A quantitative measure of the efficacy of factor VIII in hemophilia A mice

Ernest T. Parker, Pete Lollar
Winship Cancer Institute, Emory University, Atlanta, Georgia, USA

Summary
We developed a quantitative method to study the efficacy of intravenously delivered human factor VIII in hemophilia A mice. Mortality was assessed after tail transection under conditions in which there were no survivors in untreated hemophilia A mice. Blood loss was significantly greater in untreated hemophilia A mice compared to normal C57BL/6 mice, and in hemophilia A non-survivors that were treated with sub-therapeutic doses of factor VIII. The up-and-down method for small samples yielded an estimated dose of factor VIII producing survival in 50% of the mice (ED$_{50}$) of 58 units/kg (95% confidence interval: 42.4 - 78.5 units/kg). This method should be useful in the evaluation and comparison of novel factor VIII products delivered either parenterally or in a gene therapy setting.

Keywords
Hemophilia A, factor VIII, ED$_{50}$, animal model

Introduction
Hemophilia A (hem A) mice created by targeted disruption of the murine factor VIII (fVIII) gene (1, 2) have become widely used in evaluating gene therapy strategies for hemophilia A (3-9) and in studying the immune response to fVIII (10-16). The mice have undetectable fVIII activity, occasional spontaneous bleeding, and a traumatic bleeding diathesis. Additionally, compared to normal mice they have increased mortality after tail transection (3). Reduction in mortality following tail transection has been used as a primary efficacy variable in gene therapy studies.

Quantitative analysis of the efficacy of therapeutic agents typically involves the determination of the dose that produces 50% survival (ED$_{50}$). Statistical models used to calculate the ED$_{50}$, such as probit analysis or the up-and-down method, require nearly uniform mortality in control subjects that do not receive test substance (17). Conversely, animals that do not lack test substance, such as normal mice, should have near uniform survival. However, a significant fraction of hem A mice have been reported to survive in the tail transection model (2,18). In this study, we modified the tail transection model to produce a hemorrhagic insult that was uniformly lethal in hem A mice. The new protocol was used to calculate the ED$_{50}$ of recombinant B domain-deleted human fVIII. Additionally, we identified a secondary efficacy variable, decreased body weight due to blood loss, that was a predictor of survival.

Materials and methods
Materials
Exon 16-disrupted (E16) hem A mice in a C57BL/6 background (1) were obtained from Dr. Leon Hoyer and a breeding colony was established. Nine- to twelve-week old E16 male or female hem A or normal C57BL/6 mice were used in the experiments.

A B domain-deleted form of human fVIII, rh-fVIII SQ, (19), was expressed from a baby hamster kidney-derived cell line (20) using conditions described previously (21). The rh-fVIII SQ cDNA, which encodes a 14 amino acid linker...
sequence in place of the B domain, was constructed by SOE mutagenesis (22) in the ReNeo mammalian expression vector (23) using a FVIII cDNA that lacked the entire B domain (24). Rh-fVIII SQ was purified from conditioned serum-free cell culture media using a two-stage ion-exchange chromatography procedure. Briefly, rh-fVIII SQ containing media was loaded onto a 5 × 20 cm SP-Sepharose Fast Flow column equilibrated in 0.18 M NaCl, 20 mM HEPES, 5 mM CaCl$_2$, 0.01% Tween-80, pH 7.4. Rh-fVIII SQ was eluted with a linear 0.18 – 0.65 M NaCl gradient in the same buffer. Fractions containing fVIII were pooled, diluted to 0.2 M NaCl in the same buffer, applied to a mono Q FPLC column and eluted with a linear 0.2 – 1.0 M NaCl gradient. Fractions were analyzed by one-stage coagulation assay (25), A$_{280}$ and SDS 9%-PAGE. The specific activity of rh-fVIII SQ was calculated using an estimation of the molar extinction coefficient obtained by absorbance at 280 nm and the known tyrosine, tryptophan and cysteine content (26). The mean (± standard deviation) specific activity of the peak fractions from four separate preparations was 1630 ± 530 units/nmol (9870 ± 3180 units/mg).

**Experimental protocol**

Initially, we used a tail transection bleeding model in E16 hem A mice that followed the description by Connelly et al. (18) with the exception that methoxyflurane was used as an anesthetic instead of isoflurane. After anesthesia, the distal 2 cm of the tail was transected and survival at 24 h was determined. Because a significant number of mice survived this protocol, a more severe hemostatic challenge was produced as follows. After determination of an initial body weight, mice received an intraperitoneal injection of a solution of freshly mixed 1.5 mg/kg droperidol/75 mg/kg ketamine, corresponding to an injection volume of 2.1 µl/kg body weight. Approximately seven minutes later, mice were ear punched for identification, warmed under a 60- or 75-watt lamp for approximately three minutes to dilate the tail veins, and injected with rh-fVIII SQ or vehicle. The volume injected was 2 µl/kg in all mice. Thirteen minutes later, mice were placed in a methoxyflurane chamber for two minutes to deepen the anesthesia and then were placed in a 50 ml conical restraint tube. The distal 1 cm of tail was transected and the stump was placed in a 13 × 100 mm test tube containing a 7.5 ml solution of 150 mM NaCl submerged in a 37°C water bath for 2 h or until death. The tail was kept at least 4 cm from bottom of tube to avoid contact with coagulated blood. Mice were monitored for survival every 10-15 minutes for two h after tail transection and then at 2, 4, 6, and 24 h. At 2 h, survivors were placed in clean cages lined with paper towels instead of litter to absorb blood. Moistened mouse chow was placed inside each cage in addition to a water bottle and dry mouse chow.

Preliminary experiments revealed that acute blood loss, as measured directly by the weight increase of the test tube collecting the blood, equaled the body weight loss. Therefore, body weights were measured in subsequent experiments. Survivors were weighed at 2, 4, 6, and 24 h. Body weight of non-survivors was measured as soon as possible after death. Survivors were terminated after 24 h using methoxyflurane anesthesia, followed by cervical dislocation.

**Measurement of the ED$_{50}$ of rh-fVIII SQ**

The up-and-down method for small samples (27, 28) was used to calculate the ED$_{50}$ of rh-fVIII SQ. In this method, the initial dose is an a priori estimate of the ED$_{50}$. If the subject survives, another subject is tested and the dose is decreased. If the subject dies, the dose is increased in the next subject. A constant log dose increment or decrement of 0.1, corresponding to a dilution factor of 1.26 was used. Testing continued until a chosen nominal sample size of six was reached. Because survival at 24 h was assessed, testing continued sequentially on a daily basis. In addition to E16 mice receiving rh-fVIII SQ, a control E16 mouse receiving no fVIII and a normal C57BL/6 mouse were tested each day.

The up-and-down method assumes that the log of the dose of a drug that produces survival is normally distributed in the population under study with a mean equal to log ED$_{50}$. The ED$_{50}$ was calculated using the equation

\[
\text{Log ED}_{50} = \log X_f + \log d + \log k
\]

where \(X_f\) is the logarithm of final test dose, \(d\) is the log dose increment or decrement, and \(k\) is obtained from a table based on maximum likelihood estimates (27). The standard error of log ED$_{50}$, s.e., was estimated using the equation

\[
\text{s.e.} = \frac{\sigma}{\sqrt{n}}
\]

where \(\alpha\) equals 0.31 for a nominal sample size of six (30) and \(\sigma\) is the population standard deviation. It is not possible to estimate the standard deviation, \(\sigma\), of the measurement using the up-and-down method for small samples. The standard deviation in all-or-none responses such as mortality data can be estimated using probit analysis (17). Bruce found no correlation between \(\sigma\) and log ED$_{50}$ in a review of 42 studies using probit analysis (31). The average value of \(\sigma\) was 0.12, which we assumed in this study.

**Results**

In preliminary experiments, we determined the mortality in hem A mice following transection of the distal 2 cm of tail, essentially as described by Connelly et al. (3). Ten of fifty-one hem A mice survived greater than 24 h (19.6%) using this protocol. This result is consistent with the original report of 6 survivors out of 20 mice. These survival rates are too high to apply standard
statistical methods, such as probit analysis or the up-and-down method, to determine the ED$_{50}$. These methods assume no survival in the underlying population in the absence of a therapeutic test substance (17).

We modified the protocol in two ways to increase the hemostatic insult. First, ketamine and droperidol were added as anesthetic agents because rats receiving these agents have significantly greater blood loss following tail transection than animals receiving pentobarbital or no anesthesia (32). Second, tails were submerged in saline at 37° C for 2 h following transection. In rats, this produces greater blood loss than no submersion, and is even greater if submersion is done at 23° C (33). In a preliminary study, we found that only 14 of 21 normal C57BL/6 mice survived 2 cm tail transection and tail submersion in saline at 23° C under droperidol and ketamine anesthesia (data not shown). Therefore, we decreased the hemostatic insult by subjecting mice to 1 cm tail transection and tail submersion in 37° C saline. None of seven E16 hem A mice survived greater than 2 h (the average survival time was 48 min), whereas 7 out of 8 normal C57BL/6 mice survived greater than 24 h.

This protocol was used to determine the ED$_{50}$ of rh-fVIII SQ using the up-and-down method for small samples (27, 28). In addition to E16 mice receiving rh-fVIII SQ, a control E16 mouse receiving no fVIII and a normal C57BL/6 mouse were tested each day and for two additional days. None of eight control E16 mice survived, whereas 7 of 8 normal C57BL/6 mice survived (Table 1). The method yielded an ED$_{50}$ of 57.7 units/kg (95% confidence interval, 42.4-78.5 units/kg$^a$).

<table>
<thead>
<tr>
<th>Rh-fVIII SQ Group</th>
<th>Dose (units/kg)</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>50.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>63.1</td>
<td></td>
<td>X</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>79.4</td>
<td></td>
<td></td>
<td>O</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ED$_{50}$ = 57.7 units/kg$^a$
95% confidence interval: 42.4 - 78.5 units/kg$^a$

Control groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>E16 hem A</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>X</td>
<td>O</td>
</tr>
</tbody>
</table>

O - Alive    X - Dead

$^a$ see Materials and Methods

The survival times of the different experimental groups are shown in Fig. 1. All control E16 mice died within 2 h with an average survival time of 33 min. Mice receiving rh-fVIII SQ had an apparent all-or-none response. Mice died in less than 90 min until a threshold of 60-70 units/kg was reached, beyond which survival was greater than 24 h. All mice surviving 24 h returned to normal activity and appeared healthy.

Body weight loss, which acutely is equivalent to blood loss, is presented in Fig. 2. At the time of death, untreated E16 mice lost 4.37 ± 0.45% (mean ± s.d.) of their initial body weight. The average blood volume of a normal C57BL/6 mouse is 6.4% of its body weight (34). Thus, untreated E16 mice lost 68% of their blood volume. In contrast, normal C57BL/6 mice lost only 2.21 ± 1.11% of their body weight at 2 h, corresponding to 34% of their blood volume. The blood loss in the C57BL/6 group was significantly less than the in E16 control group (t test, p = 0.0001). Blood loss in rh-fVIII SQ treated mice was significantly greater in non-survivors compared to survivors (5.25 ± 0.07 vs. 2.55 ± 0.98%, t test, p = 0.02).

**Discussion**

We developed a blood loss model in E16 hem A mice to test the efficacy of intravenous fVIII therapy. Although several groups have reported that hem A mice have a bleeding diathesis, it has been difficult to establish a model to evaluate therapeutic agents. In the initial reports that described hem A mice produced by targeted disruption of the fVIII gene, an overall survival of 33% was noted 2 h after tail transection (2). Survival at 24 h, the amount of tail transected and the number of mice were not reported. Subsequently, in a gene therapy study Connelly et al. reported that survival at 24 h was 30% in twenty isoflurane-anesthetized hem A mice following 2-cm tail transection.
The bleeding time in hem A mice following tail vein incision is not prolonged compared to normal mice (35), consistent with the observation that the template bleeding time is not prolonged in human hemophilia. However, hem A mice re-bleed after tail vein incision, but normal mice do not.

Other variables have been studied in hem A mice in gene therapy studies. Fakharzadeh et al. measured the clotting time of whole blood after 1 cm tail transection (7). The clotting time in hem A mice was greater than 60 min. The clotting time in normal mice was 8 to 15 min. Balague et al. reported increased
blood loss and prolonged in vitro blood clotting and bleeding time after 2-cm tail transection in hem A mice compared to normal C57BL/6 mice (9).

In this study, we report a method for studying the efficacy of intravenous fVIII therapy in E16 hem A mice by determining the ED_{50} using the up-and-down method for small samples (27, 28). This method assumes that the log of the dose of a drug that produces survival is normally distributed and thus, that there is no survival in the untreated control population. Therefore, we modified the procedure reported by Connelly et al. (18) to increase the mortality in hem A control mice. Ketamine and droperidol, which produce increased blood loss in rats undergoing tail transection (32), were added as anesthetic agents in mice undergoing 1 cm tail transection. Additionally, the tail stump was submerged in saline at 37° C, which also produces increased blood loss in rats undergoing tail transection (33).

Comparison of the up-and-down method to probit analysis has shown that it provides reliable estimates of ED_{50} values and requires smaller numbers of animals (31). A nominal sample size of six has been employed most commonly. Use of larger sample sizes has not been associated with better estimates of ED_{50}. We obtained an ED_{50} of 57.7 units/kg for rh-fVIII using this sample size (Table 1). Because the plasma volume in mice is 49 mL/kg (36), the ED_{50} for rh-fVIII is predicted to produce peak rh-fVIII SQ levels of 1.2 units/ml. If we assume that the activity of rh-fVIII SQ is similar to murine fVIII, this level approximates the fVIII level in normal mice (1 unit/ml).

The ED_{50} is model-dependent and cannot be used to predict dosing of fVIII in human clinical trials, although it could be used in comparative efficacy studies in mice. In our model, blood loss in normal C57BL/6 mice in the first 2 h is 2.2% of the initial body weight (Fig. 2), corresponding to 34% of the total blood volume. This is a significant hemostatic insult that produces hemorrhagic shock in mice (37). Accordingly, 2 of 16 normal C57BL/6 mice died following the procedure. Thus, the fact that the ED_{50} in hem A mice is predicted to produce normal fVIII levels is consistent with the fact that the levels in normal C57BL/6 mice are marginally protective in this model.

Compared to E16 mice receiving no fVIII and treated non-survivors, there is a significant reduction of blood loss in treated E16 survivors and in normal C57BL/6 mice (Fig. 2). This secondary efficacy variable indicates that the therapeutic effect of rh-fVIII SQ is due to reduced blood loss.

**Acknowledgements**

We thank Heather N. Craddock for technical assistance maintaining the hemophilia A mouse colony.

**References**
