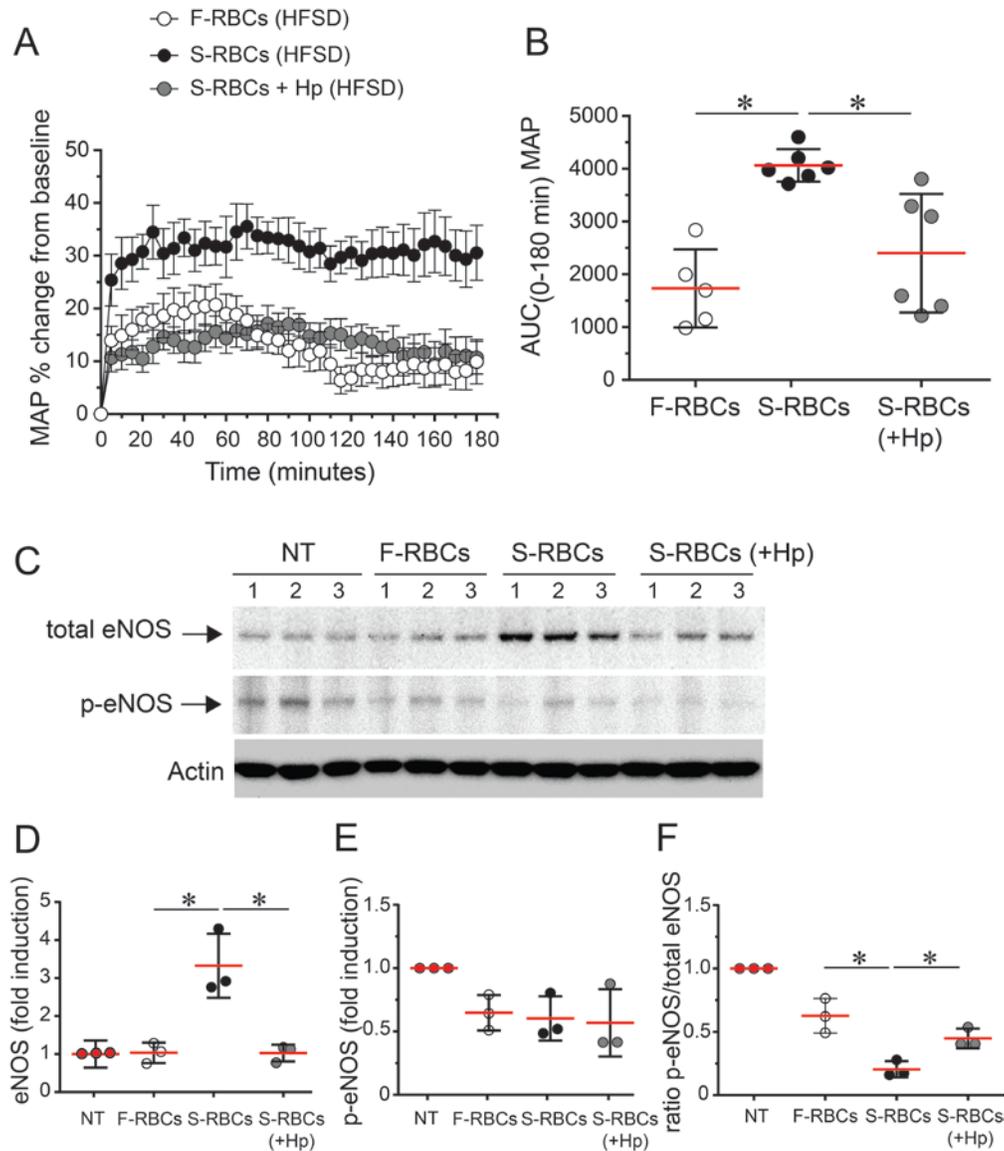


Supplemental Results

Guinea pig model of pre-existing vascular disease: Systemic blood pressure and eNOS response to fresh (F-RBCs) and stored (S-RBCs) RBC transfusion

In the present model HFSD guinea pigs with vascular/endothelial dysfunction were exchange transfused with 9 units of F-RBCs, S-RBCs and S-RBCs + Hp (300 mg/kg). Following F-RBC transfusion increased mean arterial pressure (MAP) to a maximum of 20% greater than baseline in the initial sixty minutes post transfusion then declined to an MAP 6% greater than baseline over the next two hours, consistent with exposure to Hb in the stored RBC preparation. Transfusion with S-RBCs increased MAP to 36% over basal levels and these pressures were maintained over the three hour monitoring period. Co-administration of S-RBCs with Hp maintained MAP between 10-15% greater than baseline over three hours post transfusion, similar to F-RBC (Supplemental Figure 1A). Areas under the MAP versus time curves demonstrated significant increases in MAP when comparing F-RBCs to S-RBCs ($P = 0.0080$, $n=6$) and S-RBCs to S-RBCs + Hp ($P=0.007$, $n=6$) (Figure 1B). Western blotting of eNOS and its active phosphorylated form (p-eNOS) (Figure 1C) at the end of the study demonstrated a significant increase in eNOS densitometry (Figure 1D) following S-RBC compared to F-RBCs ($P = 0.0012$, $n=3$), however the increase in eNOS was found to be an inactive non-phosphorylated form (Figure 1E). The ratio of p-eNOS/eNOS (Figure 1F) was significantly less following S-RBC transfusion compared to F-RBC transfusion ($P = 0.0013$, $n=3$). The response to S-RBC transfusion + 300 mg/kg Hp was equivalent to F-RBCs with regard to MAP, eNOS, p-eNOS and p-eNOS/eNOS ratio. These data are consistent with the MAP response in transfused diabetic mice (1), however, attenuation of S-RBC induced MAP response by Hp is inconsistent with diabetic mice dosed with premixed Hb and Hp (2). Differences in species/model may account for this observation or alternatively the higher doses of Hp, used here may be required.



Supplemental Figure 1: Guinea pig model of pre-existing vascular disease: Systemic blood pressure and eNOS response to fresh (F-RBCs) and stored (S-RBCs) RBC transfusion. (A) Blood pressure response to F-RBCs, S-RBCs and S-RBCs + Hp (300 mg/kg). (B) Shows a significantly increased MAP following S-RBCs compared to F-RBCs (n=6, P=0.008, ANOVA with a Multiple comparisons test). Hp co-infusion significantly reduced the S-RBC induced hypertensive response (n=6, P=0.0077, ANOVA with a Multiple comparisons test). (C) Shows the end of experiment western blotting of endothelial nitric oxide synthase (eNOS) and phosphorylated eNOS (active eNOS) obtained from the thoracic aorta (NT = not-transfused). Densitometry band intensity for eNOS protein is shown as fold induction over NT animals. (D) Shows a significant increase eNOS protein following F-RBC transfusion (n=6, P=0.0012, ANOVA with a Multiple comparisons test). Hp significantly reduced eNOS induction (n=6, P=0.0012, ANOVA with a Multiple comparisons test). (E) P-eNOS was unchanged across groups, however the ratio of active to total eNOS was significantly reduced following S-RBC transfusion (n=6, P=0.0013, ANOVA with a Multiple comparisons test). Hp co-transfusion restored the ratio to that of fresh blood (n=6, P=0.0312, ANOVA with a Multiple comparisons test). All data are presented as individual values with the mean ± SD.

Supplemental Materials and Methods

Materials

Antibodies - eNOS: Anti eNOS rabbit polyclonal antibody from Abcam (cat#: ab5589) and p-eNOS: Anti eNOS (phospho S1177) rabbit polyclonal antibody from Abcam (cat#: ab75639)

Animal surgeries

Animals allocated to study groups were dosed twelve hours prior to surgery with enrofloxacin (10 mg/kg) by the subcutaneous route. On days of surgery, guinea pigs were dosed subcutaneously with ketoprofen (5 mg/kg) for post recovery pain management and then anesthetized via the intraperitoneal route with a cocktail of ketamine HCl (100 mg/kg) and xylazine HCl (5 mg/kg) (Phoenix Scientific Inc.). Sterilized PE50 tubing catheters were placed in left external jugular vein, right carotid artery and left femoral artery.

Blood pressure measurements

Basal blood pressures were monitored at baseline for 30 minutes in HFSD animals (n=6 per group). (1) 9 units of F-RBCs, (2) S-RBC or (3) S-RBCs + 300 mg/kg Hp through the venous jugular catheter in conscious guinea pigs, simultaneously blood pressure was monitored from a carotid artery catheter coupled to a Gould Statham pressure transducer (Beckton Dickenson Critical Care, Singapore) with data acquired using a Biopac MP150 data acquisition system (Biopac systems Inc., Goleta Ca). Systolic and diastolic blood pressure was monitored for 3 hours post transfusion, mean arterial pressure (MAP) was calculated from systolic and diastolic pressure.

Western blot analyses

Tissue lysates from aorta were resolved on 4–12% Bis-Tris gels, transferred to nitrocellulose membranes, and blocked for 1 h in Tris-buffered saline and Tween-20 (TBS-T) with 5% nonfat dry milk. Membranes were incubated overnight at 4°C with antibodies to eNOS (1:1000 dilution, ab5589) and phospho-eNOS (1:1000 dilution, ab75639) from Abcam (Cambridge, MA). The membranes were washed and then incubated with a relevant horseradish peroxidase–conjugated secondary antibody for 1 h. The signal was developed using an ECL Plus kit and detected with the KODAK image station 4000MM pro (Carestream Health, Inc. Rochester, NY). Membranes were stripped and reprobed for β -actin. Densitometry analysis was performed using KODAK Molecular Imaging Software (Carestream Health, Inc. Rochester, NY) with normalization to actin.

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Conflict-of-interest statement (past 5 years): Donat R. Spahn

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