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ORIGINAL ARTICLE

Novel, high incidence exercise-induced muscle bleeding model in hemophilia B mice: rationale, development and prophylactic intervention

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Summary. *Introduction:* Muscle hematomas are the second most common complication of hemophilia and insufficient treatment may result in serious and even life-threatening complications. Hemophilic dogs and rats do experience spontaneous muscle bleeding, but currently, no experimental animal model is available specifically investigating spontaneous muscle bleeds in a hemophilic setting. *Aim:* The objective of this study was to develop a model of spontaneous muscle bleeds in hemophilia B mice. We hypothesized that treadmill exercise would induce muscle bleeds in hemophilia B mice but not in normal non-hemophilic mice and that treatment with recombinant factor IX (rFIX) before treadmill exercise could prevent the occurrence of pathology. *Methods:* A total of 203 mice (123 F9-KO and 80 C57BL/6NTac) were included in three separate studies: (i) the model implementation study investigating the bleeding pattern in hemophilia B mice after treadmill exercise; (ii) a study evaluating the pharmacokinetics of recombinant FIX (rFIX) in hemophilia B mice and based on these data; (iii) the treatment study, which tested therapeutic intervention with rFIX. At termination of the treadmill studies the presence of bleeds was evaluated. *Results:* Treadmill exercise resulted in a high incidence of muscle bleeds in F9-KO mice but not in C57BL/6NTac mice. Treating hemophilia B mice with rFIX before treadmill exercise prevented muscle bleeds. *Conclusion:* A novel model of muscle bleeds in hemophilia B mice, responsive to rFIX, has been developed.

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Introduction

Patients suffering from hemophilia are prone to bleeding in the musculoskeletal system [1,2]. Most research is focused on hemarthroses as these account for the majority of bleeds [3]; however, muscle hematomas are the second most common complication of hemophilia, representing 10–25% of all diagnosed bleeding episodes [3–5]. With insufficient treatment muscle hematomas may result in serious and even life-threatening complications [6]. There is limited consensus regarding optimal diagnosis and treatment of muscle hematomas in hemophilic patients.

Presently, knowledge on muscle hematoma pathophysiology, diagnosis and management can be obtained from sports medicine [7], where bleeds are present in over 90% of all sports-related injuries [7,8]. Even though parallels exist between sports-induced muscle injuries and muscle hematomas in hemophilic patients, a complete translation is not possible [7]; most importantly, sports injuries occur in healthy individuals, hence differences in etiology, progression and treatment of the bleeds between the two groups exist. Though several animal models of muscle contusions [9–13] are available, none are investigating spontaneous muscle bleeds, contusions or hematomas in a hemophilic setting. Hemophilia research would potentially benefit from a pathophysiologically relevant animal model of spontaneous muscle bleeds.

The objective of this study was to develop a model of spontaneous muscle bleeds in hemophilia B mice.

It was hypothesized that exposing hemophilia B mice to treadmill exercise would induce pathological changes (i.e. muscle bleeds) in hemophilia B mice but not in normal non-hemophilic mice and that treatment of hemophilia B mice with a recombinant factor (F) IX product

before treadmill exercise could prevent the occurrence of pathology.

Materials and methods

Animals

Hemophilia B mice (F9-KO, B6.129P2-F9 < tm1Dws>) originally obtained from D.W. Stafford (University of North Carolina) and normal control C57BL/6NTac mice were included in the studies.

All mice were purchased from Taconic, Denmark, and were between 12 and 16 weeks when included in the study. The mice were housed in standardized conditions with food and water *ad libitum*. The mice were provided with large cages with raised lids. The mice had an acclimatization period of at least 7 days at Novo Nordisk A/S, Måløv.

The studies were approved and performed according to guidelines from the Danish Animal Experiments Council, The Ministry of Food, Agriculture and Fisheries of Denmark.

Study design

Three studies were conducted: (i) the model implementation study investigating the bleeding pattern in hemophilia B mice after treadmill exercise; (ii) a study evaluating the pharmacokinetics of recombinant FIX (rFIX, BeneFIX®, Pfizer, Wyeth Pharmaceuticals Inc., Philadelphia, PA, USA) in hemophilia B mice and based on these data; (iii) the treatment study, which tested therapeutic intervention with (rFIX). Bodyweight was measured daily before treadmill exercise.

Model implementation study A total of 120 mice (60 male, 60 female) were included in the study. Ten F9-KO mice and 10 C57BL/6NTac were euthanized on day 1 without treadmill exercise, providing baseline measurements for plasma haptoglobin, plasma creatine kinase (CK), myoglobin and skeletal muscle troponin-I. In addition, the mice were thoroughly inspected for macroscopic bleeds. Fifty-two F9-KO mice and 48 C57BL/6NTac mice were subjected to treadmill running. After 1, 2, 3 or 4 weeks of treadmill running, 17–26 mice were euthanized at the end of each week (Table 1).

Pharmacokinetics Fifteen F9-KO mice (six males and nine females) were included in the study. Mice were administered a single intravenous dose of 1.5 mg/kg (306–315 U/kg) of rFIX, 5 ml/kg. Blood was sampled from the retro-orbital plexus up to 4 days after administration in a sparse sampling schedule consisting of three samples per mouse and three mice per time-point. rFIX activity (clot and chromogenic) and antigen levels were determined.

The pharmacokinetic parameters were assessed using a standard two-compartment model, including the following parameters: the time-zero intercept macro constants for the distribution phase (A) and the terminal phase (B), as well as the distribution phase macro constants (α and β). Based on these primary parameters the following secondary parameters were calculated: volume of distribution of the central compartment (V1), volume of distribution in the periphery compartment (V2), total clearance (CL), distribution half-life (α-t½) and terminal half-life β-t½ (Fig. 1A). Simulations were performed assuming that linear kinetics apply, using the model and estimated parameters as listed above. The main objective of the simulations was to guide dose levels and dosing intervals in non-clinical experiments. All modelling and simulations were performed using the PhoenixTM software (PhoenixTM WinNonlin, version 6.0.0.1648 Pharsight®, St Louis, MO, USA).

Treatment study Sixty-eight male mice were included in the study. Ten F9-KO mice and 10 C57BL/6NTac mice were euthanized on day 1 without treadmill running, providing baseline measurements. The remaining 36 F9-KO mice and 12 C57BL/6NTac mice were dosed daily (randomized in a blinded fashion) with intravenous injections of vehicle (histidine buffer, n = 12 F9-KO + 12 C57BL/6NTac) or a high dose (n = 12 F9-KO) or low dose (n = 12 F9-KO) of rFIX (5 mL/kg) for 5 days before subjected to treadmill running (Table 1). Mice were euthanized on day 5 after treadmill running.

Exclusion criteria

For ethical reasons, strict exclusion criteria were defined. The maximum speed of 15 m/min was based on a pilot study (data not shown). Electrical stimuli and gentle tail

Table 1 Treadmill exercise schedule and number and sex of the mice terminated each week during the 4-week study period in the model implementation study. The exercise schedule for week 1 was applied in the treatment study

	Week 1	Week 2									Week 3					Week 4				
Running day number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Speed/m/min	10	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15	15
Running time/min	10	10	20	25	30	30	30	45	45	45	45	45	60	60	60	60	60	60	60	60
Mice terminated	F9-KO	5♂ and 6♀				9♂ and 6♀					4♂ and 5♀					4♂ and 4♀				
	C57BL/6NTac	5♂ and 6♀				5♂ and 6♀					6♂ and 5♀					5♂ and 4♀				

touch were used if necessary to encourage the mice to run on the treadmill. Running time and all electrical stimuli were recorded.

For both studies the lowest possible current for electrical stimuli was used (0.1 mA) and only a limited number of 15 electrical stimuli per day were allowed. For C57BL/6NTac mice 15 electrical stimuli on three consecutive days throughout the study period resulted in exclusion from the study. For F9-KO mice, 15 electrical stimuli on three consecutive days during the first 2 weeks of the study period resulted in exclusion from the study. During the remaining study period 15 electrical stimuli in 1 day resulted in removal of the mouse from the treadmill and a thorough evaluation of the mouse. If no signs of bleeding or discomfort were observed the mouse was rested for the day and included in the treadmill protocol again the next day. Daily evaluations were performed on all mice and any discomfort or sign of depression, (lethargic behavior, ruffled coat and/or decreased activity) resulted in termination. Termination of a mouse before time resulted in transfer to the corresponding group. Any signs of bleeding or discomfort resulted in euthanasia of the mouse but not exclusion from the study.

Termination

In the model implementation and the treatment studies blood was sampled from the retro-orbital plexus at termination and the mice were euthanized by cervical dislocation under full Isofluran/N_2O/O_2 (0.3 L/min O_2 and 0.7 L/min N_2O) anesthesia. Blood was EDTA stabilized and centrifuged for 5 minutes at 4000 $\times g$. Plasma was frozen immediately and stored at −80 °C until analysis.

The skin was carefully removed and the body macroscopically evaluated for presence of bleeds. The right knee joint was macroscopically inspected for the presence of distension. The joint cavity was exposed and presence of blood was evaluated.

Further, the abdominal cavity was opened and macroscopically inspected for any bleeds. All observed bleedings were categorized into muscle, joint or other bleed. If muscle bleedings were present they were scored 0–3, where 0 represents no bleeding and 1, 2 and 3 represent bleedings with a summarized area of < 50 mm^2, 50–225 mm^2 and > 225 mm^2, respectively (Fig. 2).

Detection of plasma haptoglobin, myoglobin, skeletal muscle troponin-I and CK

Plasma levels of haptoglobin, myoglobin and skeletal muscle troponin-I were determined by a mouse haptoglobin ELISA test kit, a mouse myoglobin ELISA test kit and a mouse skeletal muscle troponin-I ELISA test kit, respectively (Life Diagnostics Inc., West Chester, PA, USA), as previously described [14–16]. Plasma levels of CK were determined using the Pentra 400 (Horiba, Kyoto, Japan).

Statistical analysis

Statistical analyses were carried out using GraphPad Prism (GraphPad software Inc., La Jolla, CA, USA) or JMP® (Release 8, SAS Institute Inc., Cary, NC, USA). A significance level of 0.05 was used. GraphPad was used for graphic illustrations of the results.

The number of electrical stimuli is illustrated as mean values ± SD. Results of bleeds are expressed as numbers. Haptoglobin, CK, myoglobin and skeletal muscle troponin-I were analyzed for differences from baseline values using Kruskal–Wallis with Dunn's multiple comparison test. The Mann Whitney test was used for statistical analysis of difference between F9-KO mice with or without muscle bleedings in the same parameters. Fisher's exact test was used in order to compare numbers of F9-KO and C57BL/6NTac mice with bleeds during the study period as well as numbers of F9-KO male and female mice with muscle bleeds. For comparison of average number of electrical stimuli between strains in the model implementation study a Mann Whitney test was used. For comparison of average number of electrical stimuli between the groups in the treatment study a Kruskal–Wallis test together with Dunn's multiple comparisons post-test was used. An ordinal logistic regression was used for comparison of muscle bleeding scores between groups.

Results

Pharmacokinetics

The pharmacokinetic profile of a single dose of rFIX in F9-KO mice confirmed previously published data [17] (Fig. 1A,B). A multiple dosing simulation (Fig. 1C) targeting a trough level of 3% of 1 IU/mL, simulating the conversion of severe hemophilia to high factor coverage, was performed. To reach approximately uniform plasma FIX profiles on a daily basis, it was calculated that mice should be dosed with 1.12 mg/kg (228–235 U/kg) on the first day and 1.08 mg/kg (220–227 U/kg) on the following 4 days. The lowest treatment group targeting a 0.3% trough level was dosed at 0.112 mg/kg (23–24 U/kg) on day one and 0.108 mg/kg (22–23 U/kg) on the following 4 days.

Number of electrical stimuli

Model implementation study Six C57BL/6NTac mice and eight F9-KO mice were excluded from the study due to maximum number of electrical stimuli. Seven F9-KO mice were terminated before time as these mice were found to be lightly depressed; these mice were included in the study in the group corresponding to their termination

A

	V_1 (L kg^{-1})	V_2 (L kg^{-1})	CL (mL h^{-1} kg^{-1})	α-$t_{1/2}$ (h)	β $t_{1/2}$ (h)
Mean	0.28	0.51	49.92	2.28	20.90
CV%	46	53	22	54	42

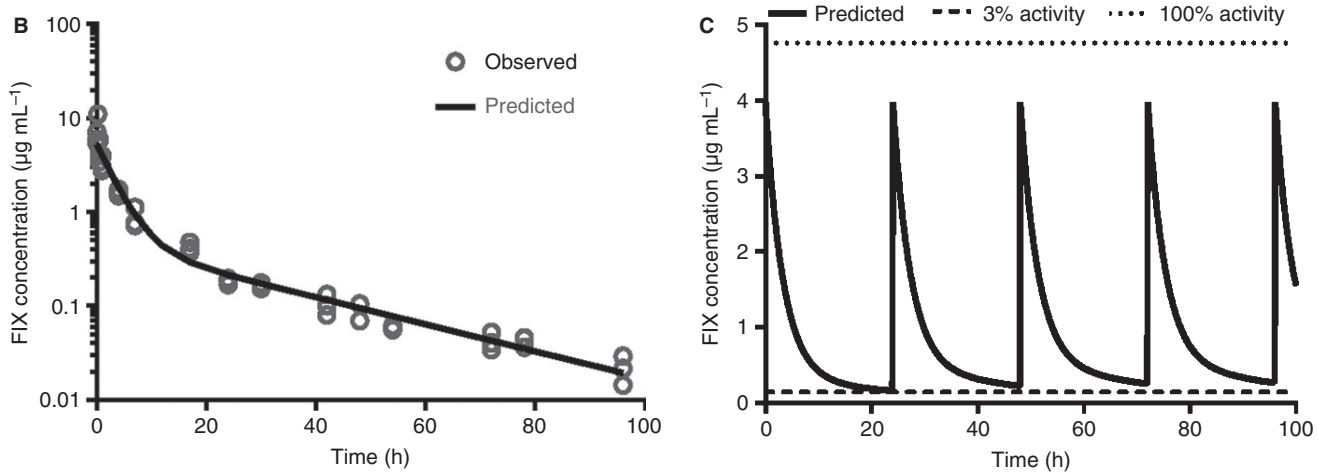

Fig. 1. Estimated pharmacokinetic parameters of rFIX in F9-KO mice after a single IV administration of 15 mg/kg (306–315 U/kg) rFIX. (A) V_1, volume of distribution of the central compartment; V_2, volume of distribution in the periphery compartment; CL, total clearance; α-$t_{½}$, distribution half-life; β-$t_{½}$, terminal half-life; SE, standard error. (B) Plasma concentrations of rFIX shown as individual measurements (grey symbols) after administration of a single dose of 15 mg/kg (306–315 U/kg) FIX to F9-KO mice (three mice per time-point). The solid line represents the fit to a two-compartmental model. (C) The fitted parameters were used in a multiple dose simulation to estimate a trough level of 3% after daily dosing. The horizontal lines represent 100% and 3% of 1 IU/mL, respectively.

week. The F9-KO mice received significantly more electrical stimuli per day compared with C57BL/6NTac mice during the study period ($P < 0.0001$) (Fig. 3A). F9-KO mice received on average 7.4 (SD = 5.0) electrical stimuli per mouse per day and C57BL/6NTac mice received on average 2.4 (SD = 3.7) electrical stimuli per mouse per day. For C57BL/6NTac mice the number of electrical stimuli tended to decrease during the study period, whereas for F9-KO mice the number of electrical stimuli per day tended to increase (Fig. 3B).

Treatment study All mice completed the study. F9-KO mice received significantly more electrical stimuli compared with C57BL/6NTac mice, independent of rFIX treatment ($P < 0.0001$) (Fig. 3C). C57BL/6NTac mice received on average 0.9 (SD = 1.3) electrical stimuli per day whereas F9-KO mice received on average 4.1 (SD = 3.9), 5.6 (SD = 4.7) and 5.8 (SD = 5.0) electrical stimuli per day when treated with vehicle, 1.1 mg/kg rFIX (220–235 U/kg) and 0.1 mg/kg rFIX (22–24 U/kg), respectively.

The mean number of electrical stimuli for the four groups of mice during the study period decreased, except for the last day, where all three F9-KO groups had a slight increase in the number of electrical stimuli (Fig. 3D). Data were comparable to observations in the model implementation study.

Clinical appearance

All mice were closely monitored during the study periods and body weight was measured daily. The body weight was slightly increased during the study period for both strains and the majority of mice showed no signs of discomfort or pain. The mice were eating, running freely in their cages and had normal appearances and behavior. Only a few mice exhibited mild depression and were terminated.

Localization of bleeding

Model implementation study: Baseline F9-KO mice had a low incidence of muscle bleeds (one out of ten; 10%) but exposing F9-KO mice to treadmill exercise resulted in a significant increase in numbers of mice with muscle bleeds (to 21 out of 43; 48.8%) ($P < 0.05$; data not shown). At neither baseline nor after treadmill exercise did C57BL/6NTac mice develop muscle bleeding.

Baseline F9-KO mice had an average muscle bleeding score of 0.1 (SD = 0.3) but exposing F9-KO mice to exercise significantly increased the average muscle bleeding score throughout the 5 weeks to 1.2 (SD = 1.4; $P < 0.05$) (Fig. 4A). After running, muscle bleedings were present in five out of 11 (45.5%), seven out of 15 (46.7), five out of nine (55.6%) and four out of eight (50%) F9-KO mice

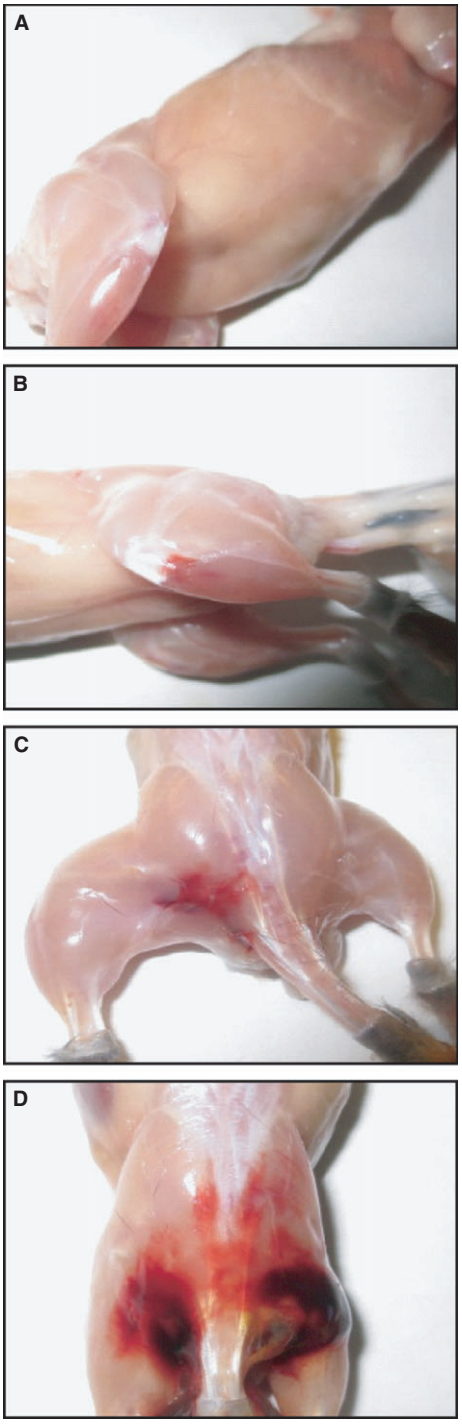

Fig. 2. Muscle bleeding score. (A) Score 0 represents no bleeding. (B) Score 1, < 50 mm^2. (C) Score 2, 50–225 mm^2. (D) Score 3, > 225 mm^2.

terminated after 1, 2, 3 and 4 weeks, respectively (Fig. 4B). The average muscle bleeding score was not significantly different between F9-KO mice exposed to treadmill running for 1, 2, 3 or 4 weeks (Fig. 4C).

Significantly more F9-KO male mice had muscle bleeds after treadmill exercise compared with F9-KO female mice ($P < 0.05$) (Fig. 4D). Fifteen out of 22 male mice (68.2%) and six out of 21 female mice (28.6%) had muscle bleedings during the study period. Furthermore, the average muscle bleeding score for F9-KO male mice (1.9; SD = 1.3) was significantly higher than that for F9-KO female mice (0.6; SD = 1.1; $P < 0.05$) (Fig. 4E).

Treatment study: Baseline F9-KO mice had a low incidence of muscle bleeds (two out of 10 mice; 20%). Exposing F9-KO mice to 5 days of treadmill running resulted in an increase in mice with muscle bleeds to nine out of 12 mice (75%). Treating F9-KO mice with both doses of rFIX (1.1 mg/kg, 220–235 U/kg and 0.1 mg/kg, 22–24 U/kg) daily before treadmill running prevented muscle bleeding and reduced the level to baseline F9-KO mice. No C57BL/6NTac mice had muscle bleeds during the study period (Fig. 5A).

Vehicle-treated F9-KO mice exposed to treadmill exercise had a significantly higher average muscle bleeding score (1.7; SD = 1.2) ($P < 0.01$) compared with both F9-KO baseline (0.2; SD = 0.4) and F9-KO mice treated with rFIX (0.25; SD = 0.6 for both doses) (Fig. 5B).

Joint

Only minor joint bleeds were observed. Six mice (two out of 42 (4.7%) C57BL/6NTac mice and four out of 52 (7.7%) F9-KO mice) in the model implementation study and one F9-KO mouse, a vehicle-treated mouse (one out of 12 (8.3%)), in the treatment study.

Other bleeds

Model implementation study—Twelve F9-KO mice were found to have a total of 14 other bleeds. Other bleeds included penile (two incidences), testicular (one incidence), paw (eight incidences), mouth (one incidence), nose (one incidence) and internal bleeding (one incidence). There was no incidence of other bleeds in any of the C57BL/6NTac mice.

Treatment study Eight F9-KO mice experienced a total of nine other bleeds. Seven of nine other bleeds were observed in vehicle-treated F9-KO mice and two other bleeds were observed in F9-KO mice treated with 0.1 mg/kg (22–23 U/kg) rFIX. Other bleeds were one testicular bleed and eight paw bleeds.

Haptoglobin, myoglobin, skeletal muscle troponin-I and CK

No significant differences were found in plasma levels of haptoglobin, myoglobin, skeletal muscle troponin-I and CK between F9-KO mice and baseline or between C57BL/6NTac mice and baseline level. In addition, no significant difference in plasma myoglobin, skeletal muscle troponin-I and CK was observed between F9-KO mice with muscle bleeds and F9-KO mice without.

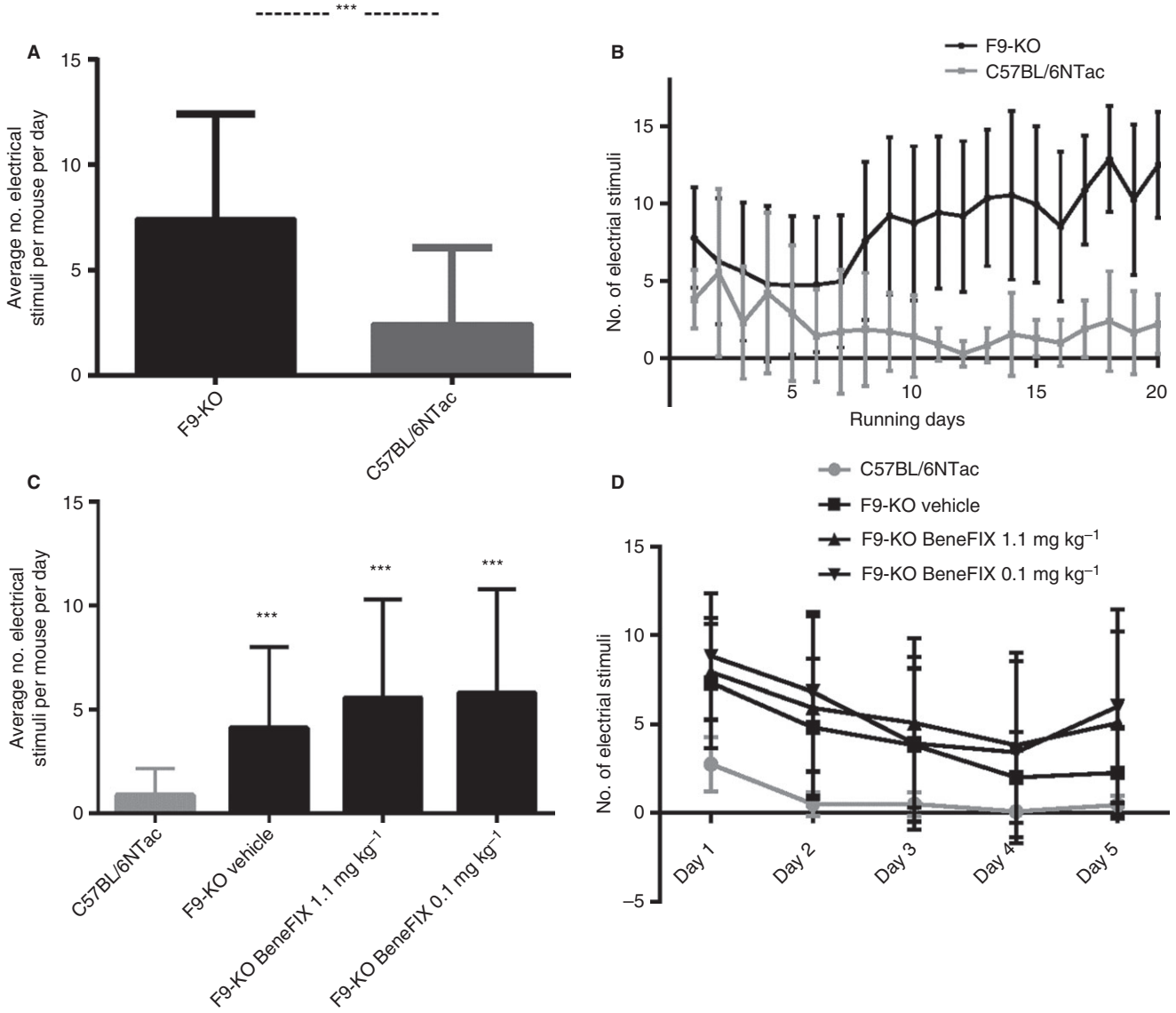

Fig. 3. Average numbers of electrical stimuli. (A) Implementation study, average electrical stimuli per mouse per day including SD. (B) Implementation study, average (+SD) electrical stimuli over time (4 weeks) in C57BL/6NTac and F9-KO mice. (C) Treatment study, average electrical stimuli per mouse per day including SD. F9-KO mice treated with vehicle, rFIX 11 mg/kg or rFIX 01 mg/kg. (D) Treatment study, average (+SD) electrical stimuli over time (5 days). Grey bars/lines, C57BL/6NTac; black bars/lines, F9-KO ***$P < 00001$.

Plasma haptoglobin levels were slightly but significantly elevated in F9-KO mice with muscle bleeds compared with F9-KO mice with no muscle bleeds ($P < 0.05$) (Table 2).

Discussion

The study demonstrated that exposing hemophilia B mice to treadmill exercise resulted in a high incidence of muscle bleeds, especially in male mice. Exposing normal non-hemophilic mice to the same exercise protocol did not result in any muscle bleeds. Thus, a spontaneous animal model addressing muscle bleeds, the second most common complication of hemophilia, has been developed.

Furthermore, treating hemophilia B mice before treadmill exercise with rFIX, aiming for daily trough levels of 3 and 0.3%, respectively (1.1 mg/kg, 220–235 U/kg or 0.1 mg/kg, 22–24 U/kg), prevented muscle bleeds and both doses were equally effective.

The number of electrical stimuli needed to keep the F9-KO mice running increased during both studies and was significantly increased compared with C57BL/6NTac mice. The decreasing tendency for C57BL/6NTac mice indicated a quick adjustment to treadmill running procedures. Previous studies have shown a large strain variation in exercise capacity and training response [18–20] and the difference in number of electrical stimuli between F9-KO and C57BL/6NTac mice could indicate a difference in performance skills between the two strains. However, the increasing number of electrical stimuli for F9-KO mice could also indicate less willingness to run

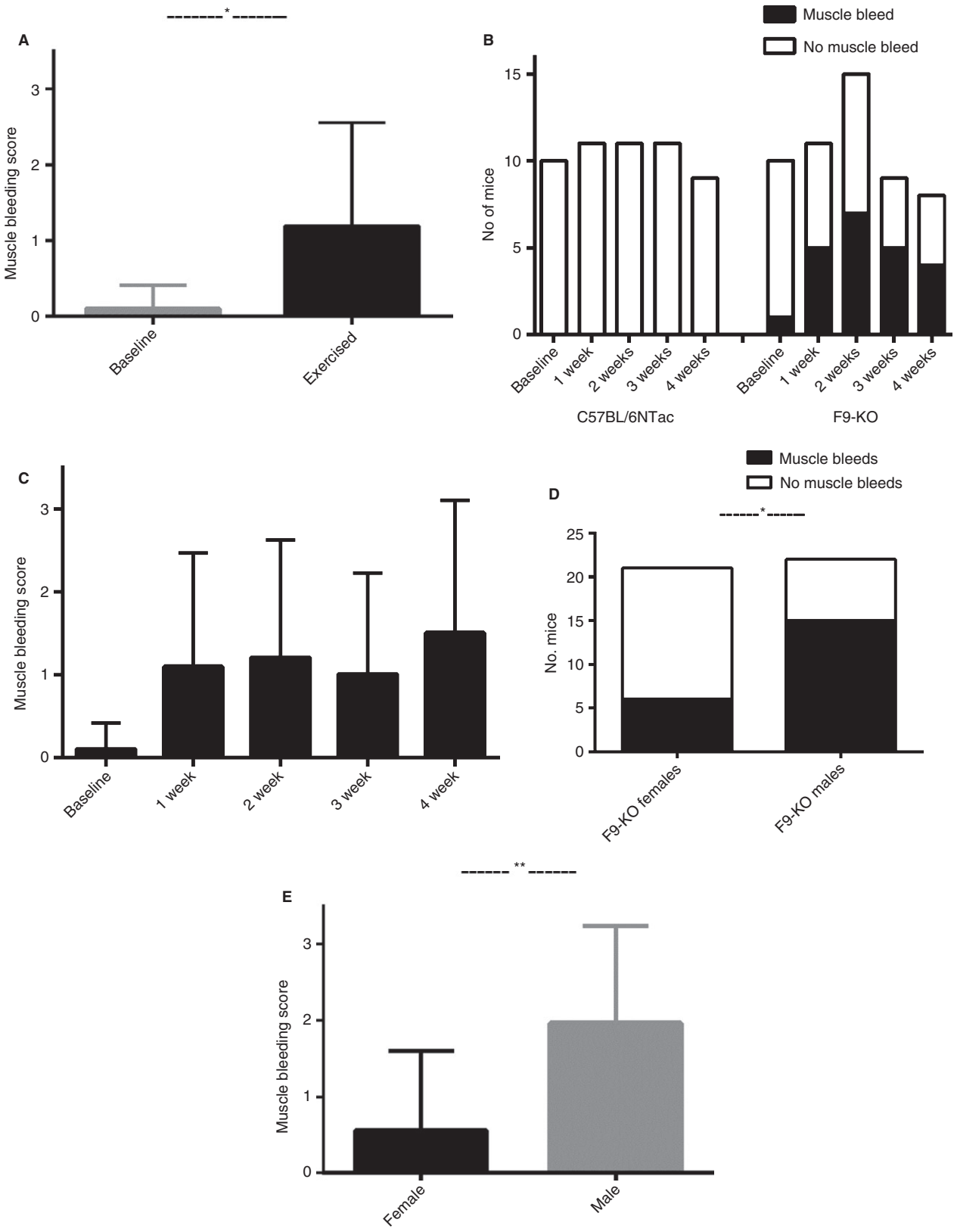

Fig. 4. Proportions of mice with muscle bleeds and the muscle bleeding score for the model implementation study. (A) Number of C57BL/6NTac or F9-KO mice with no bleeding or with muscle bleeding. White part of the bars represents no muscle bleeding and black part of the bar represents bleed. (B) The average (+SD) muscle score at baseline (grey bar) and 4 weeks accumulated (black bar). (C) Average (+SD) muscle bleeding score for F9-KO mice exposed to 1, 2, 3 or 4 weeks of exercise. (D) Number of muscle bleeds in female and male F9-KO mice. (E) Muscle bleeding score in F9-KO female mice (black bar) and male F9-KO mice (grey bar). *$P < 005$, **$P < 001$.

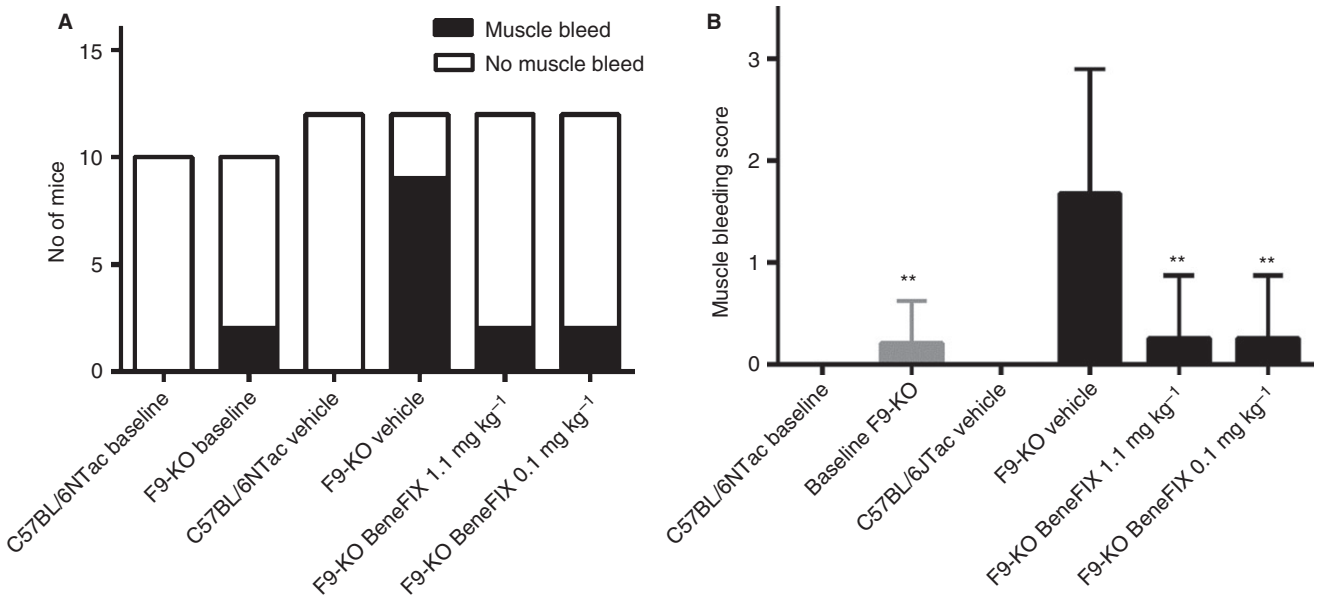

Fig. 5. Proportions of mice with muscle bleeds and muscle bleeding score in the treatment study. (A) Number of mice with or without bleeding in C57BL/6NTac or F9-KO mice at baseline, and after vehicle or rFIX treatment. White part of the bars represents no muscle bleeding and black part of the bars represents bleed. (B) Average (+SD) muscle bleeding score in C57BL/6NTac or F9-KO mice at baseline, and after vehicle or rFIX treatment. $^{**}P < 001$compared with F9-KO vehicle.

Table 2 Haptoglobin, myoglobin, skeletal muscle troponin-I and CK

	Haptoglobin (ng/mL)		Myoglobin (ng/mL)		Troponin I (ng/mL)		CK (U/L)	
	Average	SD	Average	SD	Average	SD	Average	SD
Baseline C57BL/6NTac	4.281	[a]	198	78	15	19	298	174
Baseline F9-KO	2.059	[a]	247	9	7	11	290	83
Bleeding	288.240	578217	176	90	11	17	251	164
No bleeding	40.176*	132835	196	81	6	11	279	266

[a]Only one value out of 10, all others below detection limit. $^{*}P < 005$. Bleeding mice vs. non-bleeding mice.

the treadmill due to the development of muscle bleeds. However, muscle bleeds were observed already during the first week of treadmill exercise and consequently the duration of the treatment study was reduced to 1 week.

In the model implementation study it was found that significantly more F9-KO male mice developed muscle bleeds compared with F9-KO female mice after treadmill exercise and hence, only male mice were included in the treatment study. Previous studies on exercised rats found increased muscle damage in males compared with females, suggesting that estrogen plays a protective role in muscle membrane stability through its capacity as an antioxidant [21].

Enzymes such as creatine kinase (CK) [22–25], proteins such as myoglobin [22,24] and skeletal muscle troponin-I [26,27] are widely used biomarkers for muscle damage. However, no indications of muscle damage were observed in the present study, supporting the idea that the model may mimic hemophilic bleeding and not injury to the muscles.

This is further substantiated by the fact that an increase in plasma CK levels has been reported in mice after prolonged exercise (120 min) [22] but not after mild [23] or modest (30 min) [24] exercise. In the current study, no significant increase was seen in plasma CK after exercise in either F9-KO mice or C57BL/6NTac mice, indicating the exercise schedule to be appropriate and not inducing muscle injury.

Significantly elevated levels of plasma haptoglobin were found in F9-KO mice with muscle bleeds compared with F9-KO mice without muscle bleeds. It could be speculated that the increase in plasma haptoglobin in mice with muscle bleeds is caused by an acute phase reaction triggered by the bleed. However, 13 of 22 samples from mice with muscle bleeds were below detection level and hence no consistency was observed between the presence of muscle bleeding and a high plasma haptoglobin level.

A number of animal models mimicking the hemophilic phenotype exists [28–34]. Up until the model reported

here, bleeding in hemophilic mice models has been induced by trauma [35–40]. However, except for rats and dogs, no hemophilic animal model of spontaneous muscle bleeding has previously been described.

Hence, in conclusion, it is demonstrated, for the first time, that exposing hemophilia B mice to treadmill exercise resulted in a high incidence of muscle bleeds, especially in male mice.

In addition, we have demonstrated that treatment of hemophilia B mice with a recombinant factor IX product before treadmill exercise prevented the occurrence of muscle bleeds and resulted in a normalization of the muscle bleeding score compared with untreated and unexercised hemophilia B mice.

We hope that this model can provide knowledge on initiation and progression of muscle hematomas and be a valuable tool in the evaluation and testing of new therapeutic strategies in the prevention and management of muscle hematomas in hemophilia.

Addendum

M. Tranholm designed the studies, reviewed study protocols, interpreted results, and wrote and reviewed the manuscript. M. P. Groth designed and performed the studies, did statistical analysis, and wrote and reviewed the manuscript. A. T. Kristensen reviewed the study protocols, interpreted data, and reviewed and revised the manuscript. M. L. Broberg supplied the pharmocokinetic data and models and reviewed the manuscript.

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Disclosure of Conflict of Interests

M. P. Groth, M. L. Broberg and M. Tranholm are employed at Novo Nordisk and A. T. Kristensen is employed by The University of Copenhagen.

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