HHS Public Access

Author manuscript

Transfusion. Author manuscript; available in PMC 2017 October 01.

Published in final edited form as:

Transfusion. 2016 October ; 56(10): 2571–2583. doi:10.1111/trf.13745.

Testosterone-dependent sex differences in red blood cell hemolysis in storage, stress and disease

Tamir Kanias, PhD[1,2], **Derek Sinchar, BSc**[1], **David Osei-Hwedieh, PhD**[1], **Jeffrey J. Baust, BSc**[1], **Andrew Jordan, MSc**[3,4], **James C. Zimring, MD, PhD**[5,6,7], **Hayley R Waterman, BSc**[5], **Karen S de Wolski, BSc**[5], **Jason P. Acker, PhD, MBA**[3,4], and **Mark T. Gladwin, MD**[1,2]

[1]Pittsburgh Heart, Lung, Blood and Vascular Medicine Institute, University of Pittsburgh

[2]Division of Pulmonary, Allergy and Critical Care Medicine, Department of Medicine, University of Pittsburgh

[3]Laboratory Medicine and Pathology, University of Alberta, Edmonton, Canada

[4]Canadian Blood Services, Centre for Innovation, Edmonton, Canada

[5]Bloodworks NW Research Institute, University of Washington School of Medicine, Seattle, Washington

[6]Department of Laboratory Medicine, University of Washington School of Medicine, Seattle, Washington

[7]Department of Medicine, Division of Hematology, University of Washington School of Medicine, Seattle, Washington

Abstract

INTRODUCTION—Red blood cell (RBC) hemolysis represents an intrinsic mechanism for human vascular disease. Intravascular hemolysis releases hemoglobin and other metabolites that inhibit nitric oxide signaling and drive oxidative and inflammatory stress. While these pathways are important in disease pathogenesis, genetic and population modifiers of hemolysis including sex have not been established.

MATERIALS AND METHODS—We studied sex differences in storage or stress-induced hemolysis in RBC units from the US and Canada, in 22 inbred mouse strains, and in sickle cell disease using measures of hemolysis in 315 homozygous SS sickle cell patients from the Walk-

Correspondence should be addressed to: Tamir Kanias, Vascular Medicine Institute, E1200-22B BST, 200 Lothrop Street, University of Pittsburgh, Pittsburgh, PA 15261, Phone: 412-624-5004, tak77@pitt.edu.

CONFLICT OF INTEREST DISCLOSURE: Dr. James C. Zimring has a sponsored research agreement with Immucor, and serves on the scientific advisory board for Rubious Therapeutics. The rest of the authors declare no competing financial interests.

AUTHORSHIP CONTRIBUTION

T.K and M.T.G conceived the study, executed the experimental plan, and performed data analysis. T.K wrote the manuscript, executed the experiments, and supervised affiliated staff. D.S and D.O.H performed the majority of the hemolytic assays in mice and human, storage and transfusion of orchiectomy murine RBCs, and assisted with data analysis presented in this manuscript. J.J.B helped planning and executing the mouse surgical and testosterone-repletion procedures performed at the University of Pittsburgh. J.C.Z and his research team H.R.W and K.S.de.W designed and executed the murine transfusion studies, which compared the recovery of stored female versus male RBCs in-vivo. A.J collected and analyzed the hemolysis data from the Canadian Blood Services cohort. J.P.A designed, directed and analyzed the hemolysis data from the Canadian Blood Services cohort, as well as reviewed this manuscript.

PHASST cohort. We used a mouse model to evaluate post-transfusion recovery of stored RBCs, and gonadectomy to determine mechanisms related to sex hormones.

RESULTS—An analysis of predisposition to hemolysis based on sex revealed that male RBCs consistently exhibit increased susceptibility to hemolysis than females in response to routine cold storage, under osmotic or oxidative stress, after transfusion in mice, and in sickle cell disease. The sex difference is intrinsic to the erythrocyte and not mediated by plasmatic factors or female sex hormones. Importantly, orchiectomy in mice improves RBC storage stability and post-transfusion recovery, whereas testosterone repletion therapy exacerbates hemolytic response to osmotic or oxidative stress.

DISCUSSION—Our findings suggest that testosterone increases susceptibility to hemolysis across human diseases, suggesting that male sex may modulate clinical outcomes in blood storage and sickle cell disease, and establishing a role for donor genetic variables in the viability of stored erythrocytes and in human hemolytic diseases.

INTRODUCTION

Contemporary blood banking and transfusion practices have successfully minimized the risks associated with transfused red blood cell (RBC) products. Despite the advancement in cell preservation technologies, cold storage may compromise RBC viability and may promote post-transfusion injury via multiple mechanisms including hemolysis. The risks associated with hemolysis largely stem from the redox-active nature of cell-free hemoglobin that may induce hypertension, inflammation, endothelial dysfunction, and organ failure via interference with nitric oxide (NO) signaling[1–3], and heme-mediated oxidative[4] and inflammatory stress.[5] These scenarios not only apply to RBC transfusion, but also to the pathophysiology of RBC disorders, such as sickle cell disease (SCD)[6] and thalassemia.[7]

Pre-clinical and human physiological studies suggest that transfusion of RBCs stored at the limits of FDA-approved storage time produce endothelial dysfunction, inflammation, and systemic and pulmonary vasoconstriction.[1,8,9] Conversely, recent randomized clinical studies found no significant association between RBC storage duration time and increased risk of end-organ injury.[10,11] However, both trials evaluated fresh blood versus standard of care, which in these studies averaged from 22 to 28 storage days, with limited power to evaluate the risk of blood transfusion beyond 5 weeks of storage. It is also increasingly appreciated that not all donor units are equal, and significant blood donor-manufacturing differences, and donor-specific variability exists in the rate of storage-hemolysis and post-transfusion RBC recovery.[12] We hypothesized that predisposition to hemolysis in blood donors has a genetic base, and that biological variables including sex or race may significantly determine the quality and recovery of transfused RBCs.[13]

Sex represents a biological variable that can modulate the pathophysiology and severity of human disease including cardiovascular disease, renal failure, and RBC disorders. Male sex has been associated with enhanced susceptibility to cardiac[14,15] and renal[16] ischemia-reperfusion injury or, in the case of SCD, with higher risk of early death.[17] Similarly, recent reports have indicated that stored RBCs from male donors hemolyze more than females at the end of storage or in response to applied mechanical stress.[12,18] Sex differences in

predisposition to hemolysis have been largely ascribed to premenopausal women, where factors such as menstruation, lower hematocrit and iron levels, and the protective action of female sex hormones (primarily estradiol and progesterone) may modulate rheological properties to enhance membrane deformability.[18–21]

The purpose of this study was to further characterize the effect of sex on predisposition to hemolysis using a range of stress perturbations that may predict storage stability and RBC post-transfusion recovery. We demonstrate that male sex correlates with increased susceptibility to hemolysis during routine blood banking storage, and in sickle cell disease. Our mechanistic studies in mice replicate findings in humans and confirm that this phenomenon predicts lower post-transfusion recovery in hemolysis-susceptible mouse strains and in males across strains. We further demonstrate that orchiectomy in mice, rather than ovariectomy, modulates predisposition to hemolysis in mice, and is dependent on *in vivo* exposure to testosterone. These findings implicate male sex, and specifically testosterone, as a risk factor for hemolysis in blood banking and hemolytic disease.

MATERIALS AND METHODS

Determination of storage hemolysis

Canadian Blood Services cohort—Whole blood from healthy eligible female and male donors was collected according to Canadian Blood Services (CBS) standard operating procedures. Leukocyte-reduced RBCs were suspended in approximately 110 mL saline adenine glucose mannitol (SAGM) additive solution for storage at 1–6° C for 42 days. Testing for percent storage hemolysis was performed at product expiry (day 43). Total hemoglobin (Hb) and hematocrit (HCT) were measured using an Advia 120 Hematology System (Siemens AG, Erlangen, Germany). Supernatant Hb was measured photometrically at 570 nm and 880 nm (Plasma/Low Hb Photometer, HemoCue, Ängelholm, Sweden) against tri-level hemoglobin controls (R&D Plasma Hemoglobin Hematology Controls, R&D Systems, Minneapolis, MN). Percent hemolysis was then calculated as described previously.[22]

United States ITxM Blood Bank cohort—Non leukocyte-reduced RBC units (n=77 females and 70 males) were stored (1–6° C) for 47±5 days in additive solution formula 3 (AS-3). Percent storage hemolysis was determined by comparing the supernatant Hb to total Hb concentrations and correcting for the hematocrit level of each sample. RBC unit hematocrit was measured by collecting blood samples into capillary tubes (Clay Adams, BD, NJ), which were centrifuged in a micro-hematocrit centrifuge (LW Scientific, Lawrenceville, GA), and analyzed on a digital hematocrit reader (Iris, Westwood, MA). Hb micromolar concentrations were determined using the Drabkin's method.[23]

RBC hemolytic assays

We evaluated predisposition to hemolysis by subjecting washed RBCs from human or mouse to selected stress assays including osmotic fragility, mechanical fragility, and oxidative hemolysis using 2,2′-azobis-2-methyl-propanimidamide, dihydrochloride (AAPH) or diamide, as described before[24] and detailed in the Supplemental Methods.

Experimental mice

All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pittsburgh or at Bloodworks NW. For the hemolytic propensity survey, age-matched (12–16 week old) female and male mice (n=3–5) from selected 22 strains (Supplementary table S1) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). RBCs isolated from sacrificed animals were subjected to hemolytic assays as detailed in the Supplemental Methods.

For the gonadectomy studies, 4-week old FVB/NJ pups underwent orchiectomy or ovariectomy and housed with age-matched intact males or females for 14–16 weeks, after which all animals were sacrificed, and washed RBCs were subjected to hemolytic assays as described in the Supplemental Methods. Testosterone repletion was performed by subcutaneous insertion of osmotic pumps (Alzet 2004, Alzet Cupertino, CA) into orchiectomy FVB/NJ mice. One orchiectomy group was treated with pumps containing testosterone at 1 mg/Kg body weight/day (Orch+T). The second group was treated with propylene glycol (Orch Sham; vehicle control). Intact males served as reference controls were also treated with propylene glycol. All mice were housed for 32 days, after which blood was collected for complete blood count, and hemolytic assays as described in the Supplemental Methods.

Post-transfusion recovery of stored mouse RBCs

We tested the effect of sex or orchiectomy on post-transfusion recovery of leukocyte-reduced stored RBCs using tractable transfusion mouse models.[25,26] Sex differences in post-transfusion RBC recovery were assessed in two strains (C57BL/6 and FVB/NJ) as described before[25] and in the Supplemental Methods for Figure 4. The effect of orchiectomy on RBC post-transfusion recovery was assessed by storage of RBCs from orchiectomy or intact FVB/NJ males and transfusion into males of the same strain, as described under the Supplemental Methods for figure 5G–H.

Evaluation of hemolytic component in SCD patients

We studied sex differences in hemolytic component using the University of Pittsburgh's Walk-PHASST cohort database (Clinical trial identifier # NCT00492531), which included 154 male and 161 female consented patients (hemoglobin SS, age 12 and over). We calculated a hemolytic component derived from each patient's lactate dehydrogenase (LDH), aspartate aminotransferase, reticulocyte percentage, and total bilirubin values that have been validated as a measure of the steady state rate of intravascular hemolysis.[6,27] The study protocol was approved by the University of Pittsburgh's Institutional Review Boards and ethic committees.

Statistical analyses

Sex differences in hemolysis were analyzed by unpaired *t* tests, whereas one-way or two-way ANOVA and Holm-Sidak's multiple comparison tests were used to examine the differences among multiple groups. Probabilities less than 0.05 were considered significant. Statistical analyses were performed using commercial software (GraphPad Prism version 6, GraphPad Software. CA, USA).

RESULTS

Sex differences in storage hemolysis are intrinsic to the RBC

Prior studies have suggested that plasma factors could account for sex differences in mechanical hemolysis of stored RBCs.[18,19] To evaluate this, we tested AS-3 RBC units (ITxM, Pittsburgh) for storage and stress-induced hemolysis (Figure 1). In support of our hypothesis, we found that male RBCs exhibited significantly higher levels of end-of-storage (47±5 days) hemolysis compared with females (Figure 1A; 0.96±0.68 % vs. 0.75±0.52 %, respectively, p=0.035). Furthermore, washed RBCs from male donors hemolyzed more than female RBCs in response to osmotic stress (42.8±12.9 % vs. 32.5±11.1 %, respectively, p<0.0001, Figure 1B) or mechanical stress (MFI= 11.1±2.2 vs. 10.1±2.1, respectively, p=0.042, Figure 1C). These data using washed RBCs (additive solution and plasma free) suggested a sex effect that is intrinsic to the RBC, and may relate to inherited differences in membrane integrity.

Sex differences in storage hemolysis are greatest during donor's reproductive years

We confirmed our findings by testing for sex differences in hemolysis using 42-day stored SAGM units (n=28,543) from Canada. A density distribution of storage hemolysis by sex (Figure 2A) revealed a right shift in the males' histogram, which reflects the overall increased levels of hemolysis in male RBC units. To test the hypothesis that sex hormones mediate sex differences in predisposition to hemolysis, we stratified the storage hemolysis data by age and sex (Figure 2B). Both parameters had a significant effect on storage hemolysis demonstrated by increased levels of hemolysis in RBCs from older male or female donors, whereas male RBCs hemolyzed more than female RBCs (P<0.0001) at all tested age groups. The largest differences in storage hemolysis were observed between the ages of 30 to 59. Furthermore, we observed sex differences in the effect of donor's age on storage hemolysis. RBCs from male donors over 30 years old exhibited significantly (p<0.0001) higher levels of hemolysis as compared with younger male donors ages 20–24, whereas significant differences between younger and older female donors were observed at the age of 45 or older.

Male sex determines increased susceptibility to hemolysis in mice

We further examined the effect of sex on predisposition to hemolysis using a systematic survey of 22 mouse strains that represents a wide genetic diversity (Supplementary table S1). Under the tested conditions, predisposition to hemolysis was associated with strain and sex (Figure 3 top panels), where male RBCs from the majority of the tested strains exhibited higher susceptibility to hemolysis in response to osmotic stress (17/22 strains, Figure 3A), and AAPH-induced oxidative stress (19/22 strains, Figure 3B). Furthermore, certain strains including FVB/NJ have demonstrated remarkable sex differences in hemolytic response to both stress conditions. Based on these observations, we have chosen the FVB/NJ strain for our subsequent studies.

Increased stress hemolysis predicts poor post-transfusion RBC recovery in male and female mouse strains

Following our observations of increased susceptibility of male RBCs to hemolysis *in vitro*, we investigated the effect of sex on post-transfusion recovery of stored RBCs in two mouse strains, C57BL/6J and FVB/NJ that represent low (C57BL/6J) or high (FVB/NJ) susceptibility to hemolysis.[28] Transfused RBCs from male mice of both strains exhibited poor post-transfusion recovery compared with female RBCs (Figure 4). In C57BL/6J (Figure 4D), female RBCs had 80 % 24h *in vivo* recovery versus 55% recovery of transfused male RBCs. FVB/NJ RBCs had generally lower levels of post-transfusion recovery compared with C57BL/6J, however, the sex effect was similar to that of C57BL/6J, with male RBCs exhibiting faster clearance from the circulation (Figure 4E).

Sustained in vivo physiological testosterone exposure accounts for increased stress hemolysis

In order to elucidate the effect of sex hormones on hemolytic propensity, human RBCs were stored in the presence of testosterone, progesterone, 17β-estradiol or dimethyl sulfoxide (DMSO, 0.1 %, vehicle control) at 20 nmol/L or 100 nmol/L. The lower concentration is within the physiological range of plasma testosterone in men, and progesterone in women. After 42-days of storage in SAGM, we observed no significant differences among the tested groups with regards to storage hemolysis, RBC microparticle formation, RBC phosphatidylserine exposure, and RBC elongation index (Supplementary figure S5.1). These data suggested that sex hormone exposure *in vitro* had little effect on RBC storage recovery.

As mature erythrocytes lack DNA, and are therefore indifferent to genomic actions of sex hormones, we tested the effect of gonadectomy on predisposition to hemolysis using FVB/NJ mice. RBCs from male, female, orchiectomized males (Orch-males), and ovariectomized females (OVX-females) were subjected to osmotic stress or oxidative hemolysis using AAPH or diamide. We initially investigated the effect of gonadectomy on RBC indices (Supplementary figure S5.2) and found no significant differences between females and ovariectomized females in terms of RBC count, hematocrit and hemoglobin levels, mean corpuscular volume (MCV), and red cell distribution width (RDW). Conversely, orchiectomy RBC indices including hemoglobin and hematocrit levels, were significantly ($p<0.05$) lower than that of males, and were comparable to that of ovariectomized and intact females.

With regards to hemolysis, ovariectomy had no significant effect on osmotic hemolysis (Figure 5A) or AAPH-induced oxidative hemolysis (Figure 5B) as compared with intact females. Conversely, we found significant differences between the male groups, where orchiectomy was associated with increased resistance to osmotic hemolysis (51.6±18.9% versus 67.4±20.5%, p=0.0345), and AAPH-induced oxidative hemolysis (61.1±23.5% versus 81.7±6.2%, p=0.0339, orchiectomy versus males, respectively). Consistent with a mechanism mediated by endogenous testosterone, the hemolysis levels found in orchiectomy males were similar to that of intact females or ovariectomy females (Figure 5A +B). Additionally, we found that orchiectomy modulates hemolytic response to diamide (Figure 5C), an oxidizing agent shown to compromise membrane integrity via activation of

spleen tyrosine kinase (Syk) leading to membrane protein Band 3 phosphorylation and aggregation, microparticle formation, and hemolysis.[29] Incubation of FVB/NJ RBCs with diamide revealed significant sex differences in hemolysis (70.9±4.3 % in males versus 44.3±18.1 % in females, p=0.0011). More importantly, RBCs from orchiectomy males exhibited significantly lower levels of diamide-induced hemolysis (55.1±12.8 %, p=0.0116) compared with intact males. There were no significant differences (p=0.1004) in diamide-induced hemolysis between orchiectomy males and females.

To further verify the requirement for testosterone on predisposition to hemolysis, orchiectomized FVB/NJ mice underwent testosterone repletion treatment (Orch+T; 1 mg/Kg bodyweight/day) or sham treatment (Orch Sham; propylene glycol) for 32 days. Intact males treated with propylene glycol were used as reference control. As predicted, testosterone repletion therapy significantly increased the levels of RBC count (p=0.0179), hemoglobin (p=0.0449), and hematocrit (p=0.0042) in Orch+T mice compared with Orch Sham (Supplementary figure S5.3). Remarkably, testosterone repletion increased RBC susceptibility to osmotic hemolysis (Figure 5D, 32.9±3.6 % in Orch+T versus 27.6±3.2 % in Orch Sham; p=0.0132), and AAPH-induced oxidative hemolysis (Figure 5E–F, 38.3±9.6 % in Orch+T versus 20.3±15.9 % in Orch Sham; p=0.034). In both assays (Figure 5D–F), Orch +T RBCs exhibited similar levels of hemolysis to that of male controls.

We have further confirmed our findings by assessing the post-transfusion recovery of stored RBCs from orchiectomy or intact FVB/NJ males. Leukocyte-reduced RBCs pooled from 17–20 mice of each group were stored (1–6 °C) in CPDA-1 for one or six days, and later transfused into recipient hosts (FVB/NJ males; n=7). After one day of storage, male RBCs exhibited enhanced levels of degradation compared with orchiectomy RBCs manifested by increased levels of storage and osmotic hemolysis, and enhanced (p<0.0001) 24 h clearance from the circulation (Figure 5G and Supplemental Table S2). After six days of storage, the 2 h and 24 h post-transfusion recoveries of male RBCs were significantly (p<0.0001) lower than that of orchiectomy RBCs, and measured 39.0±6.8 % versus 75.3±8.9 % at 24 h (Figure 5H and Supplemental Table S2).

Sex differences in predisposition to hemolysis are observed in patients with sickle cell disease

Male sex has been associated with increased risk of early death in SCD.[17] We hypothesized that male patients would demonstrate increased susceptibility to hemolysis. To test this hypothesis, we studied sex differences in the hemolytic component of 315 homozygous hemoglobin SS patients from the Walk-PHASST cohort[6] (Table 1). Similar to our observations in banked RBCs and mice, the hemolytic score of male patients was significantly higher than that of female patients (0.21 in men versus −0.11 in women; p=0.001). To further support findings, RBCs from male or female sickle-cell trait (SCT) mice (Berkley, hemizygous) expressing normal and sickle human hemoglobin (HbA/S) were subjected to selected hemolytic assays (Figure 6). Sickle cell disease is associated with abnormal resistance of sickle erythrocytes to osmotic hemolysis.[30] Despite the relatively low levels of osmotic hemolysis observed in male and female SCT RBCs (Figure 6A), hemolysis levels in males were significantly (p=0.02) higher than that of females. Male SCT RBCs also

exhibited increased levels of hemolysis in response to oxidative stress induced by AAPH (Figure 6B) and diamide (Figure 6C+D).

DISCUSSION

We have found in two unrelated cohorts of RBC donors from Canada and the US that sex is a significant modulator of RBCs hemolysis. Judged by end of storage percent hemolysis (Figures 1A and 2), male RBCs demonstrate worse recovery outcomes compared with female RBCs. This observation was consistent in both cohorts despite the fundamental differences in manufacturing procedures or the type of additive solution used for RBC storage. Furthermore, we found that sex differences in storage hemolysis appear to be greatest during donors' reproductive years (Figure 2B), which indicated the possible involvement of sex hormones in modulating hemolytic propensity. Above all, elimination of plasma or additive solution did not alter our observation of increased susceptibility to stress hemolysis in male donors (Figures 1(B+C) and 3).

In addition to donor's sex, our data portrayed in Figure 2B suggest that age is a confounding factor that is associated with increased levels of storage hemolysis in older male and female donors. This observation seen in both sexes may not be related to the effect of sex hormones, and may explain the higher rates of storage hemolysis found in older male donors (≥30 years of age) as compared with younger male donors (20–24 years of age) with presumably similar or higher levels of endogenous testosterone. The mechanisms by which aging modulates RBC susceptibility to cold storage are yet to be characterized, and may be related to age differences in erythrocyte characteristics, such as cell density or to the proportion of younger versus older RBCs in the donor's circulation.

Female blood on average hemolyzes 21% less than male blood (absolute reduction of mean value). Although it can be argued that the difference in storage hemolysis between male and female donors is minor (Figures 1A and 2), and may not be of clinical significance, our studies suggest that this observation is associated with impaired post-transfusion RBC recovery in two mouse strains at two extremes of hemolytic intensity (Figure 4), and also confirm measureable differences in *in vivo* hemolysis of male and female SCD patients. RBCs stored for more than 5 weeks exhibit similar increases in hemolysis and induce vasoconstriction in the human forearm circulation and pulmonary vasculature.[9] The effect of sex of as a risk factor for transfusion reactions is controversial. A recent study has suggested that cross-sex transfusions (female blood given to male patients) may increase the risk death by a mechanism related to alloimmunization.[31] Conversely, a retrospective study suggested that donor sex had no significant impact on 1-year survival of patients who required blood transfusion following cardiac surgery. Interestingly, this study has noted that female patients transfused with male RBCs were at higher risk of death (not significant), as compared with male patients receiving females' blood.[32]

Our hemolytic propensity survey in mice supports our observations in humans by demonstrating increased susceptibility of male RBCs to osmotic and oxidative stress in the majority of the tested strains (Figure 3). A few of our tested strains expressed no sex differences or reverse susceptibility to our hemolytic assays. This may have resulted from

strain-specific characteristics or pathologies that may have masked or reversed the sex differences in hemolysis. For example, in the NOD/ShiLtJ strain, we found that female RBCs hemolyzed more than males in response to osmotic stress (Figure 3A). This strain is commonly used for diabetes studies, where the onset and incidence of this disease is four times higher in females than males.[33]

In support of our *in vitro* observations, we have demonstrated sex differences in post-transfusion recovery of stored mouse RBCs from two strains with different genetic predispositions to hemolysis (C57BL/6J and FVB/NJ; Figure 4), where stored male RBCs from each strain exhibited enhanced clearance from the recipient circulation compared with female RBCs. One limitation of this observation is the sex of the recipient mice, which were all females. The effect of cross-sex transfusions on RBC recovery *in vivo* is not clear and therefore requires further investigation. Interestingly, the sex differences in hemolysis may not be unique to mammals, as a few studies have reported this phenomenon in lower vertebrates such as birds, which have nucleated RBCs, and where male RBCs exhibited higher susceptibility to osmotic or AAPH-induced oxidative hemolysis.[34,35] Taken together, our findings suggest a sex effect that is intrinsic to the erythrocyte and is conserved in mammalian evolution.

RBC storage with sex hormones added *in vitro* had little effect on RBC recovery, except for increased levels of phosphatidylserine exposure in RBCs treated with testosterone at the supra-physiological level of 100 nmol/L (Supplementary Figure S5.1C). This trend, however, was not statistically different from the other treatments. To our knowledge, progesterone is the only sex hormone whose effect on RBC storage hemolysis had been studied[36], where progesterone-treated RBCs exhibited better maintenance of ATP levels, and lower levels of storage or osmotic hemolysis. Other studies have supported the hypothesis that female sex hormones may improve membrane fluidity[21,37], which in the case of enucleated RBCs, are likely to be mediated via non-genomic mechanisms.

Contrary to the aforementioned studies, our ovariectomy data suggest that female sex hormones have little effect on RBC predisposition to osmotic or oxidative stress (Figure 5A–B). Conversely, orchiectomy not only protected against these stress conditions, but also improved RBC storage stability and post-transfusion RBC recovery (Figure 5H). The protective effect of orchiectomy against diamide-induced hemolysis (Figure 5C) may be linked to sex differences in molecular pathways, which involve Syk activation and Band 3 phosphorylation under hemolytic stress. Our testosterone repletion study (Figure 5D–F) in orchiectomized mice strongly suggests that testosterone renders male erythrocytes more susceptible to osmotic and oxidative hemolysis. The direct mechanisms by which testosterone modulates predisposition to hemolysis are yet to be resolved, however, our current data suggest that such mechanisms are likely to occur during erythropoiesis rather than affecting differentiated erythrocytes. It should be emphasized that our conclusions are based on mouse studies, and additional mechanisms may contribute to sex differences in hemolysis in human and mice.

The action of testosterone on erythropoiesis in males may be reflected by changes in blood characteristics that may affect erythrocyte rheology and susceptibility to hemolysis under

stress. Our evaluation of erythrocyte indices in orchiectomized mice (Supplementary figure S5.2) suggests that the absent of androgen resulted in RBCs with ‘female-like’ characteristics, which could be reversed by testosterone repletion therapy (Supplementary figure S5.3). A comparable study suggested that long-term high-dose administration of testosterone to orchiectomized C57BL/6J mice has no adverse effects on blood viscosity or RBC rheology; however, there was no report in regard to the effect of these treatments on hemolysis.[38]

Testosterone has been shown to exacerbate ischemia-reperfusion injury in the heart[14] or kidney[16], where the rate of injury in mice was mitigated by orchiectomy while aggravated in females infused with testosterone. In the case of SCD, testosterone may increase the risk of hemolysis and cell-free hemoglobin-associated morbidities in male patients. Likewise, the use of testosterone replacement therapies in various conditions including hypogonadism, low testosterone levels in older men or female-to-male sex change may have negative implications on RBC integrity, therefore exposing such patients to increased risk of hemolysis.

In conclusion, we have demonstrated sex differences in predisposition to hemolysis. Based on our findings, we propose that the sex effect is intrinsic to the erythrocyte, involves membrane function, and is highly conserved in mammalian evolution. Testosterone promotes sex differences in hemolysis in a mechanism likely to occur during erythropoiesis, which ultimately modulates the fragility and rheological properties of the differentiated male erythrocyte. Our findings support the growing recognition of sex as a biological variable that needs to be taken into consideration in cellular therapeutics, and in studies of human hemolytic diseases, such as sickle cell and malaria.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Dr. Gladwin receives research support from NIH grants 2R01HL098032, 1R01HL125886-01, and P01HL103455, the Institute for Transfusion Medicine and the Hemophilia Center of Western Pennsylvania. Dr. Kanias and Dr. Gladwin wish to acknowledge Dr. Darrell J. Triulzi, Dr. Mark H. Yazer, and the Institute for Transfusion Medicine for supporting this study and providing RBC units for research. They also wish to thank Mariana Hildesheim for her assistance with statistical analysis of the data presented in Figure 2. Dr. Acker would like to thank Canadian Blood Services and Health Canada for providing financial support; Tracey Turner, Adele Hansen, and Jayme Kurach at the Canadian Blood Services Centre for Innovation, Edmonton, AB, for their assistance with this study.

References

1. Donadee C, Raat NJ, Kanias T, Tejero J, Lee JS, Kelley EE, Zhao X, Liu C, Reynolds H, Azarov I, Frizzell S, Meyer EM, Donnenberg AD, Qu L, Triulzi D, Kim-Shapiro DB, Gladwin MT. Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. Circulation. 2011; 124:465–76. [PubMed: 21747051]
2. Alexander JT, El-Ali AM, Newman JL, Karatela S, Predmore BL, Lefer DJ, Sutliff RL, Roback JD. Red blood cells stored for increasing periods produce progressive impairments in nitric oxide-mediated vasodilation. Transfusion. 2013; 53:2619–28. [PubMed: 23480490]

3. Roback JD, Neuman RB, Quyyumi A, Sutliff R. Insufficient nitric oxide bioavailability: a hypothesis to explain adverse effects of red blood cell transfusion. Transfusion. 2011; 51:859–66. [PubMed: 21496047]

4. Wither M, Dzieciatkowska M, Nemkov T, Strop P, D'Alessandro A, Hansen KC. Hemoglobin oxidation at functional amino acid residues during routine storage of red blood cells. Transfusion. 2016; 56:421–6. [PubMed: 26426339]

5. Gladwin MT, Kanias T, Kim-Shapiro DB. Hemolysis and cell-free hemoglobin drive an intrinsic mechanism for human disease. J Clin Invest. 2012; 122:1205–8. [PubMed: 22446184]

6. Nouraie M, Lee JS, Zhang Y, Kanias T, Zhao X, Xiong Z, Oriss TB, Zeng Q, Kato GJ, Gibbs JS, Hildesheim ME, Sachdev V, Barst RJ, Machado RF, Hassell KL, Little JA, Schraufnagel DE, Krishnamurti L, Novelli E, Girgis RE, Morris CR, Rosenzweig EB, Badesch DB, Lanzkron S, Castro OL, Goldsmith JC, Gordeuk VR, Gladwin MT. Walk PI, Patients. The relationship between the severity of hemolysis, clinical manifestations and risk of death in 415 patients with sickle cell anemia in the US and Europe. Haematologica. 2013; 98:464–72. [PubMed: 22983573]

7. Atichartakarn V, Chuncharunee S, Archararit N, Udomsubpayakul U, Aryurachai K. Intravascular hemolysis, vascular endothelial cell activation and thrombophilia in splenectomized patients with hemoglobin E/beta-thalassemia disease. Acta Haematol. 2014; 132:100–7. [PubMed: 24525823]

8. Berra L, Pinciroli R, Stowell CP, Wang L, Yu B, Fernandez BO, Feelisch M, Mietto C, Hod EA, Chipman D, Scherrer-Crosbie M, Bloch KD, Zapol WM. Autologous transfusion of stored red blood cells increases pulmonary artery pressure. Am J Respir Crit Care Med. 2014; 190:800–7. [PubMed: 25162920]

9. Risbano MG, Kanias T, Triulzi D, Donadee C, Barge S, Badlam J, Jain S, Belanger AM, Kim-Shapiro DB, Gladwin MT. Effects of Aged Stored Autologous Red Blood Cells on Human Endothelial Function. Am J Respir Crit Care Med. 2015; 192:1223–33. [PubMed: 26222884]

10. Steiner ME, Ness PM, Assmann SF, Triulzi DJ, Sloan SR, Delaney M, Granger S, Bennett-Guerrero E, Blajchman MA, Scavo V, Carson JL, Levy JH, Whitman G, D'Andrea P, Pulkrabek S, Ortel TL, Bornikova L, Raife T, Puca KE, Kaufman RM, Nuttall GA, Young PP, Youssef S, Engelman R, Greilich PE, Miles R, Josephson CD, Bracey A, Cooke R, McCullough J, Hunsaker R, Uhl L, McFarland JG, Park Y, Cushing MM, Klodell CT, Karanam R, Roberts PR, Dyke C, Hod EA, Stowell CP. Effects of red-cell storage duration on patients undergoing cardiac surgery. N Engl J Med. 2015; 372:1419–29. [PubMed: 25853746]

11. Lacroix J, Hebert PC, Fergusson DA, Tinmouth A, Cook DJ, Marshall JC, Clayton L, McIntyre L, Callum J, Turgeon AF, Blajchman MA, Walsh TS, Stanworth SJ, Campbell H, Capellier G, Tiberghien P, Bardiaux L, van de Watering L, van der Meer NJ, Sabri E, Vo D. Investigators A, Canadian Critical Care Trials G. Age of transfused blood in critically ill adults. N Engl J Med. 2015; 372:1410–8. [PubMed: 25853745]

12. Jordan A, Chen D, Yi QL, Kanias T, Gladwin MT, Acker JP. Assessing the influence of component processing and donor characteristics on quality of red cell concentrates using quality control data. Vox Sang. 2016 In Press.

13. Kanias T, Gladwin MT. Nitric oxide, hemolysis, and the red blood cell storage lesion: interactions between transfusion, donor, and recipient. Transfusion. 2012; 52:1388–92. [PubMed: 22780890]

14. Crisostomo PR, Wang M, Wairiuko GM, Morrell ED, Meldrum DR. Brief exposure to exogenous testosterone increases death signaling and adversely affects myocardial function after ischemia. Am J Physiol Regul Integr Comp Physiol. 2006; 290:R1168–74. [PubMed: 16439666]

15. Wang M, Baker L, Tsai BM, Meldrum KK, Meldrum DR. Sex differences in the myocardial inflammatory response to ischemia-reperfusion injury. Am J Physiol Endocrinol Metab. 2005; 288:E321–6. [PubMed: 15367393]

16. Park KM, Kim JI, Ahn Y, Bonventre AJ, Bonventre JV. Testosterone is responsible for enhanced susceptibility of males to ischemic renal injury. J Biol Chem. 2004; 279:52282–92. [PubMed: 15358759]

17. Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, Klug PP. Mortality in sickle cell disease. Life expectancy and risk factors for early death. N Engl J Med. 1994; 330:1639–44. [PubMed: 7993409]

18. Raval JS, Waters JH, Seltsam A, Scharberg EA, Richter E, Kameneva MV, Yazer MH. Menopausal status affects the susceptibility of stored RBCs to mechanical stress. Vox Sang. 2011; 100:418–21. [PubMed: 21488881]
19. Kameneva MV, Antaki JF, Yeleswarapu KK, Watach MJ, Griffith BP, Borovetz HS. Plasma protective effect on red blood cells exposed to mechanical stress. ASAIO J. 1997; 43:M571–5. [PubMed: 9360109]
20. Kameneva MV, Watach MJ, Borovetz HS. Gender difference in rheologic properties of blood and risk of cardiovascular diseases. Clin Hemorheol Microcirc. 1999; 21:357–63. [PubMed: 10711771]
21. Kameneva MV, Watach MJ, Borovetz HS. Rheologic dissimilarities in female and male blood: potential link to development of cardiovascular diseases. Adv Exp Med Biol. 2003; 530:689–96. [PubMed: 14562767]
22. Acker JP, Hansen AL, Kurach JD, Turner TR, Croteau I, Jenkins C. A quality monitoring program for red blood cell components: in vitro quality indicators before and after implementation of semiautomated processing. Transfusion. 2014; 54:2534–43. [PubMed: 24805193]
23. Zwart A, van Assendelft OW, Bull BS, England JM, Lewis SM, Zijlstra WG. Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1995) and specifications for international haemiglobinocyanide standard (4th edition). J Clin Pathol. 1996; 49:271–4. [PubMed: 8655699]
24. Kanias T, Wang L, Lippert A, Kim-Shapiro DB, Gladwin MT. Red blood cell endothelial nitric oxide synthase does not modulate red blood cell storage hemolysis. Transfusion. 2013; 53:981–9. [PubMed: 22897637]
25. Gilson CR, Kraus TS, Hod EA, Hendrickson JE, Spitalnik SL, Hillyer CD, Shaz BH, Zimring JC. A novel mouse model of red blood cell storage and posttransfusion in vivo survival. Transfusion. 2009; 49:1546–53. [PubMed: 19573176]
26. Hod EA, Zhang N, Sokol SA, Wojczyk BS, Francis RO, Ansaldi D, Francis KP, Della-Latta P, Whittier S, Sheth S, Hendrickson JE, Zimring JC, Brittenham GM, Spitalnik SL. Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. Blood. 2010; 115:4284–92. [PubMed: 20299509]
27. Milton JN, Rooks H, Drasar E, McCabe EL, Baldwin CT, Melista E, Gordeuk VR, Nouraie M, Kato GR, Minniti C, Taylor J, Campbell A, Luchtman-Jones L, Rana S, Castro O, Zhang Y, Thein SL, Sebastiani P, Gladwin MT, Walk PI, Steinberg MH. Genetic determinants of haemolysis in sickle cell anaemia. Br J Haematol. 2013; 161:270–8. [PubMed: 23406172]
28. Zimring JC, Smith N, Stowell SR, Johnsen JM, Bell LN, Francis RO, Hod EA, Hendrickson JE, Roback JD, Spitalnik SL. Strain-specific red blood cell storage, metabolism, and eicosanoid generation in a mouse model. Transfusion. 2014; 54:137–48. [PubMed: 23721209]
29. Pantaleo A, Ferru E, Carta F, Mannu F, Simula LF, Khadjavi A, Pippia P, Turrini F. Irreversible AE1 tyrosine phosphorylation leads to membrane vesiculation in G6PD deficient red cells. PLoS One. 2011; 6:e15847. [PubMed: 21246053]
30. Pászty C, Brion CM, Manci E, Witkowska HE, Stevens ME, Mohandas N, Rubin EM. Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease. Science. 1997; 278:876–8. [PubMed: 9346488]
31. Middelburg RA, Briet E, van der Bom JG. Mortality after transfusions, relation to donor sex. Vox Sang. 2011; 101:221–9. [PubMed: 21477152]
32. Desmarets M, Bardiaux L, Benzenine E, Dussaucy A, Binda D, Tiberghien P, Quantin C, Monnet E. Effect of storage time and donor sex of transfused red blood cells on 1-year survival in patients undergoing cardiac surgery: an observational study. Transfusion. 2016; 56:1213–22. [PubMed: 26935140]
33. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y. Breeding of a non-obese, diabetic strain of mice. Jikken Dobutsu. 1980; 29:1–13. [PubMed: 6995140]
34. Alonso-Alvarez C, Bertrand S, Faivre B, Chastel O, Sorci G. Testosterone and oxidative stress: the oxidation handicap hypothesis. Proc Biol Sci. 2007; 274:819–25. [PubMed: 17251089]

35. March BE, Coates V, Biely J. The effects of estrogen and androgen on osmotic fragility and fatty acid composition of erythrocytes in the chicken. Can J Physiol Pharmacol. 1966; 44:379–87. [PubMed: 5914356]

36. DeVenuto F, Wilson SM. Distribution of progesterone and its effect on human blood during storage. Transfusion. 1976; 16:107–12. [PubMed: 1258111]

37. Whiting KP, Restall CJ, Brain PF. Steroid hormone-induced effects on membrane fluidity and their potential roles in non-genomic mechanisms. Life Sciences. 2000; 67:743–57. [PubMed: 10968404]

38. Guo W, Bachman E, Vogel J, Li M, Peng L, Pencina K, Serra C, Sandor NL, Jasuja R, Montano M. The effects of short-term and long-term testosterone supplementation on blood viscosity and erythrocyte deformability in healthy adult mice. Endocrinology. 2015; 156:1623–9. [PubMed: 25774550]

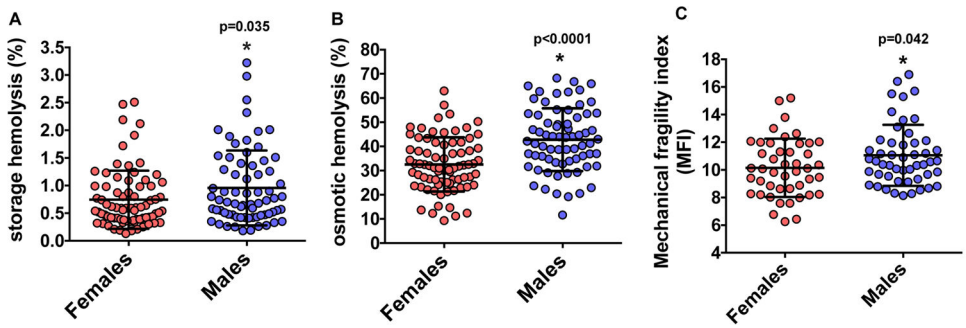


FIGURE 1. Male sex is associated with increased susceptibility to storage and stress-induced hemolysis

RBCs from male or female donors were collected from expired RBC units (ITxM, 47±5 days old) and tested for **A.** percent storage hemolysis (n=77 females and 70 men). **B.** Osmotic fragility (n=77 females and 70 men). **C.** Mechanical fragility (n=45 females and 50 men). A–C: Mean±SD, * $p<0.05$ by unpaired *t*-test.

Figure 2A

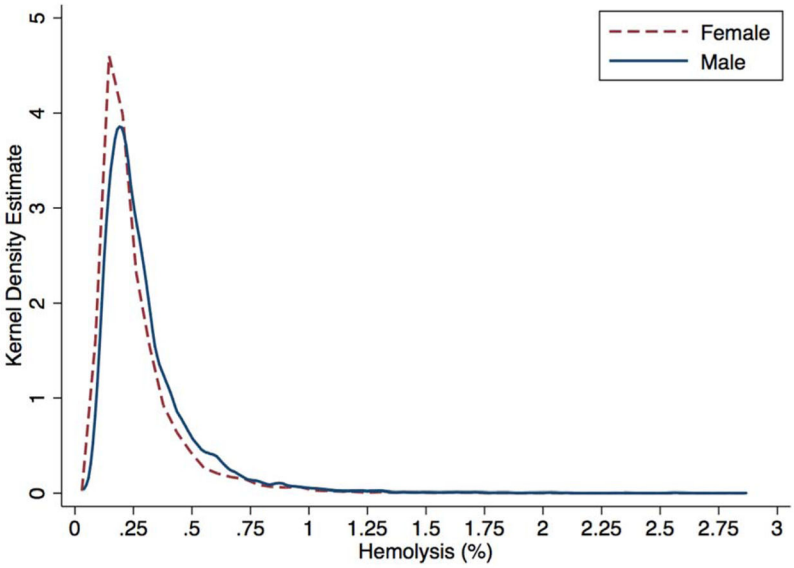


Figure 2B:

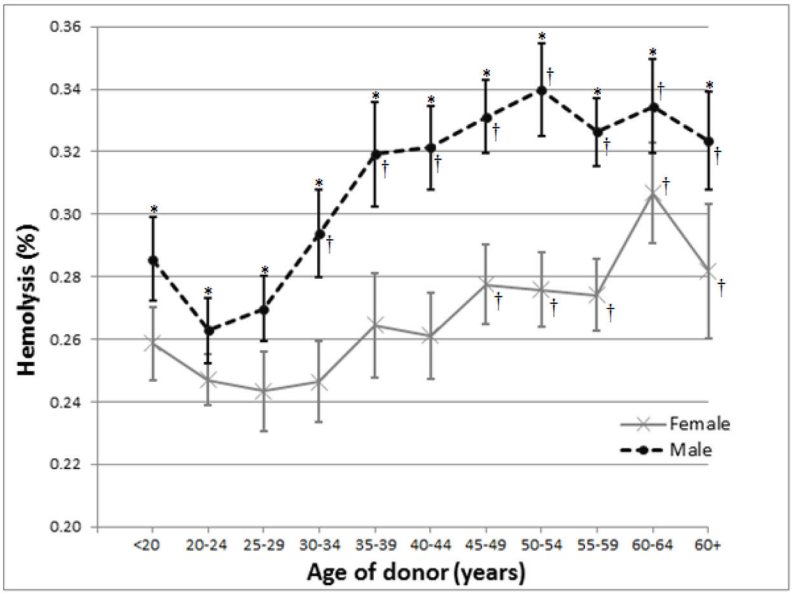
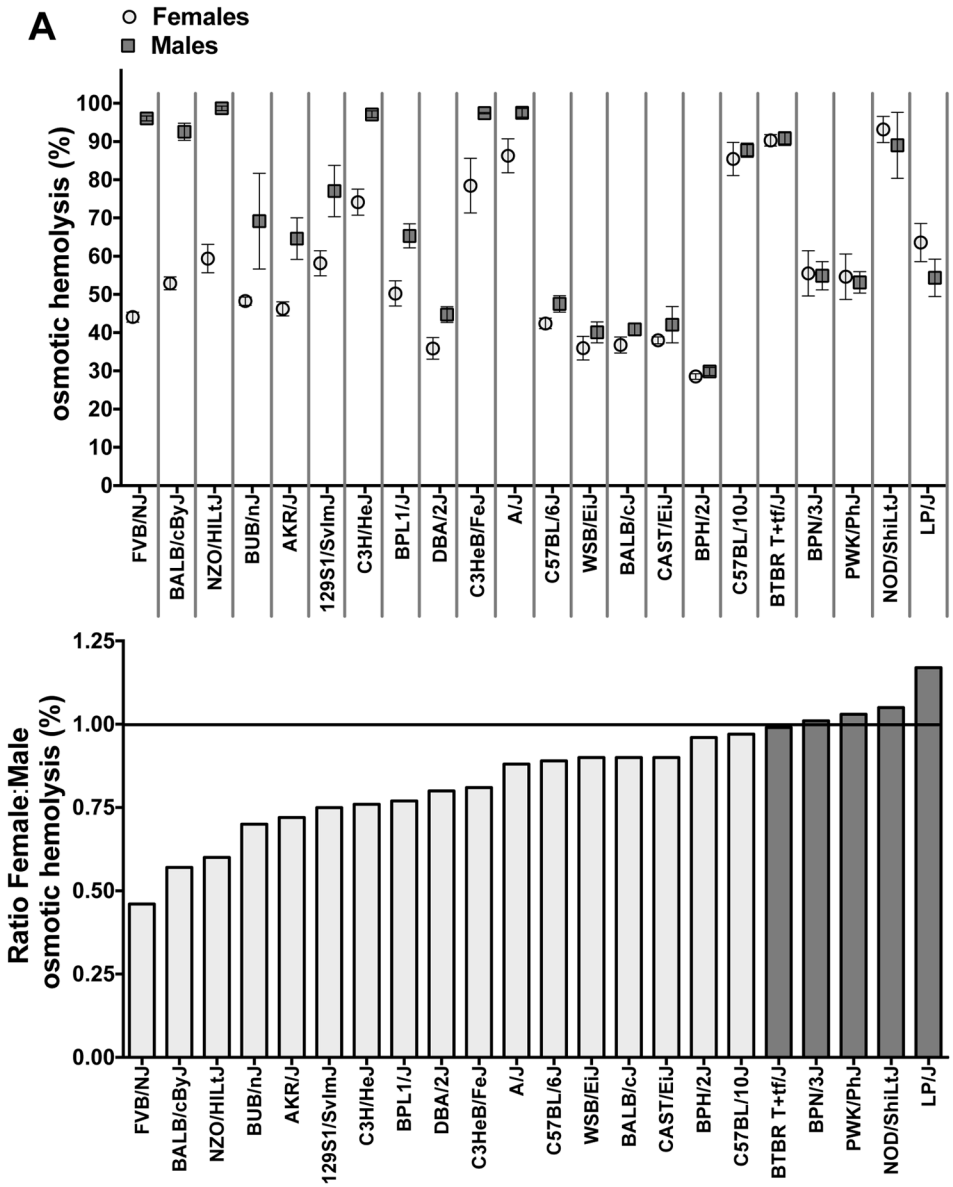


FIGURE 2. The effect of sex and age on end-of-storage hemolysis in SAGM units

Data derive from leukocyte-reduced SAGM RBC units tested within 24 h of product expiry (42 days of storage) by Canadian Blood Services. **A**. Kernel density estimate of percent storage hemolysis in male (solid blue line) versus female (dashed red line) blood donors. n= 28,543, 16,700 males and 11,843 females **B**. Distribution of storage hemolysis by sex and age (5-year intervals). Mean hemolysis values for male versus female donors are based on donor age at time of donation. Errors represent 95 % CI. n= 28,267, 16,636 males and 11,631 females. * Represents significant differences ($p<0.0001$ by one-way ANOVA) in

hemolysis between male and female donors within age range. † Represents age range at which hemolysis is significantly ($p<0.0001$ by one-way ANOVA and Games-Howell test analysis) different from donors of the same sex aged 20–24.



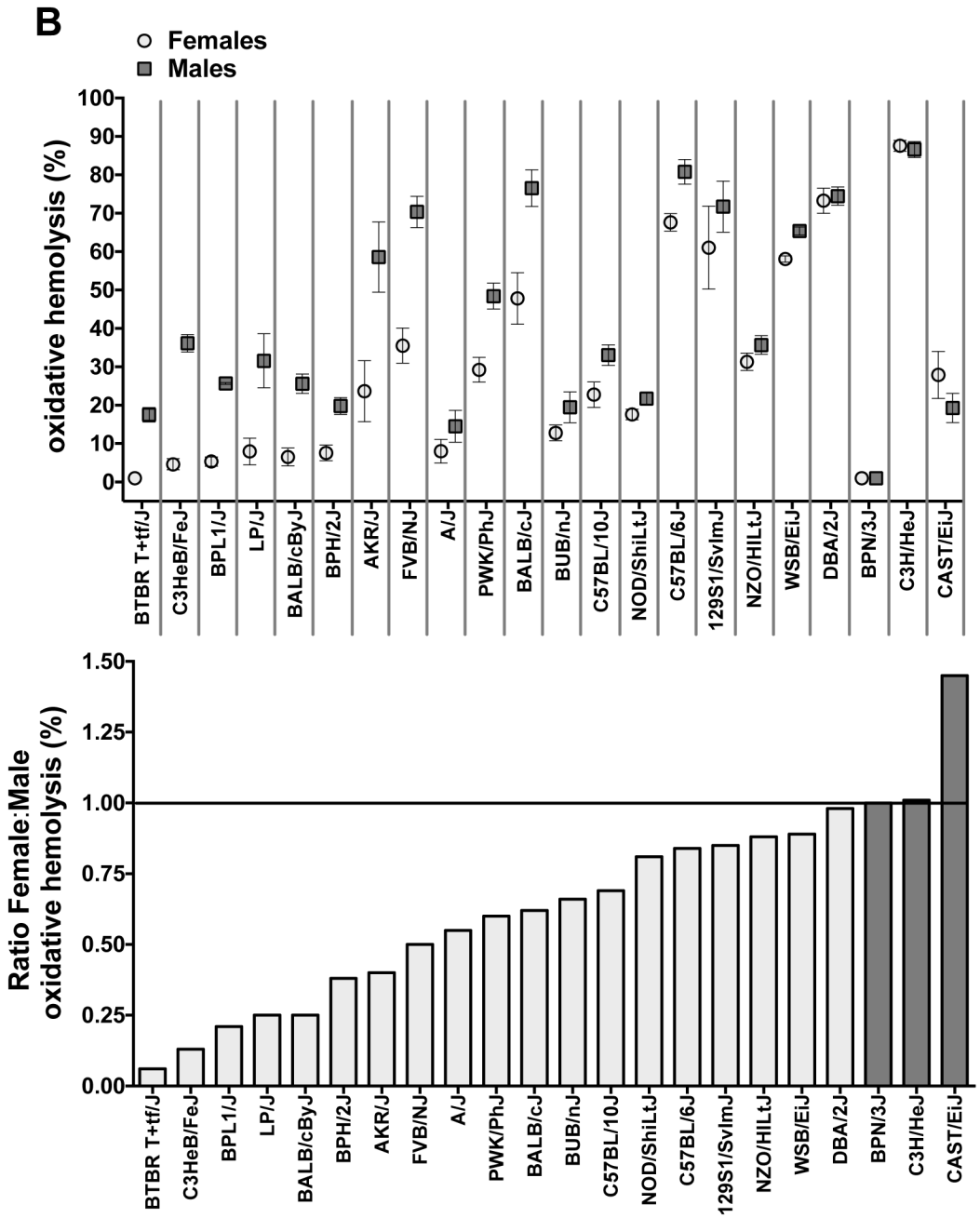


FIGURE 3. Sex differences in hemolytic propensity in 22 mouse strains

RBCs from age-matched male and female mice from 22 selected in-bred and wild-derived strains were subjected to **A.** Osmotic hemolysis (Pink test 24 h). **B.** AAPH-induced oxidative hemolysis (50 mmol/L, 3 h, 37° C). **A–B**. Top panels: Hemolytic scores are represented as Mean±SEM (n=3–5) in males versus females of each tested strain. Lower panels: Sex differences (data derived from top panels) are represented as the ratio of the females' score (mean percent hemolysis) divided by the males' score. A ratio score of "1" means no sex differences; X<1 means females hemolyzed less than males. Dark grey bars

indicate strains in which no sex differences were observed or males were more resistant to hemolysis than females.

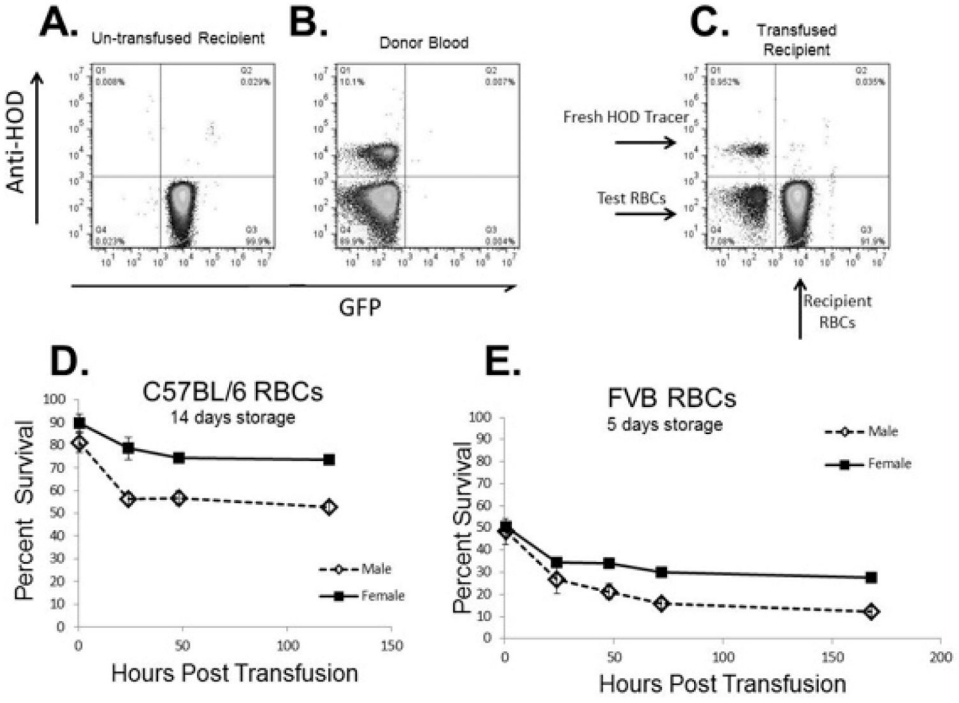


FIGURE 4. Sex differences in post-transfusion recovery of stored mouse RBCs

Mouse RBCs collected from male or female C57BL/6J or FVB/NJ strain were leukocyte reduced and stored (4° C) in CPDA-1 for 14 days or 5 days, respectively. Stored RBCs were transfused into C57BL/6J (UbiC-GFP) x FVB/NJ F1 female recipients, and post-transfusion recovery was assessed as described under Materials and Methods. **A–C.** Representative flow cytometric dot plots from **A.** Expression (Q3, 99.9 %) of green fluorescent protein (GFP) in recipient mice (C57BL/6J (UbiC-GFP) x FVB/NJ F1). **B.** Donor blood was gated as non-GFP/non-HOD positive RBCs (Q4, 89.9 %) whereas anti HOD-positive mouse RBCs, which were spiked into donors samples prior to transfusion to serve as a fresh tracer population, were gated in Q1. **C.** After transfusion, Test (donor) RBCs are visualized as HOD-negative GFP-negative RBCs, and tracer HOD RBCs are visualized as HOD-positive GFP-negative RBCs. Recipient RBCs are gated out as the only GFP-positive RBCs. **D–E.** Post-transfusion recovery of male versus female stored C57BL/6J (D) or FVB/NJ (E) RBCs. Each curve represents the average of 3 experiments.

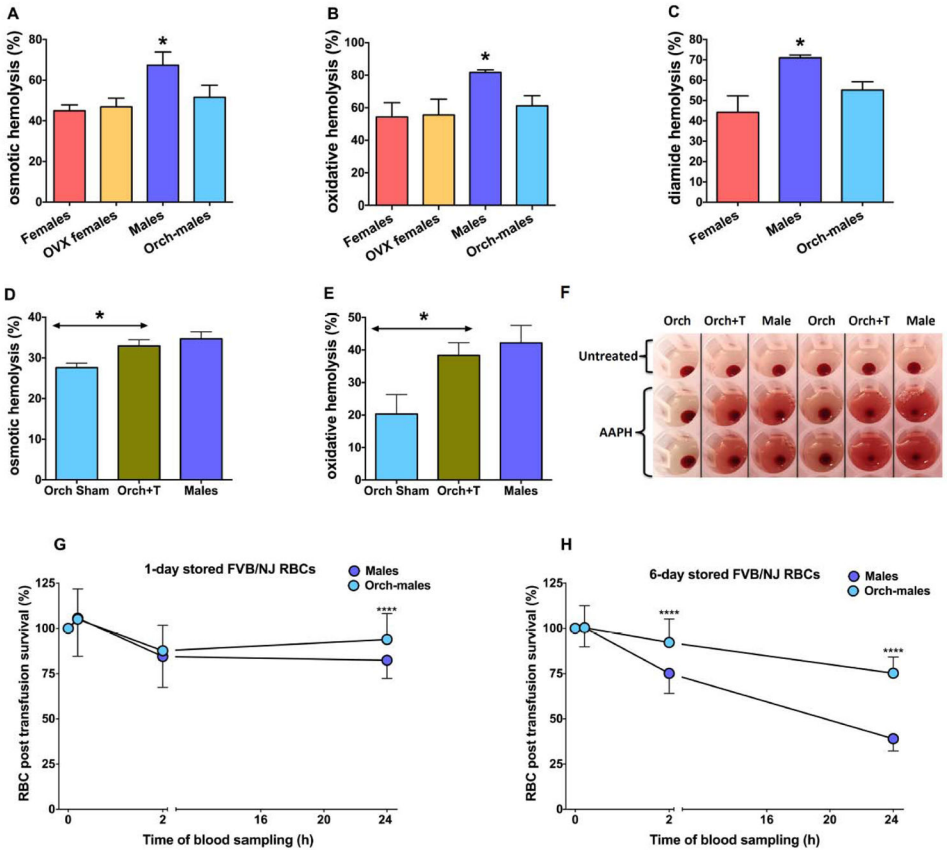


FIGURE 5. Effect of orchiectomy or testosterone repletion on predisposition to hemolysis and post-transfusion recovery in FVB/NJ RBCs

A–C. FVB/NJ pups underwent gonadectomy at 4 weeks and housed with age-matched intact males or females (n=10–15 per group) for 14–16 weeks, after which all animals were sacrificed. RBCs from each group were subjected to **A**. osmotic stress (24 h pink test), **B**. AAPH-induced oxidative hemolysis (50 mM, 3 h 37° C), **C**. RBCs from females (n=5), males (n=10), and orchiectomized males (n=10) were subjected to diamide-induced hemolysis (0.5 mM, 75 min). A–C: Mean±SEM. * designates statistically significant ($p<0.05$) differences obtained by one-way ANOVA and Holm-Sidak's multiple comparison test using males as a reference group. A. p=0.0149. B, p=0.0201. C, p=0.0008. **D–F.** Orchiectomy FVB/NJ mice (15–16 week old) were treated with testosterone (1 mg/Kg body weight/day; Orch+T) or with propylene glycol (Orch Sham, drug vehicle) for 32 days as described under Material and Methods. Age-matched intact FVB/NJ males treated with propylene glycol were used as a reference for the hemolytic assays. After treatments, RBCs were subjected to osmotic (D) and oxidative stress (E) assays. F. Representative image of AAPH-induced oxidative hemolysis (50 mmol/L, 2 h, 37° C) in RBCs from each mouse group. Mean±SEM; * designates statistical differences ($p<0.05$ unpaired *t*-test) between Orch+T and Orch Sham. D. p=0.0132; E. p=0.034. **G–H**. Percent post-transfusion recovery (Mean±SD) of leukocyte-reduced stored RBCs from orchiectomy or intact FVB/NJ males after one (G) or six (H) days of storage. **** $p<0.0001$ by repeated measures two-way ANOVA and Sidak's multiple comparisons test.

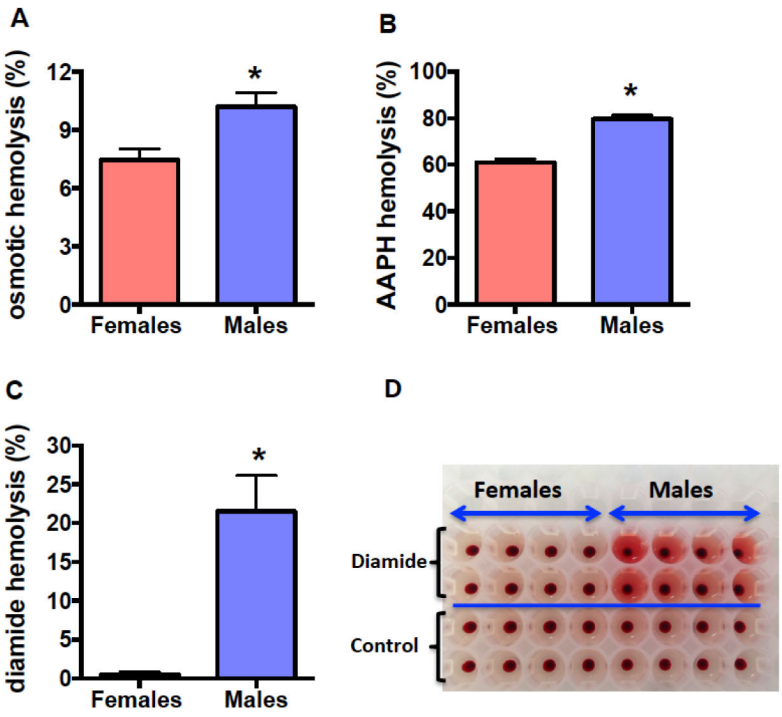


FIGURE 6. Sex differences in predisposition to hemolysis in sickle cell disease

Sex differences in BERK hemizygous (HbAS) sickle cell mice (Mean±SEM; n=4 per sex). **A**. Mean osmotic hemolysis (%) in response to 24 h Pink test. **B**. Mean oxidative hemolysis (%) in response to AAPH treatment (50 mmol/L, 3 h, 37° C). **C+D**. Mean percent hemolysis (C) and a corresponding image (D) in response to incubation with diamide (0.5 μmol/L, 90 min, 37° C). * designates significant differences ($p<0.05$, unpaired *t*-test) in hemolysis between males and females. A. $p= 0.0182$. B. $p<0.0001$, C. $p= 0.004$.

Table 1

Distribution of variables in the Walk-PHASST study

Results are represented as median and interquartile range.

	Male		Female		P value
	N	Results	N	Results	
Age	180	34 (26–44)	194	37 (28–47)	0.016
Hydroxyurea, n (%)	180	90 (50%)	194	89 (46%)	0.4
A-gene deletion, n (%)	163		180		0.7
Single deletion		49 (30%)		59 (33%)	
Double deletion		3 (2%)		5 (3%)	
Systolic blood pressure	180	120 (112–129)	193	115 (108–126)	0.1
Diastolic blood pressure	180	67 (60–73)	193	67 (61–74)	0.5
Oxygen saturation	178	96 (94–98)	192	97 (95–99)	<0.001
Hemoglobin	177	8.8 (7.6–10.0)	186	8.4 (7.5–9.4)	0.006
Hematocrit	177	25 (22–29)	187	24 (21–28)	0.045
WBC	177	9.5 (7.2–12.1)	187	9.0 (7.0–11.2)	0.3
Platelet	177	355 (265–441)	186	366 (293–471)	0.1
Hemoglobin F%	155	6 (3–14)	172	8 (5–15)	0.08
Abs. Reticulocyte count	172	267 (187–349)	177	245 (165–347)	0.1
LDH	162	434 (317–651)	177	397 (283–562)	0.022
AST	174	43 (32–59)	184	39 (28–54)	0.030
Total bilirubin	176	3.0 (2.0–4.5)	188	2.4 (1.6–3.9)	0.001
Hemolytic component	154	0.21 (−0.74–1.22)	161	−0.11 (−1.25–0.71)	0.001

	Male		Female		P value
	N	Results	N	Results	
ALT	177	22 (17–31)	188	22 (16–29)	0.6
Creatinine	178	0.8 (0.6–1.0)	189	0.6 (0.5–0.8)	0.022
Ferritin	160	195 (92–362)	174	265 (125–523)	0.028
Urine albumin	39	12.4 (1.8–70.3)	43	17.6 (5.0–44.1)	0.9
TRV	169	2.6 (2.3–2.8)	171	2.5 (2.3–2.7)	0.2