Title:
Reduced bleeding events with subcutaneous administration of recombinant human factor IX (BeneFix™) in immune tolerant hemophilia B dogs

Running title:
Prophylactic FIX reduces hemophilia B dog bleeding

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Abstract

Intravenous administration of recombinant human factor IX (rhFIX) acutely corrects the coagulopathy in hemophilia B dogs. To date, 20/20 dogs developed inhibitory antibodies to the xenoprotein making it impossible to determine if new human FIX products, formulations or methods of chronic administration can reduce bleeding frequency (Blood 1996;88:2603-2610). Our goal was to determine if hemophilia B dogs rendered tolerant to rhFIX would have reduced bleeding episodes while on sustained prophylactic rhFIX administered subcutaneously. Reproducible methods were developed for inducing tolerance to rhFIX in this strain of hemophilia B dogs resulting in a significant reduction in the development of inhibitors relative to the historical controls cited above (5/12 vs. 20/20, p < 0.001). The 7/12 tolerized hemophilia B dogs exhibited shortened whole blood clotting times (WBCT), sustained detectable FIX antigen, undetectable Bethesda inhibitors, transient or no detectable anti-human FIX antibody titers by ELISA, and normal clearance of infused rhFIX. Tolerized hemophilia B dogs had 69% reduction in bleeding frequency in Year 1 compared to non-tolerized hemophilia B dogs (P = 0.0007). If proven safe in human clinical trials, subcutaneous rhFIX may provide an alternate approach to prophylactic therapy in selected hemophilia B patients.

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Introduction

Hemophilia B is an X-linked bleeding disorder caused by a quantitative or qualitative deficiency of the FIX protein. The incidence of hemophilia B is approximately 3 in 100,000 male births.\textsuperscript{1} Bleeding episodes are usually treated on-demand with intravenous (IV) administration of recombinant human factor IX (rhFIX) or highly purified plasma-derived FIX concentrates. Strong supportive evidence indicates that prophylactic treatment at a dose of 25 to 40 IU/kg intravenously twice weekly in hemophilia B humans decreases the frequency of bleeding episodes and the associated complications.\textsuperscript{2-6} It is likely that preventing bleeds with prophylactic administration is a more physiological approach to the treatment of hemophilia than is treating bleeding once it has occurred. The clinical benefit of prophylactic treatment with FIX has not been established in an animal model of hemophilia B.

Currently, administration of FIX either as an on-demand or prophylactic basis requires venipuncture. The venipuncture procedure can be painful, difficult, and time consuming resulting in delayed treatment and considerable stress for the patient. Both intra-tracheal\textsuperscript{7} and subcutaneous administration\textsuperscript{8,9} are being tested as alternative routes of administration to circumvent the requirement for venipuncture. While both routes have been shown to provide some measure of correction of the hemophilic coagulopathy, it is unknown if either would reduce the frequency of bleeding over long periods of time when given prophylactically.

The Chapel Hill strain of hemophilia B dogs have been used in short-term preclinical studies to determine the safety and efficacy of rhFIX administered intravenously.\textsuperscript{10} These hemophilic dogs have no detectable FIX activity or antigen in
plasma and exhibit a severe bleeding phenotype that closely mirrors the severe form of the human disease. The molecular defect in this strain of hemophilia B dogs is a missense point mutation in the catalytic domain of the FIX molecule that changes Gly to a Glu residue. Bleeding episodes are effectively managed by immediate and aggressive treatment using normal canine plasma prepared from normal donor dogs. One unique feature of this colony is that dogs treated with normal canine plasma rarely develop inhibitory antibodies to infused canine factor IX. In contrast, all of the dogs that received human FIX developed inhibitory anti-human FIX antibodies within 14 days of exposure. If the immune response of these hemophilic dogs to rhFIX could be avoided, then the safety and efficacy of long-term administration of subcutaneous recombinant human FIX could be studied in this clinically relevant animal model.

Our goal was to determine if hemophilia B dogs rendered tolerant to rhFIX would enjoy a reduction in the frequency of bleeding episodes while on sustained prophylactic rhFIX administered subcutaneously. Reproducible methods were developed for inducing tolerance to rhFIX in this strain of hemophilia B dogs. For this experiment, tolerance is defined as the absence of a detectable inhibitory antibody to rhFIX despite prolonged and repeated exposure to the xenoprotein. The frequency of bleeding events per year was then determined in the tolerized hemophilia B dogs on chronic prophylactic subcutaneous rhFIX maintained for 3.5 years and compared to non-tolerized hemophilia B dogs that were treated “on-demand.”
Methods

Hemophilia B dogs

Hemophilia B dogs from the closed colony at the Francis Owen Blood Research Laboratory at the University of North Carolina in Chapel Hill were used in this study. This strain of hemophilia B dogs has been maintained continuously since 1966. All animals were treated according to standards in the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health publication No. 85-23). The Institutional Animal Care and Use Committee approved all experiments.

Recombinant human FIX (rhFIX)

rhFIX (BeneFix™) was prepared by Genetics Institute, Inc., Andover, MA as previously described. Preparations of frozen liquid and lyophilized rhFIX ranged in activity from 690 IU/ml to 14,500 IU/ml. The specific activity of these preparations ranged from 240 to 290 IU/mg protein. The rhFIX was stored and reconstituted according to the manufacturer’s specifications.

Tolerance protocol: pilot studies, induction, interruptions in therapy, intravenous challenges, and chronic maintenance

An empiric 7-step protocol for inducing tolerance was devised that consisted of the following regimen shown on a timeline on Table 1: 1. obtaining baseline pretreatment samples; 2. administering rhFIX subcutaneous daily, Days 1-60; 3. allowing first washout by administering no FIX from days 61 to 84; 4. administering a single dose of
intravenous rhFIX at 50 IU/kg on Day 85; 5. allowing second washout by administering no FIX from days 86 to 94; 6. administering a daily dose of intravenous rhFIX at 50 IU/kg for 14 days from Days 96 to 109; 7. starting Maintenance from Day 112 forward (dose of rhFIX indicated on Table 1).

Assessment of correction of hemophilic coagulopathy and phenotype

Blood and plasma samples drawn while the dogs were receiving rhFIX were obtained just before administration of rhFIX. Blood samples during interruptions in therapy were obtained at scheduled intervals indicated on Table 1.

Whole blood clotting time (WBCT)

The WBCT was performed by a two-tube procedure at 28°C. One ml of whole blood collected with a one ml syringe was distributed equally between two siliconized tubes (Vacutainer™, #6431, Becton-Dickinson, Rutherford, NJ). The first tube was tilted every 30 sec. After a clot forms, the second tube is tilted and observed every 30 sec. The endpoint is the clotting time of the second tube. The range of values for 6 non-tolerized hemophilia B dogs and 6 normal dogs was determined for reference.

Activated Partial Thromboplastin Time (APTT)

The APTT assays were determined in the ST4 coagulation instrument (Diagnostica Stago, Asnieres, France). Mixtures consisted of equal portions of partial thromboplastin (APTT reagent, bioMérieux Inc., Durham, NC), 0.025 M CaCl₂, and citrated test
plasma. The range of values for 6 non-tolerized hemophilia B dogs and 6 normal dogs was determined for reference.

**Human-specific FIX antigen ELISA**

The concentration of rhFIX antigen was determined by a double monoclonal sandwich ELISA that is specific for human FIX and does not cross react with canine FIX. Standard curves were prepared by adding known amounts of rhFIX into canine hemophilia B plasma. The lower limit of detection of the assay is 25 ng/ml.

**Anti-human FIX antibodies by ELISA and Bethesda Inhibitor Assay**

Anti-human FIX antibody assays were performed using two procedures: a direct binding ELISA with immobilized recombinant human FIX and enzyme-coupled anti-immunoglobulin reagents, and the Bethesda inhibitor assay. The ELISA titer of a given sample represents an arbitrary unit defined as the reciprocal dilution for that sample that would generate an OD value equal to the cutpoint OD of the assay. The cutpoint OD is defined as twice the mean OD of the negative control wells. Titer values were then calculated by linear interpolation from values above and below the cutpoint OD in the dilution series using the following equation:

Equation 1: \[ \text{Titer} = \text{DilnOD1} - \left( \frac{\text{OD1} - \text{ODcp}}{\text{OD1} - \text{OD2}} \right) \times (\text{DilnOD1} - \text{DilnOD2}) \]

Where:

- ODcp = cutpoint OD
- OD1 = Sample OD value above the cutpoint OD in the dilution series
OD2 = Sample OD value below the cutpoint OD in the dilution series
DilnOD1 = Sample reciprocal dilution at OD1
DilnOD2 = Sample reciprocal dilution at OD2

These anti-FIX antibody data were then reported as the log of the interpolated value. The Bethesda inhibitor assay was performed with an activated thromboplastin reagent and hemophilia B substrate at 37°C.\textsuperscript{7,10,17,18} One Bethesda unit (BU) of inhibitor per ml is defined as the residual FIX activity of 50% of the normal control. Positive controls were obtained from hemophilia B dogs with known anti-human FIX antibodies confirmed in a second laboratory. Negative controls were obtained from non-tolerized dogs hemophilia B dogs in the colony repeatedly shown to be without inhibitors.

\textit{Frequency of bleeding events in tolerized and non-tolerized hemophilia B dogs}

The frequency of spontaneous bleeds and excess bleeding from minor trauma requiring replacement FIX treatment in the 5 tolerized and in 11 non-tolerized hemophilia B dogs was determined from the treatment records. Comparisons were made for the first year of life for both groups. The frequency between the end of Year 1 and 3.5 years was also measured for the available dogs. Detection of spontaneous bleeding in hemophilia B dogs is done by clinical observation and/or monitoring dogs for a decrease in hematocrit. Clinical manifestations are variable and include swelling in joints or soft tissues, lameness, weakness consistent with bleeding in the central nervous system or entrapment of a peripheral nerve, and the development of a spontaneous hematoma. In addition, a change in behavior (e.g., lethargy or poor appetite) may be the only manifestation of an occult bleed in the retroperitoneum, mediastinum, peritoneal cavity,
or other similar location. In the case of suspected bleeding, the dog is monitored with frequent measurements of hematocrit. All bleeding events, clinical observations, and treatments are recorded daily in computer-based clinical records. In addition, two of the tolerized hemophilia B females were bred during the 3.5 year treatment period. Control of hemostasis was maintained by intravenous administration of rhFIX during surgical procedures, peripartum and post partum.

Clearance of rhFIX in tolerized hemophilia B dogs

After 3.5 years of receiving subcutaneous rhFIX, the half-life of an intravenous bolus rhFIX (50 IU/kg) was determined in two situations. The first was while the tolerized dogs were on subcutaneous prophylaxis (i.e., an intravenous dose of rhFIX was substituted for the next subcutaneous dose) and the second was after a two-month washout during which no rhFIX was given. Plasma samples were drawn at baseline, 5 min, 30 min, 1, 4, 8, 24, 48, and 72 hours. FIX activity was determined as described.\textsuperscript{7,10}
Results

Induction of tolerance in neonatal hemophilia B dogs and incidence of anti-FIX antibodies by ELISA and Bethesda inhibitor assay

Two pilot studies were performed to test the efficacy of an empiric method for inducing tolerance to rhFIX in the hemophilia B dogs and to identify a target dose for a larger study. In Study 1, hemophilia B puppies were treated with subcutaneous rhFIX at a dose of 82.6 IU/kg (n = 2) or 1650 IU/kg (n = 2) daily starting during the first 48 to 72 hours following birth through step 2 of the induction protocol (Table 1). These four puppies then had their dose of rhFIX adjusted as described to complete all 7 steps of the tolerance induction protocol. On Day 29 in Study 1, both neonatal dogs that received 82.6 IU/kg/d subcutaneously had an anti-hFIX antibody detectable by ELISA (Table 1, Dogs B02 and B04). This anti-hFIX antibody on Day 29 had no inhibitory activity detectable by Bethesda assay. Neither of these dogs had an anti-FIX antibody or detectable Bethesda inhibitor for the duration of the protocol (149 days). These 2 dogs were defined as tolerized. The two neonatal dogs in Study 1 that received 1650 IU/kg/d subcutaneously developed anti-hFIX antibodies by ELISA by Day 15 that were inhibitory by Bethesda assay (Table 1, Dogs B03 and B05). These inhibitory anti-FIX antibodies persisted in these dogs for the duration of the protocol (122 days).

In Study 2, 7-week old hemophilia B dogs were started on rhFIX at 82.6 IU/kg (n = 2) daily (Table 1). These puppies completed step 2 of the protocol. Both 7-week old dogs developed detectable Bethesda inhibitors by Day 15 and were not analyzed beyond step 2 of the treatment protocol (60 days). The results of these two studies
suggested that tolerance to rhFIX could be successfully induced in hemophilia B puppies by initiating therapy at 82.6 IU/kg/d subcutaneously in the neonatal period.

Based on the results from Studies 1 and 2, Study 3 was then initiated with 48 to 72 hour old puppies from a single litter produced by a hemophilia B sire and dam (Table 1). These neonatal hemophilia B dogs received 82.6 IU/kg/SC daily (n = 4) or 3 times a week (n = 4) for 60 days. These eight puppies then had their dose of rhFIX adjusted as described to complete all 7 steps of the tolerance induction protocol. Five of eight hemophilia B neonatal puppies completed the 7-step protocol and exhibited no neutralizing anti-human FIX antibodies and were defined as tolerized to rhFIX (Table 1). Three of these 5 tolerized dogs had detectable but low titer, self-limited anti-human FIX antibodies by ELISA between days 29 and 51 of the study (Table 1, Dogs C22, C23, and C25). However, Bethesda assays performed on these study days found no detectable inhibitory activity for these antibodies. The two other tolerized dogs had no detectable anti-FIX antibodies by ELISA and also had no detectable Bethesda inhibitors (Table 1, Dogs C20 and C26). All 5 tolerized hemophilia B dogs continued to exhibit immune tolerance through interruptions and intravenous challenge regimens (Table 1). The dose of rhFIX was adjusted to 82.6 IU/kg twice a week in all 5 tolerized dogs on Day 178 through the rest of the 3.5 years.

The remaining three hemophilia B puppies in Study 3 developed inhibitory anti-FIX antibodies during the first 60 days of exposure (Table 1, C19, C24, and C27). These inhibitor puppies also had positive ELISA results by Day 29 and developed values that were up to 2 logs higher than detected in the tolerized dogs. Anti-hFIX antibodies were still detectable by both assays at the end of step 6 (Day 108) when
these three inhibitor dogs were excluded from further study. Overall, a significant reduction in inhibitors to rhFIX was achieved in the hemophilia B dogs receiving subcutaneous rhFIX relative to the historical controls that received intravenous rhFIX (5/12 vs. 20/20, p < 0.001).\textsuperscript{10}

**Correction of hemophilic coagulopathy and phenotype**

WBCT and APTT assays were performed at baseline and over the next 3.5 years in the 5 tolerized dogs (Figures 1 and 2, respectively). Both the WBCT and APTT were shortened compared both to non-treated hemophilia B dogs and three of the tolerized dogs in whom samples could be obtained after a two-month pause in therapy. The degree of correction appears greater for the WBCT than for APTT since the WBCT is more sensitive to trace amounts of FIX.\textsuperscript{12} Both the WBCT and APTT increased during short time periods associated with the washouts described in Table 1 (data not shown). The level of rhFIX antigen varied depending on the sampling time as described for the APTT and the WBCT but was approximately 1% of normal (i.e. \~50 ng/ml) while the dogs were receiving 82.6 IU/kg/SC twice per week (Figure 3).

The average number of bleeding episodes experienced by the 5 tolerized hemophilia B dogs receiving subcutaneous rhFIX was 1.8 ± 0.5 as opposed to 5.5 ± 3.3 in the 10 non-tolerized hemophilia B dogs in the Chapel Hill colony during the first year of life for both groups, a 69% reduction (P = 0.0007, Table 2). Between years 1 and 3.5, 6 non-tolerized and 4 tolerized dogs were available for comparison. Four of the original 10 non-tolerized dogs either died of bleeding or were entered into other protocols. One of the original 5 tolerized dogs died of bleeding. The average frequency of bleeds per
year between years 1 and 3.5 was 49% lower in the tolerized dogs than that detected in the non-tolerized dogs, 2.1 ± 1.0 vs. 4.1 ± 3.6 respectively. Although the trend towards reduced bleeding was still evident between years 1 and 3.5, the difference was not significant in this small sample size.

Two tolerized hemophilia B female dogs had successful gestation and parturition during this study.19 These pregnancies were managed with IV rhFIX peripartum (50 IU/kg/day starting several days before delivery and continuing until vaginal bleeding stopped post partum). Neither required blood transfusions peripartum. In addition, several surgical procedures including dental care, spaying, and repairs of traumatized tissues were managed with additional IV rhFIX during the course of this study. None of the procedures had bleeding complications. All of the tolerized hemophilia B dogs were restarted on subcutaneous 82.6 IU/kg when judged safe by the attending veterinarian.

**Clearance of IV rhFIX in tolerized hemophilia B dogs**

The percent recovery and half-life of infused rhFIX (50 IU/kg) were estimated while the tolerized dogs were on subcutaneous therapy and after two months without rhFIX (Figure 4). Respectively, the percent recovery (~28.6 ± 6.9 and 34.8 ± 7.4%) and half-life (between 18 and 20 hours in both cases) appeared comparable under these two conditions and similar to what has been reported in the non-tolerized hemophilia B dogs.10
Discussion

Our data show that sustained prophylactic administration of rhFIX subcutaneously to tolerized hemophilia B dogs significantly reduced the incidence of bleeding events during the first year of treatment consistent with an improvement in phenotype. This study became feasible with the development of reproducible methods for inducing tolerance to rhFIX in ~60% of the neonatal animals treated (i.e. 7/12 including 2/4 from Study 1 and 5/8 from Study 3). This constituted a significant reduction in Bethesda inhibitor formation relative to historical controls (5/12 vs. 20/20, p < 0.001).\textsuperscript{10} Remarkably, these hemophilic dogs maintained tolerance to rhFIX for over 3.5 years including relatively short interruptions in therapy (i.e., up to two weeks), intravenous challenges, gestation and parturition, and surgical procedures. In addition, the subcutaneous route and dose used consistently shortened the WBCT, APTT, and yielded FIX antigen levels of approximately 1%. This level of correction is generally associated with moderately severe hemophilia. Indeed, the significant reduction in bleeding events observed in the tolerized dogs on sustained subcutaneous prophylaxis is consistent with a proportional or dose responsive improvement in phenotype. These findings in a relevant animal model of hemophilia B reinforce the rationale for administering prophylactic factor replacement in humans when feasible and for considering a subcutaneous route of administration with a target trough FIX level of greater than 1%.

The choice of administering rhFIX subcutaneously in this study was a practical one based on the ease of administering medications by this route. The advantage of the subcutaneous route was particularly evident in the 1-3 day old neonatal puppies
where venous access was limited. The high concentration of rhFIX used in these studies (690 IU/ml – 14,500 IU/ml) was also critical to the success of the subcutaneous route. These formulations were 7 – 150 times higher concentrations of FIX than is found in commercial products. This high concentration allowed very low volumes of injection throughout the study period. The subcutaneous route was well tolerated, circumvented the challenges associated with venipuncture, and partially corrected the hemophilic coagulopathy. Moreover, these data underscore the advantages of prophylactic therapy for decreasing hemorrhages and associated complications in an animal model.

A potential pitfall of extravascular administration of hFIX could be increased immunogenicity of the therapeutic protein. Although the incidence varies among reports, an estimated 7.5% of patients with hemophilia B develop measurable Bethesda inhibitors at some point during their lifetime.\textsuperscript{20-22} Clearly, monitoring any change in the expected incidence of inhibitors is an essential component of any clinical trial in hemophilia treatment. A series of studies that administer the FIX protein subcutaneously have been reported in animals and humans with dosing that ranges from 15 to 200 IU/kg.\textsuperscript{23-25} All these studies detected measurable FIX in the recipient’s plasma following subcutaneous administration. In the Liles et al study, a single adult hemophilia B patient (cross-reacting material negative, CRM-) given a high purity plasma derived hFIX subcutaneously developed a transient, low titer inhibitor (1-2 Bethesda units) months after the clinical study while being treated for a traumatic bleed.\textsuperscript{25} Currently, even after rechallenge, he has no detectable inhibitor. This patient had been extensively treated in the past, and had not shown evidence of developing an inhibitor though he had not been as rigorously monitored as he was in the clinical protocol.
These results raise the issue of whether or not extravascular administration of FIX protein could result in an immune response with a greater frequency than intravascular administration. This important issue cannot be fully addressed with data from the one patient who received plasma-derived FIX given that host response can vary considerably. However, at least two points can be raised. First, many patients probably receive some FIX protein in the extravascular space when intravenous lines infiltrate, a common occurrence in any clinical setting. Whether hemophilic patients are more likely to develop an inhibitor with extravasation of FIX protein is unknown. Second, FIX has been shown to have binding sites in the extravascular space and on endothelium. Stafford et al. built on the studies of Heimark and Stern to document that FIX binds to type IV collagen via lysine at position 5 or valine at position 10 identifying the mechanism of extravascular and endothelial binding of hFIX. The published data and this report support the hypothesis that FIX can traverse not only from the extravascular space to the systemic circulation but also from the systemic circulation to the extravascular space. Whether or not FIX in the extravascular space has a function beyond preventing bleeding into joints and soft tissues so characteristic of hemophilia B patients is unknown. However, to test the relative immunogenicity of FIX given subcutaneously versus intravenously in a rigorous fashion would require additional studies such as administering species-specific FIX to hemophilic dogs and mice.

The extravascular distribution of FIX and its binding to collagen IV and endothelium suggests that the clearance of FIX from plasma would be altered by this complex compartmentalization. Remarkably, the half-life of infused rhFIX did not appear
to be altered by the dose used in our subcutaneous protocol. This observation suggests that the clearance of rhFIX from plasma does not change when the extravascular space is occupied by FIX to the extent achieved at this dose.

Our data support considering subcutaneous administration of rhFIX as an alternative to IV infusions if proven safe and efficacious in clinical trials in human hemophilia B patients. Twice weekly injections of 82.6 IU/kg as used in our study would require ~$86,000 per year for each 10 kg of body weight assuming a cost of ~$1 per unit of rhFIX. The total cost would then be $258,000 over the first three years of life for a hemophilic child with an average weight of 10 kg. Alternatively, our dose simulation estimates\(^8\) suggest that the combination of an 86 IU/kg intravenous loading dose and a 15 IU/kg rhFIX subcutaneous daily prophylactic dose would maintain rhFIX activity above 2% at the lowest cost. A target trough level of 2% would be higher than that achieved in our study in hemophilic dogs and therefore might produce a greater reduction in the frequency of bleeding. Again assuming a cost of ~$1 per unit of rhFIX, daily injections of 15 IU/kg would cost ~$54,600 per year for each 10 kg of body weight. This would translate to ~$164,000 over the first three years of life for a hemophilic child with an average weight of 10 kg. While the costs in both cases are prohibitive in adult patients with easily accessible veins, pediatric patients with difficult or absent vascular access sites might have the greatest potential benefit. Such a clinical trial would require careful monitoring of the immune response to rhFIX.
Acknowledgements

The authors gratefully acknowledge Ms. P. McElveen and Ms. M. Fleishman and their staff for the outstanding care given to these hemophilic dogs. Ms. Kate Leitermann is thanked for skillful technical assistance. Nicholas Warne, Chandra Webb and Elizabeth Bartlett are acknowledged for their preparation of the high concentration rhFIX formulations. SP Khor is thanked for the dose simulation estimates.
References


bioavailability of recombinant factor IX (BeneFix™) in healthy beagle dogs and cynomolgus monkeys. Blood. 1996;88:68b


Figure Legends

Figure 1. WBCT in tolerized hemophilia B dogs. The average WBCT values obtained quarterly from the five tolerized dogs are listed. Four dogs survived for 3.5 years and the fifth died during Year 2. At baseline (first value shown) and after 3.5 years of subcutaneous prophylaxis, the average value was markedly prolonged. The * denotes a sample drawn during a rhFIX free wash out period. The horizontal lines marks the WBCT range for normal dogs (8-12 min) and for untreated hemophilia B dogs (>60 min). Maintenance with subcutaneous rhFIX was started at Day 112 as described on Table 1 and adjusted to 82.6 IU/kg twice weekly at Day 178. This treatment markedly shortened the WBCT. All samples were drawn just prior to the next dose.

Figure 2. APTT in tolerized hemophilia B dogs. The APTT values from the five tolerized dogs are listed as described in Figure 1 and show some shortening while on subcutaneous rhFIX prophylaxis. Horizontal lines mark the APTT range for normal dogs (22-30 sec) and for untreated hemophilia B dogs (70-90 sec). Maintenance with subcutaneous rhFIX was started at Day 112 as described on Table 1 and adjusted to 82.6 IU/kg twice weekly at Day 178. The * denotes a sample drawn during a rhFIX free wash out period. The APTT is less sensitive than the WBCT to low levels of plasma FIX. All samples were drawn just prior to the next dose.

Figure 3. rhFIX antigen level in tolerized hemophilia B dogs. The level of rhFIX antigen is shown from just before initiation of maintenance through Day 475 in the five tolerized hemophilia B dogs. Maintenance with subcutaneous rhFIX was started at Day
112 as described on Table 1 and adjusted to 82.6 IU/kg twice weekly at Day 178. All samples were drawn just prior to the next dose.

**Figure 4. Clearance of intravenous rhFIX with or without concurrent subcutaneous rhFIX prophylaxis** The peak recovery and half-life of infused rhFIX (50 IU/kg) were estimated while the tolerized dogs were on subcutaneous rhFIX therapy (●) and after two months without rhFIX (■). Both appeared comparable under these two conditions at this dose.
### Table 1. Anti-Human Factor IX Antibody Titers (first column) and Bethesda Inhibitors (second column) for Each Hemophilia B Dogs Receiving Recombinant Human FIX*

<table>
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<th>Study No.</th>
<th>Neonatal Puppies (48 to 72 hours old)</th>
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<th>Neonatal Puppies (48 to 72 hours old)</th>
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*Anti-human FIX antibody titers detected by ELISA are listed in the first column and the Bethesda Inhibitor levels are listed in the second column for each dog. The "-" means not detected.

†C23 had one litter at 1.4 years into the study and C26 had litters at 1.3 and 2 years into the study. The pregnancies were uncomplicated and both dogs received only rhFIX to prevent bleeding.

§Dogs C20, C22, C23, C25, and C26 were switched to 82.6 IU/kg/2 x week from Day 178 through the remainder of the 3.5 years.
Table 2. Frequency of bleeding events per year in non-tolerized hemophilia B dogs treated on demand and in tolerized hemophilia B dogs on chronic subcutaneous prophylactic rhFIX replacement therapy

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<thead>
<tr>
<th>Hemophilia B dogs</th>
<th>Bleeding events per year</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 – 1 year*</td>
</tr>
<tr>
<td>Non-tolerized (n)</td>
<td>5.5 ± 3.3 (10)</td>
</tr>
<tr>
<td>Tolerized (n)</td>
<td>1.8 ± 0.5 (5)</td>
</tr>
</tbody>
</table>

Percent reduction 69% 49%
P value§ 0.0007 0.44

* Average number of bleeding events per year in the non-tolerized and tolerized hemophilia B dogs during the first year of life.
† Average number of bleeds per year in the tolerized hemophilia B dog between the end of Year 1 and the end of the study at 3.5 years.
§ P = by exact Wilcoxon rank sum test.
Subcutaneous rhFIX Maintenance started on Day 112, Dose adjusted to 82.6 IU/kg, 2 times/week on Day 178

Figure 1
Subcutaneous rhFIX Maintenance started on Day 112, Dose adjusted to 82.6 IU/kg, 2 times/week on Day 178

Figure 2
Subcutaneous rhFIX Maintenance started on Day 112, Dose adjusted to 82.6 IU/kg, 2 times/week on Day 178

Figure 3
Figure 4
Reduced bleeding events with subcutaneous administration of recombinant human factor IX (BeneFix™) in immune tolerant hemophilia B dogs


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