[8]

An FXa chromogenic-based kit (Chromogenix; Diapharma, West Chester, OH, USA) was used to measure hFVIII activity in sodium citrate-anticoagulated murine plasma samples. The chromogenic FXa assay indirectly measured the total FVIII activity resulting from both murine endogenous FVIII and hFVIII-SQ produced from AAV5-hFVIII-SQ.

In the first stage of the assay, an excess of tenase complex factors (purified bovine FIXa, FX, phospholipids, and calcium) except FVIII was added to plasma samples at the minimum required dilution (MRD) of 1:320 in buffer. The mixture was then incubated at 37°C for 2 min to allow tenase complex stabilization. During the second stage, the FXa chromogenic substrate S-2765 and synthetic thrombin inhibitor I-2581 were then added to the mixture followed by 14 min of incubation at 37°C. The FXa reaction generated chromophore peptide nucleic acid (pNA) and was stopped by addition of 20% acetic acid. The color intensity, which was linearly proportional to the amount of FVIII present in the samples, was detected by light absorbance at 405 nm with a SpectraMax M2.0E instrument. The levels of FX activity in the samples were extrapolated from an 11-point standard curve generated by spiking reference normal human plasma (George King Bio-Medical, Overland Park, KS, USA) in FVIII-deficient human plasma with the range of 0.087%–89% normal human FVIII activity. The quantitative range for this assay was 5.56%–89% normal human FVIII activity. If samples were above the upper limit of quantification (ULOQ), samples were diluted in neat human FVIII-deficient plasma and re-measured.

Assessment of Plasma Liver Enzymes

Terminal plasma levels of ALT were determined using 2 μL of plasma/well with the ALT (MAK052) activity assay kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions for fluorometric measurements. Fluorometric detection was performed with a FlexStation 3 multi-mode microplate reader (Molecular Devices, Sunnyvale, CA, USA). Terminal plasma levels of AST were determined using 5 μL of plasma/well with an AST activity assay kit (MAK055; Sigma-Aldrich) according to the manufacturer's instructions for colorimetric measurements. Colorimetric detection was performed with a FlexStation 3 multi-mode microplate reader (Molecular Devices).

Data Analysis

Results are presented throughout as mean ± SD or mean ± SEM. *Per1* and *Tat* transcript levels were normalized to the control group using the protein coding gene *Rplp0* (ribosomal protein lateral stalk subunit P0). All statistics were analyzed in GraphPad Prism 6 (GraphPad, La Jolla, CA, USA) using one-way ANOVA. Variance testing for immunohistochemical evaluation in the mice given AAV5-hFVIII-SQ at 6×10^{13} vg/kg employed the Student's t test.

AUTHOR CONTRIBUTIONS

CONFLICTS OF INTEREST

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