

Genetic influences on survival time after severe hemorrhage in inbred rat strains

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Klemcke HG, Joe B, Calderon ML, Rose R, Oh T, Aden J, Ryan KL. Genetic influences on survival time after severe hemorrhage in inbred rat strains. *Physiol Genomics* 43: 758–765, 2011. First published April 12, 2011; doi:10.1152/physiolgenomics.00245.2010.—To find a genetic basis for differential ability to survive severe hemorrhage, we previously showed eightfold differences in survival times among inbred rat strains. We assumed that rat strains had similar normalized blood volumes (NBV; ml/100 g body wt). As NBV might vary among strains and constitute one genetic variable affecting survival time to hemorrhage, in *experiment 1* of the current studies we first measured total blood volumes and calculated NBV in specific inbred rat strains (Brown Norway/Medical College of Wisconsin, BN; Dark Agouti, DA; Fawn Hooded Hypertensive, FHH; Lewis, LEW; and Dahl Salt-Sensitive, SS) previously found to be divergent in survival time. NBV differed by 20% ($P < 0.01$; $BN > SS > FHH = LEW = DA$) and had a heritability (h^2) of 0.56. Hence, differential survival times in our previously published study might reflect strain-dependent differences in NBV. Then studies were conducted wherein rats were catheterized and, ~24 h later, 47% of their blood volume was removed; these rats were observed for a maximum of 4 h. In *experiment 2*, blood volumes were measured the day prior to hemorrhage. Percent survival and survival time did not differ among strains. To obviate possible confounding effects of blood volume determination, in *experiment 3* the average NBV for each strain was used to determine hemorrhage volumes. Percent survival ($P < 0.01$) and survival times ($P < 0.001$) were different with DA demonstrating the best (62.5%, 190 ± 29 min) and BN the worst (0%, 52 ± 5 min) survival responses. These data indicate that both blood volume and survival time after hemorrhage in rats are heritable quantitative traits, and continue to suggest that genetic assessment of these phenotypes might lead to novel therapeutics to improve survival to hemorrhage.

inbred rats; quantitative trait; blood volume; epigenetic

DIFFERENCES HAVE BEEN NOTED in individual ability to survive severe hemorrhage in both humans (e.g., Refs. 36–37 and animals Refs. 25, 39–40). We have hypothesized that differences in survival time to severe hemorrhage ultimately would reflect the presence of different genetic polymorphisms and that identification of these genetic differences might ultimately provide therapeutics to enhance survival (25). Moreover, such genetic information would provide an initial database for a “personalized medicine” approach to treatment of traumatic hemorrhage.

In our initial study with 15 inbred rat strains, we measured an ~8-fold difference in survival time to a controlled hemorrhage between the longest-lived [Brown Norway/Medical Col-

lege of Wisconsin (BN)] and the shortest-lived [Dark Agouti (DA)] strains. In that study, we assumed that all rat strains had similar blood volumes, and hence rats were hemorrhaged based on their body weight and an assumed blood volume of 5.83 ml/100 g body wt. We noted that a potential caveat to our interpretation was the possibility that different inbred rat strains had different blood volumes, and if so “then this would be construed as one of the genetic variables affecting survival time to hemorrhage” (25). If this supposition were to be true, then differences in survival might in part reflect differences in blood volumes. To address this question, the current series of studies was conducted with the objective of first measuring blood volumes in select inbred rat strains. If blood volumes were both significantly and clinically different, we would remeasure survival times to hemorrhage based on a fixed percentage of the now known blood volumes in an attempt to impose the same degree of ischemic hypoxia on all strains. The inbred rat strains chosen from our previous study (25) were those with the longest (BN) and shortest (DA) survival times. Other strains tested had divergent survival times and had been used previously to construct available genetic tools (consomic and congenic rat strains) that might expedite discovery of genes associated with survival time to hemorrhage [Lewis (LEW), Dahl Salt-Sensitive (SS), Fawn Hooded Hypertensive (FHH)].

MATERIALS AND METHODS

Animals. All rats were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. These studies were approved by the Institutional Animal Care and Use Committee of the US Army Institute of Surgical Research, Fort Sam Houston, TX. Furthermore, these studies were conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals (7th edition, 1996). For our first two studies, Charles River Laboratories (Wilmington, MA) supplied the BN and SS rats. Physiogenix (Wauwatosa, WI) supplied FHH. Harlan (Indianapolis, IN) supplied DA/OlaHsd, and Lew inbred rat strains. For our third study, BN rats were no longer available through Charles River, which had closed its colony. Hence, BN were obtained directly from the University of Wisconsin (Milwaukee, WI). All rats were males that were shipped at ~10 wk of age and were held for an 18- to 24-day acclimation period in our facility before experimentation. Rats were maintained individually in plastic cages at 19–23°C, with lights on from 06:00 to 18:00, and food (Harlan Global Teklad 2018; Madison, WI) and water were constantly available. Rats were randomly assigned to day of surgery, order of surgery on each day, and order of hemorrhage on each day.

Experimental design. Three experiments were conducted. For each study a randomized block design was attempted wherein each strain

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was represented during each ~2-wk period (block). However, because of nonavailability of the different rat strains, such an optimal design was not always achieved. Nevertheless, each strain was randomly distributed throughout the entire study period for all studies. Surgeries and hemorrhage procedures in all studies were conducted between 07:00 to 12:00 to avoid any potential diurnal rhythms in blood volumes or responses to hemorrhage. As we could not measure blood volumes on the day of hemorrhage in un-anesthetized, unrestrained rats, the first study (*experiment 1*; $n = 3\text{--}5$ rats per strain) was conducted to determine if blood volumes measured on the day of surgery would adequately reflect those measured ~24 h later on the day of hemorrhage. The second study (*experiment 2*; $n = 8\text{--}10$ rats per strain) was conducted to measure survival time after hemorrhage in five inbred rat strains as a fixed percentage of the blood volume determined 24 h previously in each rat. The third study (*experiment 3*; $n = 8\text{--}10$ rats per strain) was conducted to measure survival time after hemorrhage in the same five inbred rat strains using the average normalized blood volume (NBV; ml/100 g body wt) previously determined for each strain. Therefore, rats of *experiment 3* did not have their blood volume measured following catheterization on *day 1* to obviate potentially confounding effects of procedures used for measuring blood volumes.

Surgical procedures. All surgical procedures were conducted under aseptic conditions and were described in detail previously (25). At the end of surgery, rats were injected with buprenorphine (25 µg/kg body wt sc) and with 10 ml of 0.9% saline (sc)/400 g body wt to provide analgesia and hydration respectively during recovery.

Blood volume determination. Blood volumes were measured using the Evans blue technique (10, 41) immediately after insertion of the carotid catheter. A second incision was made in the skin in the medial aspect of the thigh to expose the femoral vein for subsequent injection of Evans blue dye. In *experiment 1*, a second blood volume determination was made 24 h later on *day 2*. In that study, rats were again anesthetized, and the contralateral femoral vein was exposed. The contralateral carotid was also exposed and manipulated, but was not catheterized. For each rat on *day 2* of this study, the time from initiation of anesthesia until blood volume determination was matched with that same time period on *day 1*.

A baseline 400 µl (600 µl, *experiment 1*) blood sample was taken via the carotid catheter immediately prior to blood volume determination and a comparable volume of sterile saline infused. Then, a bolus of Evans blue dye (Sigma, St. Louis, MO) solution (0.5% in sterile saline, 100 µl per 100 g body wt) was injected into the femoral vein using a 1 ml syringe and 1/2-inch 30-gauge needle (Becton Dickinson, Franklin Lakes, NJ). Five minutes later (1, 6) a second 400 µl (600 µl, *experiment 1*) blood sample was taken and a comparable volume of sterile saline again infused. A portion of all blood samples ($3 \times \sim 10$ µl) was used for hematocrit determinations via centrifugation of microcapillary tubes (Hemat-STAT-II, Separation Technology). Blood samples were then centrifuged at 1,900 g for 30 min. The plasma supernatant was transferred to a second tube and recentrifuged at 14,000 g for 1 min. Then 2×50 µl fractions of plasma were diluted 20-fold with sterile saline, and these dilutions were analyzed spectrophotometrically (A_{605}). Standard curves were prepared with Evans blue dye in sterile saline, and the plasma volume was calculated. Concomitant measurements of hematocrit and use of documented correction factors allowed for calculation of blood volume (10, 41). Our calculations included a correction term to account for plasma trapped between erythrocytes (0.96), and the F_{cell} factor for rats (0.74, Ref. 41). Hence, the formula used to calculate blood volume (BV) from the plasma volume (PV) was: $BV = (PV \times 100)/[100 - (\text{hematocrit} \times 0.96 \times 0.74)]$ (10, 30).

An in vitro validation of this procedure was conducted using the following procedures. A pool of heparinized swine blood was collected and divided into duplicated aliquots of 15, 20, 25, and 30 ml. For each sample, Evans blue dye solution (0.5%) was injected (at a ratio of 100 µl/10 ml) directly into the blood, which was gently

vortexed. These blood samples were then processed as above for in vivo blood volume measures. The average accuracy of blood volume estimates in this in vitro system was 98.9%. The average coefficient of variability (CV) of the duplicate samples across the four different blood volumes was 1.8%. The slope of the line relating measured blood volume to known blood volume was 1.075 and did not differ from 1 ($P > 0.05$).

Hemorrhage procedures. Approximately 24 h after surgery, conscious, unrestrained rats were weighed and then bled as described in detail previously (25). In the current studies, however, 47% of each rat's measured blood volume (*experiment 2*) was withdrawn according to the following schedule: 25% of the blood volume to be removed was withdrawn at a constant rate during the first 4 min while the remaining 75% of the blood volume to be removed was withdrawn at a constant rate during the next 22 min. A 47% hemorrhage was used as initial results obtained in *experiment 1* indicated that this was the actual hemorrhage used for rats with the shortest survival times (DA) in our previous work (25). We chose to use this percent hemorrhage as it was deemed essential that as the DA rats to be used in the current studies at least survived the actual hemorrhage procedure of 26 min. In *experiment 3*, this same hemorrhage protocol was used, but 47% of the previously determined average NBV for the rat strain was removed, as blood volume was not determined on *day 1* in these rats. After hemorrhage rats that displayed irregular gasping breaths or apnea or reached 4 h postinitiation of hemorrhage were euthanized with an intravascular injection of sodium pentobarbital (150 mg/kg body wt).

Statistics. Data were analyzed using the Statistical Analysis System package (SAS, Cary, NC). Surgical and bleeding-associated measures were analyzed using a single-way analysis of variance (PROC ANOVA). Differences among individual means were examined using the a posteriori Student-Newman-Keuls test. However, when two-way ANOVA were used, multiple means comparisons were conducted using the PDIFF procedure of SAS adjusted via control of the false discovery rate (7). Correlation analyses were conducted using PROC CORR of the SAS system. When appropriate, blood volume data were analyzed using a repeated measures procedure associated with the PROC MIXED program of SAS. Analysis of covariance was conducted using PROC GLM procedures of SAS. All data were tested for homogeneity of variance (Levene's test) and normality of distribution (PROC Univariate Normal with associated Kolmogorov-Smirnov test). Data were transformed where necessary to meet assumptions of ANOVA. When rats survived the complete 4 h and were euthanized, the true survival time is unknown. Such data are said to be "censored". These survival data with censored observations were analyzed using either PROC LIFETEST or PROC PHREG. PROC LIFETEST with associated Kaplan-Meier procedure for estimating survivor functions and log-rank test for determining differences among survivor functions was used to compare all inbred rat strains without covariates. PROC PHREG, which incorporates Cox regression and its associated proportional hazards model (2), was used with covariates. All covariates considered (i.e., age at hemorrhage, duration of surgery 24 h prior to hemorrhage, duration of restraint time to attach catheter extension just prior to hemorrhage, room temperature throughout the hemorrhage procedure, and percent hemorrhage) were initially included in the overall model. Subsequently, through an iterative process, covariates that were found not to be significant were dropped from the model. Indeed, no covariate was significantly associated with survival time. Probabilities of survival curve comparisons were adjusted for multiple comparisons via controlling the false discovery rate (7). Differences in percent survival were determined using PROC FREQ and associated χ^2 -test. Homogeneity of CV were tested using a χ^2 -test suggested by Zar (44). Heritability of blood volume was determined using with and between variance components from ANOVA (21). Heritability of survival times was obtained through the estimate of a pseudo- R^2 value from the partial likelihoods corresponding to the Cox proportional hazards regression model (29). Data are

Table 1. *Body weights and MAP in inbred rat strains for experiments 2 and 3*

Experiment	Inbred Rat Strain	Body Weight at Hemorrhage, g	Prehemorrhage MAP, mmHg	Posthemorrhage MAP, mmHg	MAP Change
2	BN (9)	268 ± 8 ^{c,*}	119 ± 2 ^b	67 ± 11 ^b	53 ± 13 ^{b,c}
	SS (10)	384 ± 6 ^a	142 ± 4 ^a	47 ± 4 ^{b,c,*}	93 ± 5 ^{a,*}
	LEW (9)	294 ± 12 ^b	102 ± 5 ^c	33 ± 7 ^c	70 ± 7 ^{a,b}
	FHH (9)	315 ± 7 ^{b,*}	146 ± 3 ^a	68 ± 8 ^b	79 ± 9 ^{a,b}
	DA (9)	266 ± 6 ^c	147 ± 5 ^a	113 ± 14 ^a	34 ± 12 ^c
ANOVA Probability		$P < 0.0001$	$P < 0.0001$	$P < 0.0001$	$P = 0.0008$
3	BN (9)	293 ± 4 ^{b,*}	111 ± 5 ^b	62 ± 10 ^{b,c}	51 ± 10 ^a
	SS (9)	388 ± 10 ^a	150 ± 5 ^a	95 ± 15 ^{a,b,*}	57 ± 17 ^{a,*}
	LEW (9)	309 ± 8 ^b	107 ± 7 ^b	42 ± 10 ^c	59 ± 17 ^a
	FHH (10)	294 ± 5 ^{b,*}	146 ± 4 ^a	84 ± 7 ^b	63 ± 9 ^a
	DA (8)	254 ± 6 ^c	143 ± 6 ^a	125 ± 8 ^a	20 ± 9 ^a
ANOVA Probability		$P < 0.0001$	$P < 0.0001$	$P = 0.0003$	$P = 0.32$

Inbred rat strains represented are: Brown Norway/Medical College of Wisconsin (BN), Dahl Salt-Sensitive (SS), Lewis (LEW), Fawn Hooded Hypertensive (FHH), and Dark Agouti (DA). Each datum point represents the mean ± SE of the number of observations noted in parentheses next to the inbred rat strain. Means with different letter superscripts within a measure and Experiment are significantly different ($P < 0.05$). Means with an asterisk superscript within a measure for a given strain are different ($P < 0.05$) between experiments for that strain. Mean arterial pressure (MAP) was recorded at 1 min prior to hemorrhage, and at 1 min after the end of hemorrhage. MAP change is the difference between pre and posthemorrhage MAP.

presented as arithmetic means ± SE. However, as some rat strains studied had censored data, survival times and associated SE are underestimates (2).

RESULTS

Initial blood volume determinations (experiment 1). Blood volumes were measured on the day of surgery (*day 1*) and 24 h later (*day 2*) to determine if measures on the day of surgery could adequately estimate blood volumes on the subsequent day. These rats were not subjected to hemorrhage procedures. Total plasma volumes were not statistically different between days of measure for any strain. However, total blood volumes decreased between *days 1* and *2* for DA and FHH rats (6.3 and 10.3% decrease, respectively, $P < 0.05$; Supplemental Table S1).¹ Statistically there were no differences among strains in the percent change of total blood or plasma volume between *day 1* and *day 2* ($P > 0.05$, data not shown). Across all strains there was a high correlation between blood volumes measured on day of surgery vs. day of hemorrhage ($r = 0.93$, $P < 0.0001$).

Survival time to severe hemorrhage in rats with measured blood volumes (experiment 2). Blood volume was measured in each rat during the catheterization procedure. Body weights differed among inbred rat strains (Table 1) as previously noted (25). Plasma and blood volumes normalized to body weight also differed ($P < 0.01$, Fig. 1) among strains. NBV was greatest in BN (7.39 ± 0.07 ml/100 g body wt) and least in DA rats (6.29 ± 0.07). There were no correlations between body weight and NBV either within or across all rat strains ($r = 0.15$, $P = 0.32$). There was, however, a high correlation between body weight and total blood volume both within and across all rat strains ($r = 0.93$, $P < 0.0001$). Percent blood volume removed was constant ($P = 0.12$) among strains and averaged $47.2 \pm .1\%$. There were no statistical differences in average survival times ($P = 0.94$), percent survival ($P = 0.87$) (Fig. 2A), nor in survival patterns, as illustrated by Kaplan-Meier graphs (Fig. 2B, Supplemental Fig. S1), among rat strains. Both baseline and post hemorrhage mean arterial pres-

sures (MAP) differed among inbred rat strains as did the decrease in MAP produced by hemorrhage (Table 1).

Survival time to severe hemorrhage in rats with calculated blood volumes (experiment 3). To obviate potentially confounding influences of procedures involved in blood volume determinations, another study was performed in which blood volume was not measured prior to hemorrhage. Rather, an average NBV for each strain in this study was calculated based on all successful blood volume determinations available at that time. As the source of the BN rats of necessity was changed (see MATERIALS AND METHODS), blood volume was measured in five such rats and included in the average. These NBV averages (BN, $n = 25$, 7.77 ± 0.13 ml/100 g body wt; DA, $n = 17$, 6.49 ± 0.09 ; FHH, $n = 15$, 6.89 ± 0.12 ; LEW, $n = 17$, 6.77 ± 0.10 ; SS, $n = 15$, 7.26 ± 0.11) were used for hemorrhage

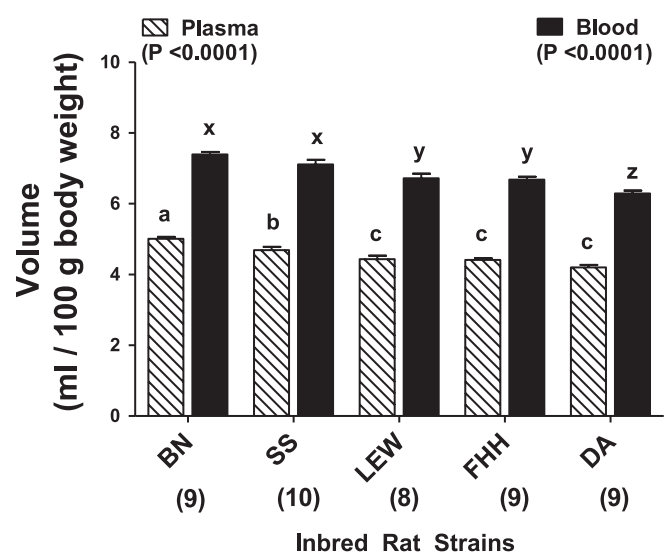


Fig. 1. Plasma volume, hematocrit, and blood volume in inbred rat strains for experiment 2. Each bar represents the means ± SE of the number of rats noted in parentheses below bars. Within a measure, bars with different letter superscripts (a, b, c, d, e; or x, y, z) are significantly different ($P < 0.05$). BN, Brown Norway/Medical College of Wisconsin; SS, Dahl Salt-Sensitive; LEW, Lewis; FHH, Fawn Hooded Hypertensive; DA, Dark Agouti.

¹ The online version of this article contains supplemental material.

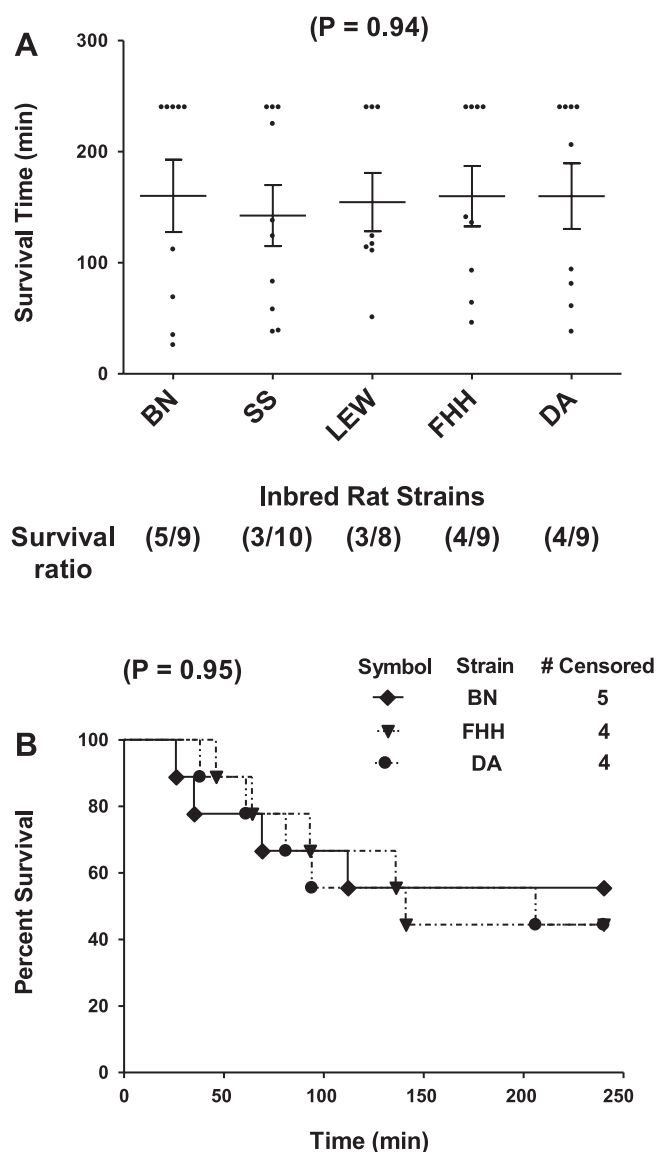


Fig. 2. Survival to severe hemorrhage for *experiment 2*, wherein each rat underwent a blood volume determination on the day prior to hemorrhage. **A**: each set of horizontal and vertical lines represents the means $\pm$ SE of the number of rats noted in the denominator of the survival ratio in parentheses below each rat strain. Survival time for each individual rat is represented as a dot. The numerator of the survival ratio represents the number of rats surviving to 240 min. **B**: Kaplan-Meier survival curves for inbred rats after hemorrhage in *experiment 2*. For clarity, only 3 strains are presented. Censored data reflect the absence of a true survival time as these rats were euthanized at 4 h after initiation of hemorrhage. There were no differences among inbred rat strains for the % hemorrhage ($47.2 \pm 0.1\%$ across all strains), survival ratios, survival times, or survival patterns ($P > 0.05$).

of rats in a strain-specific manner. Variances associated with these means were homogeneous as measured by Levene's test ($P = 0.18$) and by Bartlett's test ($P = 0.08$). Coefficients of variation associated with each mean were also not statistically different (8.33% vs. 5.6% vs. 6.95% vs. 5.94% vs. 5.68%, respectively; $P > 0.05$). Using blood and plasma volume data for each strain, we determined that the heritability (h^2) of both blood ($h^2 = 0.56$) and plasma ($h^2 = 0.58$) volumes was relatively high (21, 28). The percent hemorrhage did not differ statistically among strains and averaged $47.3 \pm 0.1\%$ ($P =$

0.29). In this experiment body weights for BN were greater than those in *experiment 2* and those for FHH were less, although ages of hemorrhage did not differ ($P = 0.85$). With the exception of SS rats, MAP measures were comparable between studies (Table 1). There were no convincing correlations between survival times and any measures of MAP in either experiment. Unlike the previous experiment, survival times, patterns, and percent survival differed among inbred rat strains (Figs. 3, A and B; Supplemental Fig. S2; $P < 0.01$) with DA rats ranking highest for both characteristics. Heritability (h^2) of survival time was determined to be 0.44.

DISCUSSION

These studies confirm our previous findings (25) that survival time after hemorrhage is a genetic trait with relatively high heritability. Further, they extend our previous observations as we now have shown that blood volume differs among inbred rat strains and also constitutes a heritable quantitative trait. The ultimate objective of our work is to identify one or more genes that regulate these complex traits. Complex quantitative traits that show continuous variation from low to high values, such as blood volume and survival time after hemorrhage, reflect the expression of multiple genes and the interaction among those genes as well as gene $\times$ environment interactions (34). Hence, determination of involved genes is a difficult process. Inbred rat strains were chosen for the current studies from our initial sampling of 17 strains (25, 26) both because they had the maximal and minimal survival times, and because of the availability of congenic and consomic rat strains derived from several of these strains (BN, SS, LEW) that would potentially expedite gene discovery (13).

To assist in such discovery, there are physiological measures that distinguish survivors from nonsurvivors to severe hemorrhage in humans (36–37) and in rodents (39–40). Moreover, studies involving organ-specific ischemia in inbred rat strains (4, 24) and in humans (e.g., Refs. 19–20) suggest genetic influences on tissue responses to ischemia. Also, previous research with the currently used inbred rat strains may reveal characteristics that give some direction to future gene discovery. For example, DA rats appear to have a high aerobic running capacity and cardiac output (5) and a greater sympathetic regulation of arterial blood pressure than other inbred rat strains (27). A genome-wide scan for loci associated with aerobic running capacity unveiled quantitative trait loci containing several genes that code for proteins involved in aspects of cellular energy production (42) that may well serve as potential candidate genes for survival time after severe hemorrhage. An *in vitro* model of myocardial ischemia (4) indicated that the infarct size was least in hearts from BN, whereas hearts from SS rats demonstrated the most adverse consequences of ischemia. Finally, data from trauma patients have demonstrated that differences in single nucleotide polymorphisms in mitochondrial DNA (9), complement (31), and β_2 -adrenergic receptors (32) are associated with differences in mortality.

In the current series of studies, *experiment 3* evolved because the absence of differences in survival time to hemorrhage in *experiment 2* were in contrast to observations from our previous work (25). We now have used known blood volumes and applied them to blood removed in that initial study (25) to

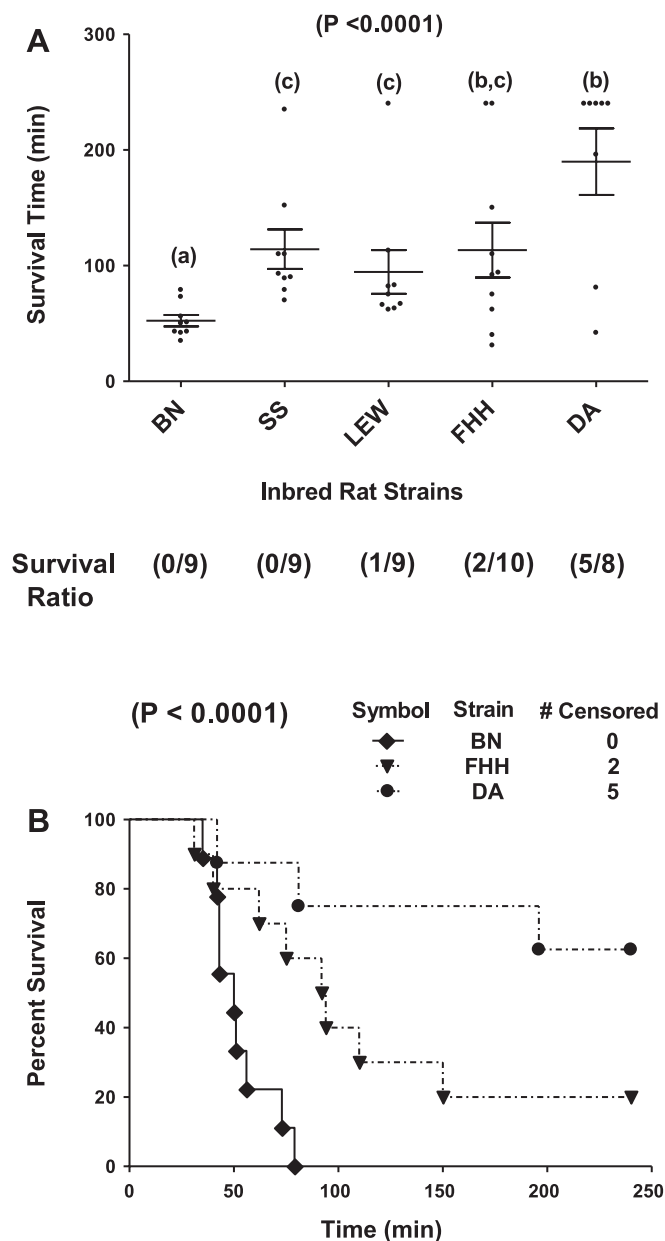


Fig. 3. Survival times to a severe 47% hemorrhage in inbred rat strains for *experiment 3*, wherein the strain-specific average normalized blood volume (NBV; ml/100 g body wt) was used as an estimate of the NBV for each rat. A: each set of horizontal and vertical lines represents the means $\pm$ SE of the number of rats noted in the denominator of the survival ratio in parentheses below each rat strain. Survival time for each individual rat is represented as a dot. There were no differences among inbred rat strains in the % hemorrhage, but survival ratios ($P = 0.0004$) and survival times were different ($P < 0.05$). Strains that do not share a common letter superscripts (a, b, c) are significantly different ($P < 0.05$). B: Kaplan-Meier survival curves for inbred rat strains after the severe 47% hemorrhage in *experiment 3*. For clarity, only 3 of the most divergent strains are presented. Censored data reflect the absence of a true survival time as these rats were euthanized at 4 h after initiation of hemorrhage.

calculate percent blood removed for inbred rat strains of interest (Table 2). Careful evaluation and comparison of blood removed and survival times in that study and *experiment 2* (data not shown) revealed contradictions that led us to speculate that one or more aspects of the blood volume determination process might have influenced survival to hemorrhage 24

h later. These conflicting observations also suggested some intriguing hypotheses: 1) Evans blue dye injected to measure blood volumes might influence survival time; and 2) Blood samples (~ 0.8 ml) removed for blood volume determinations might constitute a preconditioning stimulus that improves survival time.

Evans blue is not completely inert but, rather, has multiple biological effects such as an ability to increase activity of calcium-dependent potassium channels associated with hyperpolarization and a decrease in cellular excitability (23, 43). It also blocks the action of purinoceptors (mediate the action of extracellular ATP) in endothelial and vascular smooth muscle cells (16). Such actions could potentially influence survival after hemorrhage.

Preconditioning refers to a procedure wherein a noxious stimulus, in this case hemorrhage, near to but below the threshold of irreversible damage is applied to a tissue or organism. Shortly after preconditioning, the tissue or organism develops a certain resistance or tolerance to that or even a different noxious stimulus (15). Thus, blood removal for measurement of blood volumes 24 h earlier might precondition the rats to survive better after a more severe hemorrhage. However, there is evidence with anesthetized dogs indicating that a sublethal hemorrhage performed 48 h previously has no effect on subsequent survivability to a lethal hemorrhage (14). Nonetheless, at this time we do not have evidence to either accept or reject the above-noted hypotheses in our rat model.

Experiment 3 shows that differences in survival time to comparable severe hemorrhages do indeed exist among inbred rat strains when the above-noted concerns about confounding influences of Evans blue dye and putative preconditioning are removed. Moreover, *experiment 3* clearly demonstrates that when strain and genetic-dependent blood volumes are taken into account, DA rats survive ~ 3.7 -fold longer than do BN rats. Such results improve our understanding of our previous data, wherein it was assumed all rat strains had comparable volumes. Furthermore, these differing blood volumes were undoubtedly partly responsible for some differences in survival times measured previously (25) and for differences between that study and current studies. For example in *experiment 3*, based on calculated blood volumes, both BN and SS rats were bled ~ 1 ml more than previously (Table 2) and survived a significantly shorter time compared with previous results (25).

In addition to differences in the percent hemorrhage, other factors might also engender differences among studies. For example, FHH rats were bled more in *experiment 3* vs. our previously published study yet lived longer (Table 2). This apparent discrepancy might be accounted for in part by the obligatory difference in suppliers (Physiogenix vs. Charles River) with the concomitant possibility of generation of phenotypically different substrains (8). In *experiment 3* DA rats were bled modestly less yet lived longer (Table 2). This observation might suggest that DA is very sensitive in terms of survival time to small changes in hemorrhage volume. However, one must be cautious in making comparisons among studies as minor changes in experimental procedures, changes in personnel, and unknown environmental differences could potentially influence outcome. Genetic studies are further confounded by epigenetic mechanisms that might alter phenotype in a strain-dependent manner (18).

Table 2. A comparison of blood volumes removed and survival times between our previously published study and experiment 3 of the current report

Rat Strain	Blood volume removed (E-3), %	Blood volume removed (I-E), %	E-3 vs. I-E, ml**	E-3 vs. I-E, min#	Comparison of Survival Times, <i>P</i>
BN	47.24 ± 0.08	42.00 ± 0.30	1.147 more	161 less	0.0002
SS	47.13 ± 0.05	43.66 ± 0.38	0.921 more	84 less	0.0042
LEW	47.07 ± 0.11	47.06 ± 0.61	0.001 more	3 less	0.4100
FHH	47.29 ± 0.20	45.96 ± 0.46	0.301 more	113 more	0.0641
DA	47.60 ± 0.33	48.92 ± 0.39	0.219 less	150 more	0.0002

**Average difference in % of total blood volume removed (expressed in ml) for the experiment comparisons noted. #Average difference in survival times (min) for the experiment comparisons noted. The percent of the total blood volume removed for our previously published study (25), the initial experiment (I-E), was determined by first calculating the blood volume for each rat in that study using average strain-dependent normalized blood volumes: (BN, $n = 25$, 7.77 ± 0.13 ml/100 g body weight; SS, $n = 15$, 7.26 ± 0.11 ; LEW, $n = 17$, 6.77 ± 0.10 ; FHH, $n = 15$, 6.89 ± 0.12 ; and DA, $n = 17$, 6.49 ± 0.09) and the known weight of each rat. Then, based on the known volume of blood actually removed from each rat, the percentage of the total blood volume removed was calculated. For experiment 3 of the current report (E-3), the total blood volume was actually measured in each rat, and the percentage of the total blood removed was calculated from that measure as well as the known volume of the blood removed.

A new finding associated with our current work is that blood volume appears to be a heritable quantitative trait in inbred rats. This observation not only indicates that differences in blood volume among inbred rat strains undoubtedly contributed to differences in survival times after hemorrhage in our previous study (25), but also that blood volume provides another phenotype that can be studied and potentially altered to improve survival time after hemorrhage. Although the concept appears almost naive and oversimplified, one might suggest that an individual with a greater blood volume should be able to survive the same loss of blood longer than would an individual with a smaller blood volume. At first glance our current data for blood volumes (*experiment 2*) and survival times (*experiment 3*) do not support such a hypothesis. However, in *experiment 3* all rats were bled to the same percentage of their total blood volumes, and this hypothesis was therefore not systematically tested. This hypothesis can instead be examined using data from our previously published work (25), wherein rats were assumed to have the same NBV (5.83 ml/100 g body wt). Based on our improved knowledge of their true NBV, we now know that differential percent hemorrhages occurred. Hence, associations occurred between survival time and percent hemorrhage ($r = -0.93$, $P = 0.02$) and between survival time and the calculated NBV ($r = 0.92$, $P < 0.03$) across inbred rat strains, thereby providing evidence for this hypothesis.

Interestingly, blood volume heritability (28) was 56%. Such heritability reflects a mathematical calculation that estimates how much of the phenotypic (blood volume) variability is attributable to genes transmitted from the parents. It is thus a measure of the relative importance of heredity in determining phenotype (17). We have calculated this narrow sense heritability in two ways: 1) Using measured variances within and between strains (21) for blood volume; and 2) Using more complex calculations with survival times that contained censored data (29). The remaining ~44% of phenotypic variability are assumed to be due to environmental and possibly epigenetic factors. Indeed, there is substantial evidence for at least two environmental factors that have the ability to increase blood volume: exercise and ambient temperature (11, 12). Hence, we have demonstrated that one characteristic, blood volume, which has both genetic and environmental regulatory components, differs among inbred rat strains and may be a

useful characteristic to modify and thereby improve survival to severe hemorrhage.

There are multiple potential limitations to our model and to our interpretation of the results. First, variability in changes in blood volumes between the 2 days among rats suggest the potential that some variability in response to hemorrhage may reflect inaccuracies in predicting blood volumes present 24 h after their measurement. However, concern for potential strain-dependent differences in response to anesthesia that might result in confounding strain-dependent differences in response to hemorrhage prevented our measurement of blood volumes on the day of hemorrhage in anesthetized rats. Second, in *experiment 3*, use of average NBV for each strain rather than measured individual blood volumes for each rat may also have contributed to within strain variability in percent hemorrhage and subsequently in survival times. Hence, the validity of our accepting results of *experiment 3* rests on the assumption that one can use a strain-dependent average NBV to predict an individual's blood volume with reasonable accuracy. Indeed, statistical differences in variability of NBV estimates among strains did not exist as measured by homogeneity of variances and coefficients of variation. One could anticipate, therefore, that deviations from the actual blood volume of each rat would be randomly distributed among all rats, and should not create a bias in the ultimate results. Nonetheless, although we are proffering *experiment 3* as the most valid responses to hemorrhage in terms of survival time, at this point we cannot totally exclude the possibility that results obtained in *experiment 2* are the most nearly accurate. Third, BN rats were obtained from a different colony for *experiment 3*. The BN strain that originated from the Medical College of Wisconsin and was sent to Charles River laboratories in 2002 was used in our initial study (2005–06)(25) and in *experiment 1* and 2 of the current work (2008–09). It is possible that the different colonies constituted substrains for those BN rats that might have altered genetic or epigenetic characteristics that could influence survival time after severe hemorrhage. Fourth, hemorrhages were conducted ~24 h postsurgery to minimize confounding influences of isoflurane and buprenorphine on survival time. The half-life for isoflurane (<30 min, Ref. 22) and buprenorphine (2.1–3 h, Ref. 33) would suggest a clearance from the blood compatible with our assumption. However, one cannot totally exclude the possibility that residual effects might remain that could influ-

ence survival time after hemorrhage in a strain-dependent manner (3, 35). For isoflurane, delayed effects have been documented (38, 45). Finally, our rat models and approach will not discover all genetic mechanisms controlling variability of either blood volume or survival time after hemorrhage. Only genes that are different among these inbred strains studied will ultimately be identified. Other rat strain combinations might use different genes. But then, as above noted, our goal is only to identify sufficient genes or genetic mechanisms such that their regulation will improve survival time after hemorrhage.

In summary, differences in blood volumes among inbred rat strains undoubtedly accounted for differences in survival times for some of the rat strains in our initial study (25). However, when the percentage of blood volume removed was standardized among strains, then underlying differences in abilities to withstand global ischemia were revealed. Hence, our current results provide further evidence that survival time to severe hemorrhage and blood volume are both heritable, quantitative traits. Both phenotypes are worthy of investigation as potential vehicles to improve survival to traumatic hemorrhage and attendant shock via both genetic and environmental mechanisms.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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