Published in final edited form as:
J Control Release. 2008 December 18; 132(3): 252–259. doi:10.1016/j.jconrel.2008.06.019.

Gene transfer to hemophilia A mice via oral delivery of FVIII-chitosan nanoparticles

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Abstract

Effective oral delivery of a non-viral gene carrier would represent a novel and attractive strategy for therapeutic gene transfer. To evaluate the potential of this approach, we studied the oral gene delivery efficacy of DNA polyplexes composed of chitosan and Factor VIII DNA. Transgene DNA was detected in both local and systemic tissues following oral administration of the chitosan nanoparticles to hemophilia A mice. Functional factor VIII protein was detected in plasma by chromogenic and thrombin generation assays, reaching a peak level of 2–4% FVIII at day 22 after delivery. In addition, a bleeding challenge one month after DNA administration resulted in phenotypic correction in 13/20 mice given 250–600 μg of FVIII DNA in chitosan nanoparticles, compared to 1/13 mice given naked FVIII DNA and 0/6 untreated mice. While further optimization would be required to render this type of delivery system practical for hemophilia A gene therapy, the findings suggest the feasibility of oral, non-viral delivery for gene medicine applications.

Keywords

nonviral gene delivery; hemophilia therapy; chitosan; oral delivery; gene medicine

Introduction

Hemophilia A is a disorder of the blood coagulation cascade caused by defective factor VIII (FVIII), a protein that normally circulates in the plasma at 100–200 ng/ml. The disease is a popular target for gene therapy due to the low threshold for therapeutic value and wide therapeutic window of FVIII [1]. Gene therapy for hemophilia A using viral vectors has demonstrated high transfection efficiency and supra-physiologic FVIII levels in mice [2–4]. Despite these successes, different viral vector systems have suffered from limitations including

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random integration [5], immunogenicity [6], packaging constraints [7], and difficulties with the production of high vector titers [8]. These limitations on viral delivery systems led us to consider designing a non-viral system that could be therapeutic and safe.

The design of an effective non-viral vector for gene medicine applications, however, faces many formidable challenges. The levels of transgene expression from such systems are generally low and transient [9–11]. In treating a chronic disease such as hemophilia A, repeated administration of the non-viral vector is likely to be required to achieve sustained protein production. This consideration makes oral delivery a clearly attractive strategy. However, the harsh environment of the gastrointestinal (GI) tract requires that the plasmid DNA be effectively protected from degradation. Poor absorption of such a hydrophilic macromolecule also requires effective formulation. We opted to apply chitosan as a cationic polymeric gene carrier in this study [12,13].

Chitosan is a biodegradable polysaccharide that has been widely studied for gene delivery, particularly for mucosal delivery [14–16]. Depending on the molecular weight and the degree of deacetylation, chitosan can confer effective protection of the complexed DNA against enzymatic degradation [17], and protonated chitosan in solution has the ability to increase trans-and para-cellular intestinal permeability [18–21]. Chitosan nanoparticles were effective in oral vaccination against peanut and dust mite allergens [22,23]. A recent report also indicates the ability of chitosan DNA nanoparticles to generate expression of the much smaller blood protein erythropoietin, as indirectly evidenced by increased hematocrit after gavage delivery [24].

Developing an oral formulation for gene medicines is attractive, but challenging. In this study, we attempted to evaluate the feasibility of this concept using a complex therapeutic gene such as FVIII as well as a realistic, oral delivery method in a relevant animal model.

Materials and Methods

Preparation of Nanoparticles

DNA-chitosan nanoparticles were prepared as previously described [17]. Briefly, equal volumes of filtered 0.02% chitosan in 5mM sodium acetate buffer and 100 μg/mL plasmid DNA in 50mM sodium sulfate were heated to 55°C and mixed under vortex for 20 seconds. The chitosan was a gift of Vanson (Redmond, WA) and had a MW of 390 kDa and a degree of deacetylation of 83.5%. Chitosans with a MW of 390 kDa and degrees of deacetylation of 70% and 62% were prepared by acetylation with acetic anhydride and DNA nanoparticles were formed as described [25]. Particle size and charge characterizations were carried out using a Zetasizer 3000 (Malvern Instruments, Malvern, UK). Measurements of mean hydrodynamic diameter were obtained by photon correlation spectroscopy at 25°C using a 90° scattering angle in automatic mode. Zeta potential measurements were made in a capillary cell by laser Doppler anemometry.

Factor VIII Plasmids

The plasmid hFVIII BDD MLP consisted of B-domain deleted human factor VIII (4.6 kb) [26] cloned into the pMT2 expression plasmid, driven by the adenovirus major late promoter [27,28]. The plasmid cFVIII BDD TBG consisted of B-domain deleted canine FVIII (4.5 kb) driven by the liver-specific thyroxine-binding globulin promoter [3] and human B-domain deleted FVIII cDNA was also cloned into this backbone (hFVIII BDD TBG).

In vitro Transfection

Cells were grown at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS, 50 units Penicillin/50 μg Streptomycin (final concentration), and L-glutamine (2 mM final concentration) and plated 24 hours prior to transfection. One hour before transfection, the medium was replaced with serum-free Opti-MEM. At the time of transfection Opti-MEM was removed and polymer or Lipofectamine-DNA complexes (prepared in Opti-MEM) were incubated with cells in antibiotic-free media for 24 hours, after which cells were washed and complete media added. Supernatant was assayed for FVIII activity at 72 hours using Coatest VIII: C/4 kits (Chromogenix, Milan, Italy) following manufacturer's instructions for low-range FVIII. The standard curve was generated by diluting normal human plasma (FACT; George King BioMedical, Inc., Overland Park, KS) in DMEM. Total protein in cell lysate was measured by BCA assay (Pierce, Rockford, IL). DNA and RNA were isolated from cell lysate using the DNeasy Tissue Kit and RNeasy Mini Kit, respectively (Qiagen, Valencia, CA).

Oral Delivery of FVIII Nanoparticles

Chitosan-FVIII DNA nanoparticles (84% DA chitosan) or naked plasmid DNA were orally administered to groups of 10–12 week old FVIII exon 16 knock-out mice. Three doses of 50, 250, or 600 μg DNA were tested. Nanoparticles or naked DNA were mixed with 0.24 g/mL strawberry Jell-O brand gelatin. Solutions were incubated for several minutes at 55°C to melt the gelatin, measured into sterile 35 mm Petri dishes, and allowed to harden at 4°C. Mice previously acclimated to strawberry Jell-O were given individual dishes of FVIII Jell-O overnight. For all groups day +1 was considered to be the last day of feeding. At the low dose, eight mice per group received a single feeding containing 50 μg DNA. One mouse per group was sacrificed at day 4 and at day 14 for tissue analysis. Plasma was collected from the remaining mice prior to the study (pre-bleed) and on days 4 and 28. At the high dose, eleven mice per group received five consecutive daily doses of 120 μg DNA, for a total of 600 μg DNA per mouse. Six mice were sacrificed at day 4 for tissue analysis and plasma was collected from the remaining mice pre-study and at days 4, 8, 15, 22, and 29. At the intermediate dose, seven mice per group received five consecutive daily doses of 50 μg DNA for a total of 250 μg DNA. Plasma was collected pre-study and at days 1, 4, 15, 22, and 29. Mice (n=8) were also given an intermediate-dose feeding of chitosan-DNA nanoparticles formed with 70% DA chitosan. Mice received five consecutive daily doses of 50 μg DNA and were re-fed with a single dose of 50 μg DNA at 2 months. Plasma was collected at day 8, 15, 29, and at 2-week intervals thereafter. All procedures were carried out in accordance with institutional guidelines under approved protocols at the University of Pennsylvania.

Plasma Collection

Mice were anesthetized with methoxyflurane. Whole blood was collected from the tail vein into microfuge tubes with 0.38% (wt/vol) sodium citrate. Blood was centrifuged at 2000g for 10 min and the plasma transferred to fresh tubes and stored at −80°C until assayed. At the final time-point phenotypic correction was determined. Mice were anesthetized and approximately 2 cm of tail was transected. Mice were monitored over 48 hours using survival, normal movement around the cage, and cessation of bleeding as evidence of phenotypic correction. Although the determination of correction was made at 48 hours, mice that did not exhibit phenotypic correction generally did not survive past 24 hours.

Genomic DNA Isolation and PCR

Genomic DNA was harvested from tissue samples using the Wizard Genomic DNA Purification kit (Promega, Madison, WI) and digested with chitosanase and lysozyme (Sigma-Aldritch, Saint Louis, MO). PCR for the factor VIII transgene was carried out with primers specific for human or canine FVIII sequences. The sense primer 5′GGC TTC TGG ACA CAT

TAG AGA TTT TCA G 3′ and antisense primer 5′AGA GGT CCT GTG CCT CGC AGC CC 3′ amplified a 913 bp product from human factor VIII cDNA. For the canine FVIII, the sense Primer 5′GGA GAG TAA AGC AAT ATC AGA TGC TCA G 3′ and antisense primer 5′TCA GGC GGG CTG CTG GGT GTC G 3′ amplified a 500 bp fragment. Quantitative real-time PCR was performed at the Vector Core, University of Pennsylvania using primers and a probe targeted to the SV40 poly A region of the plasmid DNA. The primers and probe sequences were as follows: forward primer: 5′AGC AAT AGC ATC ACA AAT TTC ACA A 3′, reverse primer: 5′CCA GAC ATG ATA AGA TAC ATT GAT GAG TT 3′ and probe: 6FAM-AGC ATT TTT TTC ACT GCA TTC TAG TTG TGG TTT GTC-TAMRA. RT-PCR was carried out using the SuperScript First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) and the DNA-free DNase Treatment and Removal Kit (Ambion, Austin, TX) following manufacturer's protocols. Control samples without reverse transcriptase (-RT) were run for all samples to verify that there was no DNA contamination.

Thrombin Generation

A Fluorogenic Thrombin Generation Test (FTGT) was used which is based on the use of a fluorogenic thrombin substrate that allows thrombin generation to be determined continuously in a more physiological system than prior thrombin generation assays, without the need for sub-sampling or defibrination of plasma samples. This method is sensitive to low levels of FVIII (<0.001 IU/mL) (James et al., 2005), significantly below the level that is detectable by current coagulation factor assays. Thrombin generation was initiated by the addition of one part non-defibrinated plasma (40 μl) to 2 parts substrate mixture (80 μl) [fluorogenic substrate (0.238 mM), FIXa (5 nM), phospholipid (3 μg/mL) and Ca^{2+} (7 mM)] in a black microtitre plate (Greiner, Germany). The plate was read in a Spectramax Gemini XS Fluorimeter (Molecular Devices) at 30°C, at 30 sec intervals for 1 hour with excitation at 390 nm and reading at 460 nm. The data was exported into an Excel file and the amount of thrombin generated was calculated according to the method of Hemker [29]. This method allows determination of free thrombin in a continuous thrombin generation test with chromogenic and fluorogenic substrates. The data was analyzed and quantified for the area under the curve (AUC) and peak height parameters. Thrombin generation measured in pre-treatment plasma samples for each mouse was subtracted from all post-treatment data points. The thrombin values were converted to percent FVIII values based on a standard curve constructed by diluting normal human plasma in pooled exon 16 knock-out hemophilia A mouse plasma.

Chromogenic Assay

Plasma samples were assayed for human FVIII activity using Coatest VIII: C/4 kits (Chromogenix, Milan, Italy) following manufacturer's instructions for low-range FVIII. The standard curve was generated by diluting normal human plasma (FACT; George King BioMedical, Inc., Overland Park, KS) in pooled exon-16 knock-out mouse plasma such that the 0% value on the curve represented the reading obtained from 100% pooled hemophilia A knock-out plasma.

Anti-human FVIII Antibody Levels

Immulon high binding microplates were coated overnight at 4°C with purified B-domain deleted human FVIII protein (50 ng/well). All wash steps were carried out with 0.5% PBS/Tween. Plates were blocked with Superblock PBS (Pierce, Rockford, IL) at room temperature for 30 min, washed, and incubated for 2 hr at room temperature with plasma samples diluted 1:5 in Superblock. After washing, goat-anti mouse IgG-HRP secondary antibody (Caltag, Burlingame, CA) was bound for one hour at RT and plates were read at 490 nm using the detection substrate O-Phenylenediamine (Pierce, Rockford, IL). The standard curve was constructed of mouse monoclonal anti-human FVIII heavy chain (Abcam, Cambridgeshire,

UK). Duplicate samples of pooled knock-out plasma were included on all plates. Where possible, anti-human FVIII antibody levels are reported as levels at day 28 minus pre-bleed.

Statistical Analysis

Student's t-tests and chi-squared tests were performed on sample data. $P < 0.05$ was considered significant (indicated by *) and $p < 0.01$ was considered highly significant (indicated by **).

Results

Characterization and in-vitro evaluation of FVIII-chitosan nanoparticles

Although the FVIII plasmids evaluated ranged in size from 8.5 to 9.6 kb, the particles formed from all FVIII plasmid constructs were of similar size and charge, with an average diameter around 300 nm and a zeta potential of +10 mV at pH 5.7. In contrast, particles formed with partially sheared salmon sperm DNA (average size of less than or equal to 2 kb) were smaller and were approximately 200 nm in diameter (Figure 1A). Chitosan-FVIII nanoparticles formed with the plasmid hFVIII BDD MLP were able to transfect Cos-7 cells, a cell line previously used for the production of recombinant factor VIII protein [30]. Transfection was carried out in the presence of 10% fetal bovine serum to provide the necessary von Willebrand factor to stabilize the secreted factor VIII. Detection of FVIII plasmid DNA and FVIII mRNA in cell lysates and of functional FVIII protein secreted into the conditioned cell media confirmed the efficacy of the nanoparticle transfection (Figure 1B–D). In contrast, no functional FVIII could be detected in cells transfected with naked plasmid DNA. Although FVIII protein levels achieved with chitosan nanoparticles were lower than those achieved with the commercial lipid reagent Lipofectamine 2000, cell toxicity was also significantly less with chitosan than with Lipofectamine. Typical protein levels measured by a bicinchoninic acid total protein assay 72 hours after chitosan transfection were 90–110% of levels measured in untransfected control cells, versus levels of approximately 60% following Lipofectamine 2000 transfection (data not shown).

Biodistribution of FVIII plasmid DNA following oral administration of chitosan nanoparticles or naked DNA to mice

DNA transfer into tissues was examined by studying the *in vivo* biodistribution of FVIII plasmid 72 hours after oral delivery of high (600 μg) and low (50 μg) dose DNA-chitosan nanoparticles or naked DNA (Figure 2). Following high dose delivery, DNA was detected in seven of eight tissues examined. The strongest signals appeared to be in stomach and ileum, as might be expected from an oral delivery system. However, DNA was also detected in liver tissue, the normal site of FVIII synthesis, and in multiple systemic tissues including kidney, lung, and spleen. The detection of plasmid DNA in non-gastrointestinal tissues indicates that at least a fraction of DNA is able to cross the intestinal barrier and reach the circulation, either by blood or lymph. FVIII DNA was also detected in multiple tissues of mice receiving doses of naked plasmid DNA.

Plasmid copy numbers were determined by real-time PCR for the tissue samples that qualitatively appeared to contain the highest levels of FVIII plasmid DNA, namely stomach, ileum, Peyer's patch, liver, and spleen. DNA was extracted from tissue samples collected 72 hours after the final dose of the high dose feeding of 600 μg DNA (n=6 mice/group). Using a probe to the SV40 polyA region, factor VIII plasmid DNA was detected in all five tissues assayed at levels of up to approximately 0.1 copies per cell. The copy numbers detected in mice given naked or chitosan-condensed FVIII DNA were generally above background levels from untreated hemophilia A mice (Figure 3). High-dose oral delivery of chitosan-DNA nanoparticles yielded higher plasmid copy numbers in Peyer's patch tissue compared to naked DNA delivery, as might be expected for a particulate delivery system. On the other hand, naked

DNA delivery yielded generally higher DNA copy numbers in liver and spleen. None of these results achieve statistical significance, however, due to the high variability observed in the data (Figure 3A).

After the biodistribution experiment with high dose at day 4, an additional experiment was conducted to examine if the low dose would still yield a detectable level of plasmid DNA and to determine its persistence. Delivery of a high DNA dose (600 μg) resulted in approximately one to four times higher average tissue copy numbers than a single, low dose of 50 μg of DNA (Figure 3). However, the dose response relationship is not linear; high dose feeding of 12 times the DNA compared to low dose did not result in an equivalent fold increase in the copy numbers in the various tissues. Even at two weeks after delivery of a single low dose, plasmid DNA was still detectable in liver tissues of both naked DNA and chitosan-DNA fed mice, indicating some persistence of the plasmid DNA *in vivo* (Figure 3B).

Detection of functional FVIII protein following oral administration of FVIII DNA

The production of factor VIII protein and its activity in the plasma of mice given chitosan-DNA nanoparticles or naked DNA was analyzed by two different functional assays, a chromogenic FVIII test for the conversion of factor X to factor Xa, and a fluorogenic thrombin generation assay. Both assays revealed low levels of FVIII protein (Figure 4). By chromogenic assay, FVIII levels after high dose feeding reached 2–4%. The levels began to rise at day 15, peaked at day 22, and began to decline thereafter. In contrast, high dose delivery of naked plasmid DNA never achieved FVIII levels above 1%. The thrombin generation assay showed that relatively large amounts of thrombin were generated following high dose oral delivery of FVIII DNA-chitosan nanoparticles in the hemophilia A mice, with levels of FVIII also approximating 2–4%. Despite individual variation, there was a significant difference in factor VIII production between chitosan and naked DNA treatments at days 22 and 29.

A DNA dose response was also observed in the mice. Delivery of a low or medium dose of DNA administered as chitosan nanoparticles did not result in average factor VIII levels over 1.5%. By contrast, delivery of the high dose of 600 μg DNA produced peak factor VIII levels of approximately 3%. Both assays were based on a standard curve of normal human plasma diluted in pooled hemophilic mouse plasma, to mimic the situation of delivering human FVIII DNA to hemophilia A mice. While levels of circulating FVIII protein were clearly detectable, oral delivery of chitosan-FVIII nanoparticles did not lead to the production of high titers of anti-FVIII antibodies. Anti-human FVIII antibodies were all less than 3 ng/mL based on a 1:5 dilution of plasma (data not shown).

The low, medium and high dose chitosan nanoparticle feedings were all carried out using chitosan with a molecular weight of 390 kDa and a degree of deacetylation (DA) of 84%. A comparison at the medium DNA dose was also made using chitosan-DNA nanoparticles formed from chitosan having the same molecular weight, but with a degree of deacetylation of 70%. Transfection in Cos-7 cells of chitosan-FVIII nanoparticles formed from 84%, 70%, and 62% deacetylated chitosans resulted in lower factor VIII expression as the percent of deacetylation decreased (Figure 5A). It has been previously reported, however, that a decrease in degree of deacetylation resulted in improved protein production following intramuscular injection in mice [25]. Average plasma factor VIII levels were similar between mice given the 84% DA chitosan nanoparticles and those given the 70% DA chitosan nanoparticles over the first month, reaching approximately 1.5% FVIII (Figure 5B). Following the first month, the levels of FVIII in the mice given the 70% DA chitosan nanoparticles rose, reached a second peak of approximately 2.5% at 6 weeks, then declined. At eight weeks, the mice were re-fed with a single dose of 50 μg DNA. However, this could not arrest the decline in factor VIII levels (Figure 5C).

In addition to generating low levels of thrombin, the FVIII protein processed from both the 84% DA and 70% DA chitosan-DNA nanoparticles was able to partially correct the bleeding diathesis in hemophilia A mice. A phenotypic tail-clip assay resulted in significantly increased survival rates (13/20) for the chitosan nanoparticle-fed mice compared to mice fed with naked DNA (Figure 6A). By dose, a clear difference in survival between nanoparticle- and naked DNA- treated mice was observed at high and intermediate dose feedings. For mice fed the highest dose of DNA (600 μg), 4 out of 5 of the nanoparticle-fed mice survived the tail clip at one month, while only 1 out of 5 of the naked DNA mice survived. At the intermediate DNA dose, 3 of 7 (43%) of the 84% DA chitosan-treated mice were able to survive a phenotypic challenge at one month. Despite the recorded decline in factor VIII levels in mice given the intermediate dose of the 70% DA chitosan nanoparticles, 6 out of 8 (75%) of these mice survived a phenotypic challenge at 4 months. None of the intermediate-dose naked DNA-treated mice survived. Additionally, none of the untreated mice (0/6) survived the tail-clip assay (Figure 6B).

Discussion

While we have previously demonstrated oral gene transfer in a prophylactic vaccine model using chitosan as a gene carrier [23], in this study we explored the potential of chitosan nanoparticles to deliver gene medicines in a therapeutic disease model.

Preparation and In Vitro Evaluation of the Chitosan-DNA Nanoparticles

The chitosan used in these studies is able to condense factor VIII-encoding plasmid DNA into ~ 300nm nanoparticles. Studies evaluating the uptake of both non-biodegradable polystyrene and biodegradable poly(lactide-*co*-glycolide) (PLGA) particles of various sizes following oral administration have generally demonstrated greater uptake as particle size decreases, with 300 nm particles in the size range compatible with internalization through the gastrointestinal tract [31,32]. The FVIII nanoparticles produced in this study also demonstrated successful *in vitro* transfection and production of FVIII mRNA and functional FVIII protein, even in the presence of 10% serum. The ability of the chitosan nanoparticles to transfect in the presence of serum is encouraging as the transfection efficiency of many cationic lipids is strongly serum-inhibited. The chitosan nanoparticles also displayed low cellular toxicity, consistent with prior reports indicating the safety of chitosan as a biomaterial [33,34]. Based on our *in vitro* evaluations, the FVIII DNA-containing chitosan nanoparticles thus appeared promising as a delivery system for hemophilia A gene transfer.

Levels of Gene Transfer in Hemophilia A Mice

Although *in vitro* testing is useful, an effective gene delivery system must be able to mediate successful expression in a relevant *in vivo* model. Following oral administration of chitosan nanoparticles to hemophilia A mice, plasmid DNA was detected in local gastrointestinal tissues (stomach, ileum, and Peyer's patch) as well as in liver, spleen, and additional systemic tissues (Figure 3). Although the copy numbers detected following oral delivery were low (~0.02 copy per cell), similarly low copies have previously been reported after injection of an AAV 2 vector containing murine factor VIII DNA [7]. In this instance, 0.001 to 0.1 copies per cell in liver were sufficient to achieve plasma factor VIII levels of 2% and partial phenotypic correction several months after vector injection.

Unexpectedly, the observed tissue plasmid copy numbers were similar following delivery of both naked DNA and DNA-nanoparticles to the mice. We suspect that the gelatin base in which the nanoparticles and the naked DNA were administered may have contributed to this effect and may have served to partially protect the naked DNA or to increase its uptake into cells. Indeed, gelatin nanoparticles have previously been tested as gene carriers, and have shown

limited DNase protection as well as *in vitro* transfection efficiency [35]. The method of quantitative DNA detection employed in the study may also have contributed to the similar plasmid copy numbers observed. Copy numbers were measured via quantitative PCR using binding of a small probe to a sequence in the DNA. This method may fail to distinguish between intact and partially degraded plasmid. Therefore, it is conceivable that plasmid DNA in the form of nanoparticles remains in a more intact form than naked plasmid DNA, leading to the improved protein production and increased rates of phenotypic bleeding correction that were observed in mice (Figures 4 and 6)

The method of oral feeding likely contributes to variability in the transfection efficacy as well. In our protocol, chitosan-DNA nanoparticles or naked plasmid DNA were mixed into a gelatin base and consumed overnight by the mice. We chose this delivery method to reduce trauma associated with the use of a feeding needle, and also because the volumes produced using the current method of nanoparticle formation were too large to be successfully delivered via a feeding needle. However, different mice would digest the gelatin over a period of hours and this variation may affect the extent of DNA uptake into the tissues. Improvement to the method of chitosan-DNA nanoparticle synthesis that would allow easier encapsulation and delivery of large quantities of DNA would be useful. Successful transfection of HEK 293 cells following freeze-drying and storage of chitosan-DNA nanoparticles formed using Luciferase plasmid has previously been demonstrated [17]. In this instance, decorating the nanoparticle by poly (ethylene glycol) (PEG) and inclusion of sugars such as trehalose were required to stabilize the particles and prevent aggregation. Further studies will probably have to adopt such an approach because it is necessary to suspend a large quantity of nanoparticles in a small volume, without producing particle aggregation, for in vivo evaluation.

Despite individual variability, chitosan-FVIII DNA nanoparticles generated a significantly higher factor VIII protein level than naked DNA (Figure 4). However, the production of FVIII protein alone is not sufficient. The ultimate goal of increasing factor expression is to achieve activation of the coagulation cascade and sufficient thrombin generation to produce a hemostatic effect. Although a fraction of the thrombin response detected may be due to activation of the extrinsic coagulation cascade during the process of blood collection, we attempted to correct for this effect by subtracting any background thrombin generation that was detected in pre-treatment plasma. As a result, the FVIII levels we achieved, as measured by both chromogenic and fluorogenic assays, were 1–4% of normal plasma levels. Although these levels are modest, FVIII levels as low as 1–2% of normal have been shown to ameliorate clinical symptoms in severe hemophilia A [36].

Despite the modest FVIII levels achieved in the mice, detectable FVIII protein persisted for one month and phenotypic bleeding correction was observed in 65% of the mice given high or medium doses of chitosan-DNA nanoparticles (Figure 6). These levels are particularly encouraging given the processing and translational requirements of FVIII. Expression of high levels of FVIII protein is complicated by poor mRNA accumulation, retention of the protein in the endoplasmic reticulum, poor transport from the endoplasmic reticulum to the Golgi, and short plasma half-life in the absence of von Willebrand factor [37–39]. Even in cell culture, FVIII production is reportedly 2–3 orders of magnitude lower than other proteins [37].

Challenges to Hemophilia A Gene Transfer via Oral Delivery

Successful oral gene delivery requires initial protection of the DNA from degradation in the harsh environment of the gastrointestinal tract. Transport of FVIII DNA to the liver, a major site of FVIII protein synthesis, via an oral delivery route also requires transcytosis into the bloodstream and uptake into liver cells. Intracellular protection from endosomal degradation, release of DNA from the carrier, access to nuclear transcription machinery, and translation and processing into a functional protein are further required before a therapeutic effect can be

achieved. However, despite these extra- and intra- cellular barriers, there is evidence that genes delivered orally can mediate expression locally and systemically [22,23,40–42].

Hemophilia A may additionally be an appropriate disease to investigate for therapy via oral gene delivery, since functional FVIII can reportedly be processed from a variety of non-hepatic cell types including fibroblasts [43], keratinocytes [44], and gut epithelium [45]. These results suggest that hemophilia A may be amenable to therapeutic gene transfer using an oral route of administration, even if the non-viral carrier cannot cross the gastrointestinal wall and reach liver cells. If the secreted protein is able to reach the basal blood supply, a vector that transfects intestinal tissue may still result in the processing and secretion of sufficient levels of functional FVIII protein to effect a phenotypic correction.

Following oral delivery of DNA chitosan nanoparticles to hemophilia A mice, plasmid was detected in local gastrointestinal tract tissues. This finding implies that internalization of some fraction of the administered DNA dose is possible. However, the tissue copy numbers achieved were quite low, highlighting the inefficiency of the process and the challenges that exist to obtaining successful gene expression from an oral delivery route. Even with the protection afforded by complexation into chitosan nanoparticles, some plasmid DNA is likely to be degraded during the passage through the GI tract and into the tissues. Using a Caco-2 monolayer system, we have previously observed partial degradation of plasmid DNA in chitosan nanoparticles subsequent to transport across the cells (data not shown). The low plasmid copies and modest levels of FVIII production achieved in the hemophilia A mice orally administered the chitosan-DNA nanoparticles may be partly explained by such incomplete DNA protection.

Conversely, once nanoparticles are internalized by cells, the plasmid DNA must be released from the cationic carrier to allow gene expression to proceed. Poor intracellular DNA release from the chitosan nanoparticle complex has previously been identified as a potential barrier to gene expression [25]. Despite uptake of FVIII plasmid DNA into tissues (Figure 3), only low levels of protein were detected in the mice (Figure 4). Inefficient DNA release, in addition to partial DNA degradation, may thus represent other substantial barriers to high-level gene expression from the chitosan DNA nanoparticle delivery system.

The detection of transgene DNA in systemic tissues after oral delivery suggests that a fraction of the administered nanoparticles internalized in the GI tract are able to cross the gastrointestinal wall and reach the circulation. The possibility that particles degrade in gastrointestinal tissue and release un-bound DNA into the bloodstream cannot be discounted, however. Further studies on the uptake and macroscopic transport of chitosan-DNA nanoparticles are needed to resolve this issue.

Approaches to Address Barriers to Effective Delivery and Gene Expression

As discussed above, improvements to the DNA nanoparticle formulation will be required to achieve more effective gene transfer following oral administration. In order to partially explore the barrier that might be posed by insufficient DNA release from the chitosan nanoparticle complex, we evaluated nanoparticles formed from two types of chitosan administered to mice at the intermediate DNA dose: DNA chitosan nanoparticles formed with 84% deacetylated (DA) chitosan and particles formed with 70% DA chitosan.

A decrease in the degree of chitosan deacetylation is associated with reduced DNA binding efficiency and the formation of less stable nanoparticles. Our lab has previously reported decreased Luciferase gene expression in several cell culture lines when transfected with chitosans having decreased degrees of deacetylation [25]. Similarly, transfection of chitosan-FVIII DNA nanoparticles into Cos-7 cells resulted in decreased levels of FVIII as the degree of deacetylation decreased. However, a lower degree of deacetylation resulted in enhanced

Luciferase expression following intra-muscular injection in mice [25], suggesting that the results achieved *in vivo* may not always be predicted by the results of *in vitro* testing. When we administered both 84% DA and 70% DA chitosan-FVIII nanoparticles to the hemophilia A mice, we observed similar levels of plasma factor VIII over a one month period. These levels reached a maximum of approximately 1.5% FVIII (Figure 5). However, longer-term follow-up of the mice given the 70% DA nanoparticles revealed that while there was a peak of approximately 1.5% FVIII at day 15, there was a second, larger peak in FVIII levels around 6 weeks. This peak occurred much later than we expected and may reflect slow intracellular DNA release from the chitosan nanoparticles. A wide range of chitosans of varying molecular weights and degrees of deacetylation are available, allowing room for future optimization. In an effort to address the problem of effective plasmid protection and subsequent release we are also currently exploring the use of a more readily degradable, synthetic cationic polymer.

Many important questions remain regarding the effectiveness of therapeutic gene delivery via oral administration of polymer nanoparticles. It will be particularly valuable to elucidate the cell types transfected and to determine at which stage the DNA becomes released from the chitosan polyplex. Additional understanding of the gene transfer barriers and further optimization of the polymer carrier are clearly necessary. Simply feeding larger amounts of DNA using the current formulation is unlikely to address the problem of increasing FVIII levels, as delivery of 12 times more DNA (600 μg versus 50 μg) only led to marginal increases in tissue plasmid copy numbers (Figure 3).

The potential to express protein after oral delivery of a non-viral DNA vector is a strategy worth further examination, despite the many challenges that remain. Although modest protein levels and high variability in gene transfer were observed, the production of a detectable amount of a secreted, therapeutic protein such as factor VIII after oral nanoparticle delivery is noteworthy, given the many advantages of such a gene delivery system. Thrombin generation and phenotypic correction could be achieved in 77% of the hemophilia A mice administered the highest dose of nanoparticles, although low levels of plasma FVIII were measured. While further optimization will be required, the possibility of achieving protein replacement therapy via oral administration is an exciting prospect and a worthy goal of nonviral gene delivery.

Acknowledgments

We wish to thank Tina Kiang for providing the 62% and 70% deacetylated chitosans, Haig Kazazian for support, the Vector Core at the University of Pennsylvania and Julio Sanmiguel for help with real-time PCR, and Renée Tetreault for assistance with the animal work. Support from National Institute of Health (EB002849, P30 DK047757-11) is also acknowledged.

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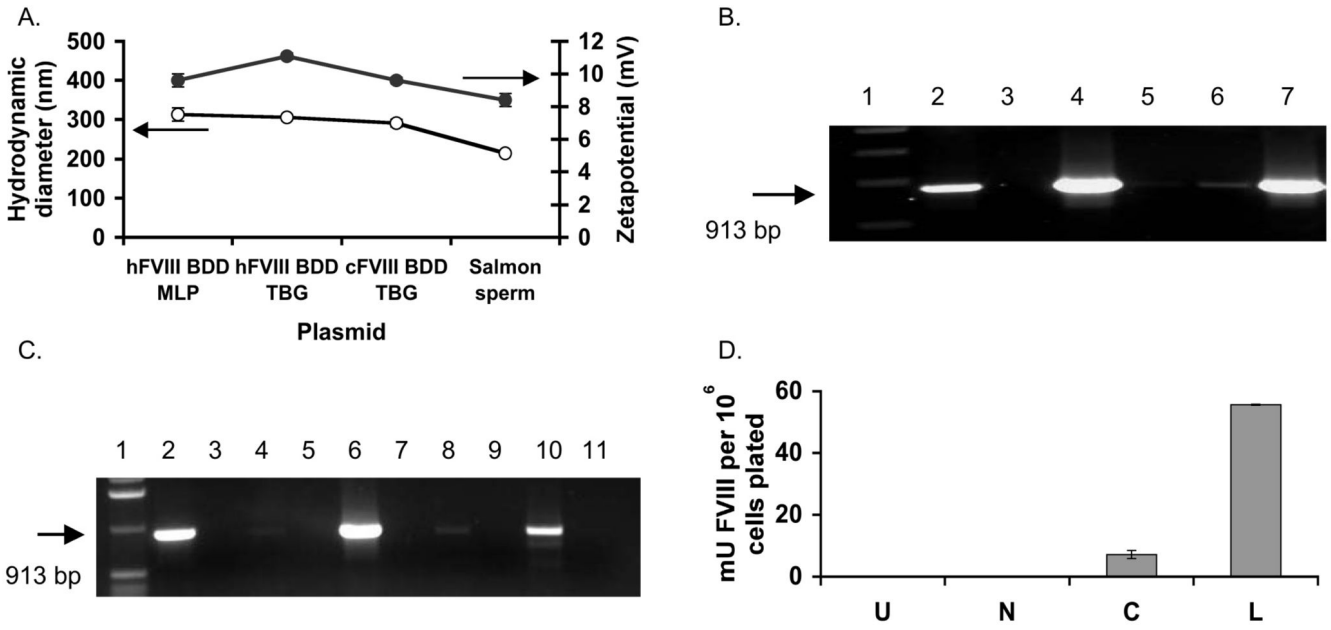

Figure 1. Characterization and *in vitro* evaluation of FVIII chitosan nanoparticles

(A) Comparison of the average size and zeta potential of chitosan nanoparticles formed with different plasmids. Measurements are given as mean +/− standard deviation (n=3). (B) Detection of 913 bp fragment of FVIII plasmid DNA in Cos-7 cells 72 hours after transfection with chitosan nanoparticles, naked DNA, and Lipofectamine 2000. The plasmid used was hFVIII BDD MLP. Lane 1: ladder, 2: positive control, 3: water, 4: Lipofectamine 2000, 5: untransfected, 6: naked DNA, 7: chitosan. (C) Detection of FVIII mRNA. Lane 1: ladder, 2: positive control, 3: water, 4: untransfected +RT, 5: untransfected −RT, 6: Lipofectamine 2000 +RT, 7: Lipofectamine 2000 −RT, 8: naked DNA +RT, 9: naked DNA −RT, 10: chitosan +RT, 11: chitosan −RT. Controls without reverse transcriptase (−RT) were included to verify that bands were not due to DNA contamination. (D) Evaluation of functional FVIII protein in conditioned media by chromogenic assay. U: untransfected control cells, N: naked DNA, C: chitosan NP, L: Lipofectamine 2000. Error bars represent standard deviation (n=3).

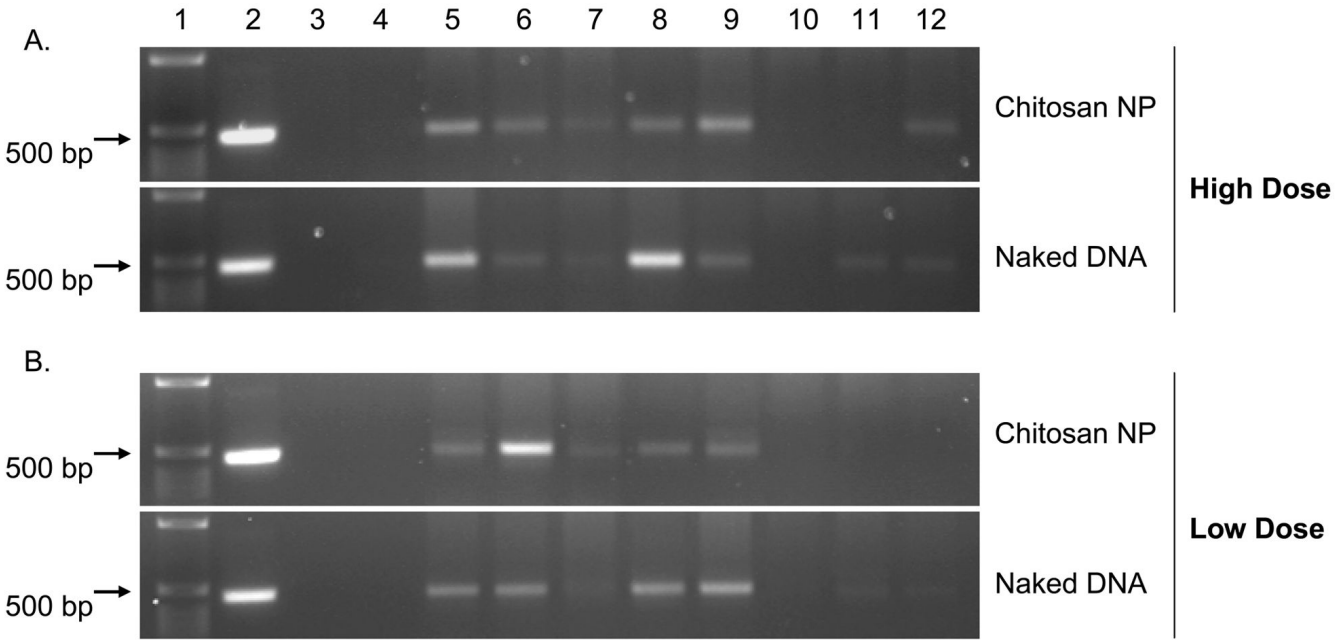

Figure 2. Tissue biodistribution of FVIII plasmid DNA after oral delivery of high or low doses of chitosan-DNA nanoparticles or naked DNA

Mice were sacrificed and tissues collected at 72 hours after feeding. Samples are 1: ladder, 2: plasmid control, 3: water, 4: untreated KO liver, 5: stomach, 6: ileum, 7: kidney, 8: liver, 9: spleen, 10: heart, 11: Lung, 12: gonad. (A) The high dose feeding consisted of 600 μg of DNA. (B) The low dose feeding consisted of 50 μg of DNA.

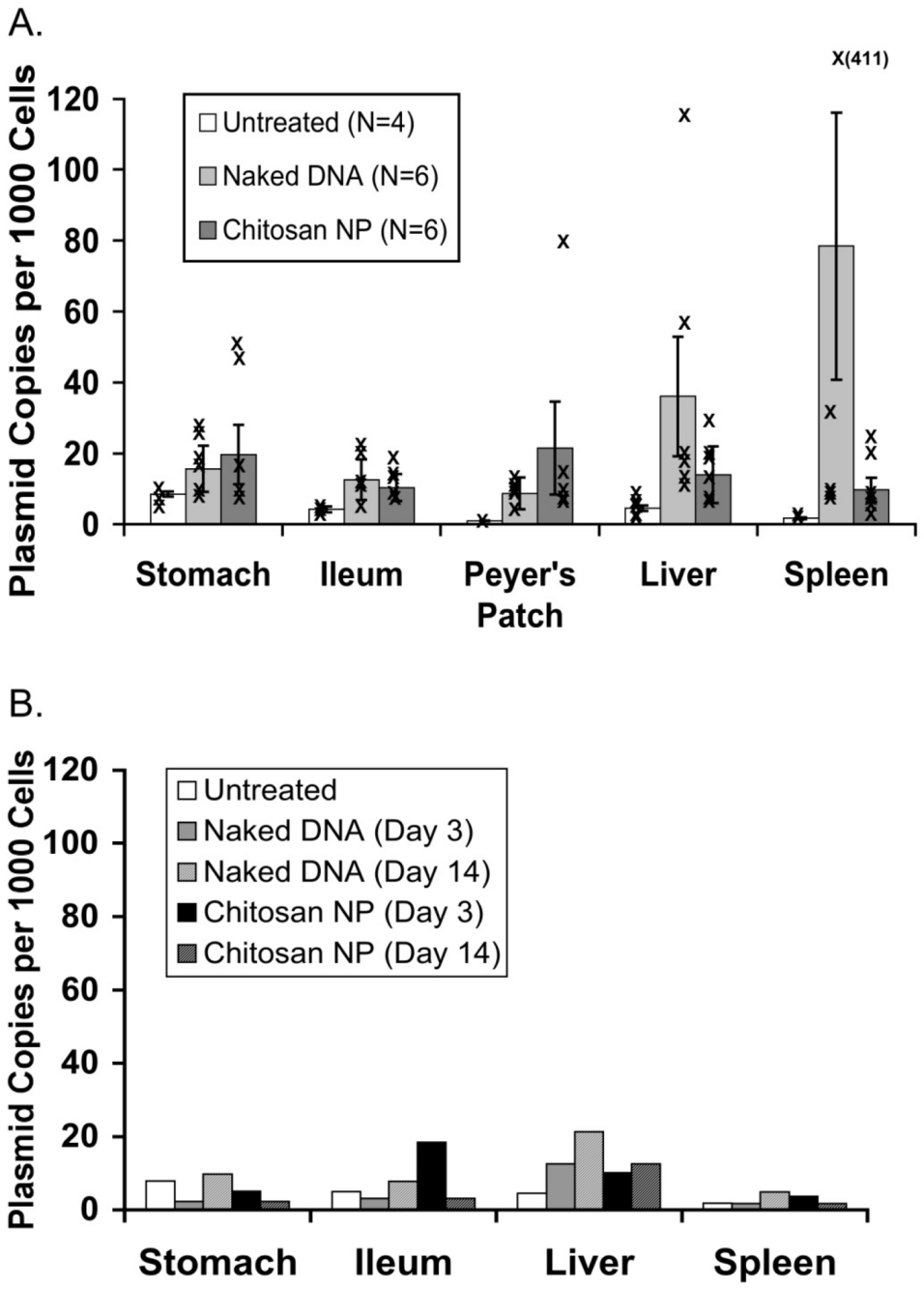

Figure 3. Plasmid copy number in tissues as determined by quantitative PCR following oral delivery of FVIII DNA-chitosan nanoparticles or naked DNA

(A) Mice were fed for 5 consecutive days with 120 μg of DNA per day and sacrificed 72 hours after the final feeding. The x's represent the plasmid copies detected in individual mice. Several outlying data points contribute to the large variation observed. Data are mean +/− SEM; n=6 for chitosan and nanoparticle-fed mice, n=4 for untreated control mice. (B) Mice were fed with a single dose of 50μg DNA and sacrificed 72 hours and 14 days after feeding (n=1).

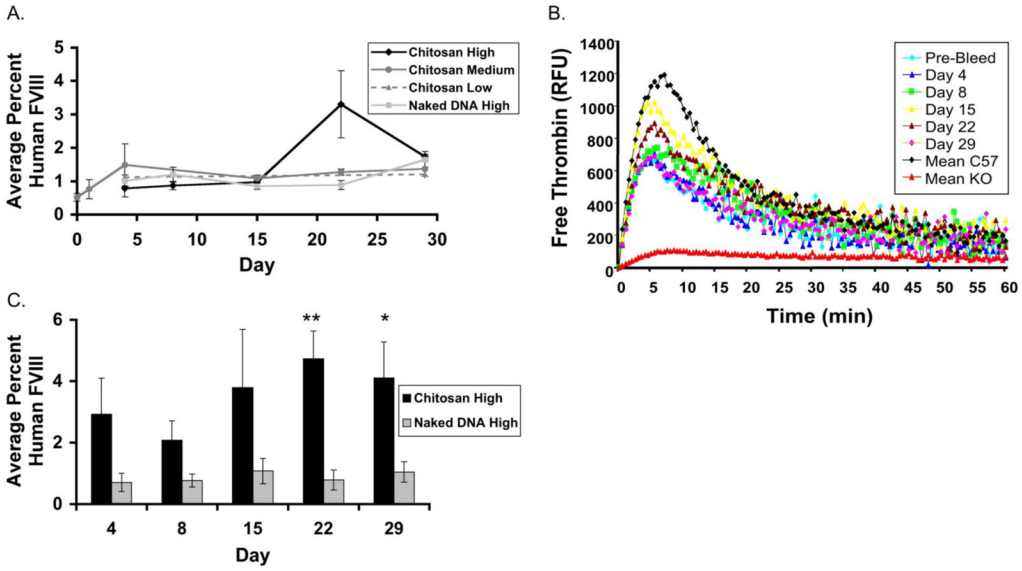

Figure 4. Production of functional FVIII protein in plasma following oral delivery of chitosan-DNA nanoparticles or naked DNA

(A) Chromogenic assay on plasma of mice given low (50 μg DNA), medium (250 μg DNA) and high (600 μg DNA) doses. Shown is mean +/−SEM (n=5–7/group). (B) Representative thrombin generation curves obtained from one mouse given high dose chitosan-DNA nanoparticles. (C) Thrombin generation assay. Mice were fed with a total of 600 μg of DNA. Shown is the mean +/− SEM (n=5). Background thrombin generation in pre-treatment plasma has been subtracted from all samples. The (*) indicates a significant difference between the average percent FVIII in plasma of mice treated with chitosan nanoparticles compared to naked DNA, $p<0.05$ and the (**) a difference of $p<0.01$.

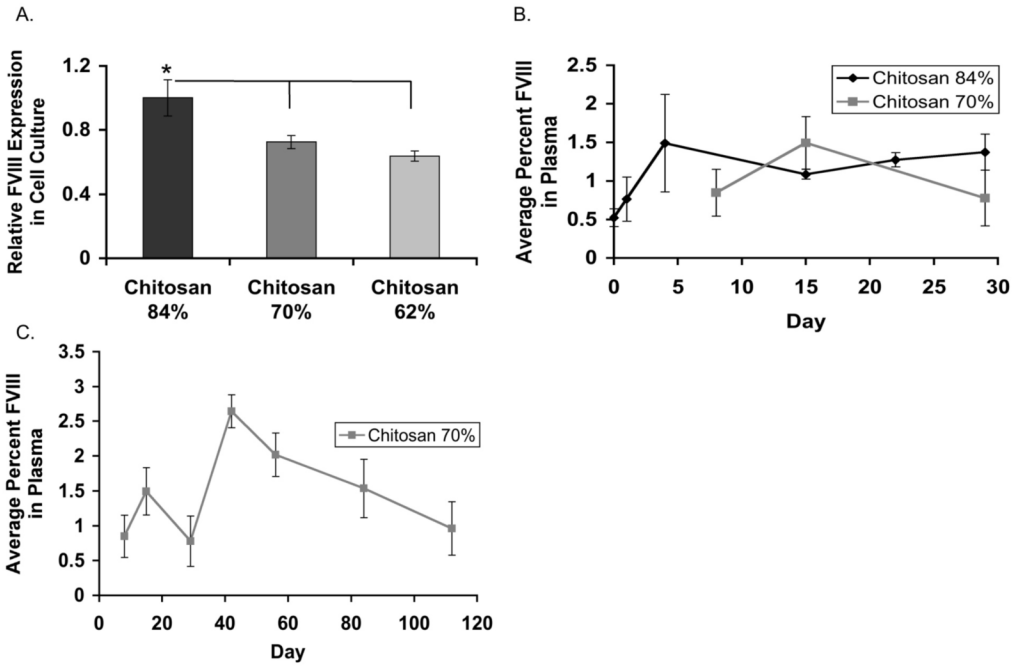

Figure 5. Comparison of chitosans having different degrees of deacetylation on factor VIII gene expression

Shown is mean +/− SEM. (A) Factor VIII gene expression 72 hours after transfection of Cos-7 cells. Expression is normalized to levels obtained from transfection of 84% DA chitosan nanoparticles (n=3). (B) Factor VIII protein in plasma of mice given medium doses of 84% DA chitosan nanoparticles and 70% DA chitosan nanoparticles (n=7–10/group). (C) Factor VIII protein in plasma of mice given 70% DA chitosan nanoparticles followed over four months (n=8–10).

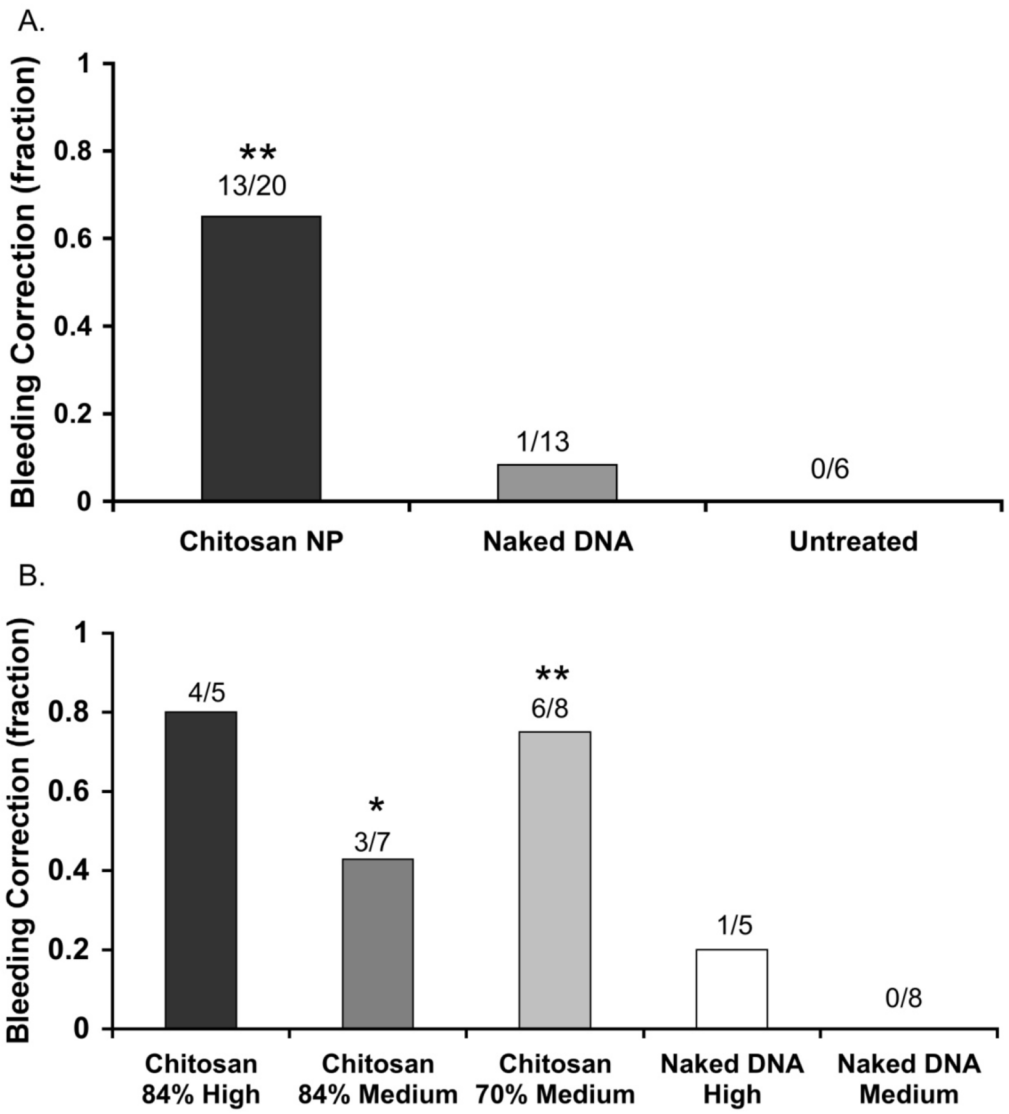

Figure 6. Phenotypic correction of hemophilia A mice in a tail clip assay following oral administration of chitosan nanoparticles or naked DNA

Numbers of mice in each group are given above each column. (A) Correction at high (600 μg) and medium (250 μg) doses of DNA. The (**) indicates a significant difference between chitosan and naked DNA treatments, $p<0.01$. (B) Phenotypic correction by formulation and DNA dose delivered. There is a significant difference in bleeding correction between the medium dose of 84% DA chitosan nanoparticles and the medium dose of naked DNA (*, $p<0.05$) and between the medium dose of 70% DA nanoparticles and the medium dose of naked DNA (**, $p<0.01$). For the high dose, there is a significant difference between chitosan-treated and untreated mice. The difference between high dose chitosan and naked DNA treatments approaches but does not reach significance ($p=0.058$).