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Suggested Citation:
Yogathasan, V., Wischmann, P., Solga, I., Jäger, L., Becher, S., Cortese-Krott, M. M., Gerdes, N., Kelm, M., Jung, C., & Chennupati, R. (2025). Divergent roles of endothelial and red blood cell nitric oxide synthase in regulating cardiovascular function during anemia. Nitric Oxide, 159, 1–10. https://doi.org/10.1016/j.niox.2025.08.004

Nitric Oxide 159 (2025) 1–10

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

Divergent roles of endothelial and red blood cell nitric oxide synthase in regulating cardiovascular function during anemia☆

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ARTICLE INFO

Keywords:
Red blood cells
Endothelium
Nitric oxide synthase
Myocardial infarction
Flow mediated dilation

ABSTRACT

Background: Red blood cells (RBCs) express functional endothelial nitric oxide synthase (eNOS), which regulates blood pressure (BP) independently of eNOS in endothelial cells (ECs) and provides cardioprotection during acute myocardial infarction (AMI). The functional role of RBC- and EC- eNOS in anemia remains unknown. This study evaluated the effects of RBC- or EC-specific eNOS deletion on hemodynamics and cardiac function in blood loss anemia.

Methods and results: Anemia was induced in EC- or RBC-specific eNOS knockout (KO) mice and their respective controls. In vivo flow-mediated dilation (FMD) was preserved in RBC-eNOS-KO mice under both baseline and anemic conditions but was impaired in EC-eNOS-KO mice compared to their respective controls. Wire myograph analysis of aortic rings showed preserved endothelium-dependent relaxation (EDR) in anemic RBC-eNOS-KO mice, while EDR was abolished in anemic EC-eNOS-KO mice relative to controls. Miller catheter BP measurements revealed elevated systolic and diastolic BP in EC-eNOS-KO mice under both baseline and anemic conditions. Both systolic and diastolic BP were increased in RBC-eNOS-KO mice compared to controls, whereas these parameters remained unchanged in anemic RBC-eNOS-KO mice compared to their respective controls. Echocardiography demonstrated preserved cardiac function across all genotypes at baseline, 3 days post-anemia, and 24 h post-reperfused AMI. However, infarct size was significantly increased in anemic RBC-eNOS-KO mice compared to controls.

Conclusions: Anemia mitigates the BP elevation caused by RBC-eNOS deletion, while hypertension persists in the absence of endothelial eNOS, highlighting vascular eNOS as the predominant regulator of BP under anemic conditions. RBC-eNOS limits infarct size under anemic conditions.

1. Introduction

The main functional role of red blood cells (RBCs) is to transport oxygen to the tissues. However, in the last two decades, the erythrocrine functions of RBCs have been explored in more detail. RBCs are known to carry or/and release different vasoactive factors such as ATP [1], sphingosine-1-phosphate [2], and nitric oxide (NO) metabolites [3]. A previous study showed that transplanting bone marrow from endothelial nitric oxide synthase eNOS global mutants to WT mice increased the blood pressure (BP) hinting at the role of hematopoietic cells in BP regulation [4]. In addition, RBCs carry a functionally active eNOS [5] and deletion of eNOS in RBCs using a constitutive erythroid-specific Cre recombinase-line resulted in a hypertensive phenotype concluding the possible role of RBC eNOS in BP regulation [6]. Circulating NO

☆ Given her role as Editor-in-Chief, Miriam Cortese-Krott had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to another journal editor.".

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https://doi.org/10.1016/j.niox.2025.08.004
Received 16 July 2025; Received in revised form 21 August 2025; Accepted 24 August 2025
Available online 25 August 2025

metabolites are reduced in RBC-eNOS deficient mice demonstrating the role of RBC eNOS in regulating circulating NO metabolites [6]. A recent study also demonstrated that RBC-eNOS-KO mice show an increased infarct size after acute myocardial infarction (AMI). In contrast, the infarct size in EC-eNOS deficient mice remained unchanged [7] demonstrating the protective role of RBC-eNOS in AMI.

Anemia is often observed in patients with cardiovascular diseases (CVD) and associates with poor prognosis [8–10]. Our previous study showed that acute blood loss anemia leads to an initial compensatory increase in endothelial eNOS and flow mediated dilation (FMD) responses to support the sufficient perfusion of the peripheral tissue despite limited oxygen-carrying capacity of blood [11]. Simultaneously acute blood loss anemia induces RBC dysfunction by reducing NO bioavailability, increasing ROS formation, reducing membrane integrity, and increasing NO scavenging by free plasma haemoglobin (Hb) [11]. In the same study, we also demonstrated that anemia yields a compensatory increase in heart function despite of unchanged infarct size [11]. Prior investigations indicated that dysfunctional RBCs from patients with AMI lose their cardioprotective properties in ischemia/reperfusion *ex vivo* [12]. However, it is unclear how anemia affects the vascular and heart function in case of eNOS deficiency in the endothelium and in the RBCs. Using cell type-specific eNOS deficient mouse models, we explore the effects of endothelial- and RBC-eNOS ablation on cardiac and vascular function in anemia.

2. Materials and methods

2.1. Animals

All procedures were approved and performed in accordance with the guidelines of LANUV (Landesamt für Natur, Umwelt-und Verbraucherschutz Nordrhein-Westfalen, Germany). Mice were treated by following the European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe Treaty Series No. 123). The approved permits for the animal experiments are 84-02.04. 2020.A073 and 84-02.04.2018.A234.

To evaluate the effect of endothelial eNOS deletion on vascular function under anemic conditions, we used tamoxifen-inducible, endothelial cell-specific eNOS knockout (EC-eNOS-KO; $eNOS^{fl/fl}$/Cdh5-cre/$ERT2^{pos}$) mice and the respective Cre-negative ($eNOS^{fl/fl}$/Cdh5-cre/$ERT2^{neg}$) littermate controls. These were previously generated and genotyped by our research group [6]. To induce endothelial cell-specific activation of Cre recombinase, both EC-eNOS-KO mice and their respective control littermates were treated with tamoxifen (50 mg/kg/day, i.p.) for five consecutive days. To study the effect of RBC eNOS deficiency under anemic conditions, we used constitutive red blood cell-specific eNOS knockout (RBC-eNOS- KO; RBC-$eNOS^{fl/fl}$/Hbb-Cre^{pos}) mice and their respective Cre-negative littermate controls (RBC-$eNOS^{fl/fl}$/Hbb-Cre^{neg}). The mice were previously generated in our research group by crossing founder line floxed eNOS ($eNOS^{flox/flox}$) [6] with erythrocyte-specific Hbb-Cre^{pos} mice (C57BL/6-Tg(Hbb-Cre)12Kpe/J; MGI: J:89725; [13]). All mice were housed in standard cages under controlled room temperature and humidity, maintained on a 12-h light/dark cycle, and provided with free access to standard pelleted chow and tap water. Mice were randomly assigned to experimental groups, and all procedures, outcome assessments, and data analyses were performed under blinded conditions.

2.2. Induction of acute anemia

To induce anemia, EC-eNOS-KO, RBC-eNOS-KO and respective littermate control (cre-negative) group of male mice aged 10–11 weeks were used in all experiments. The mice were randomized into two groups: a control group without anemia, and a group with induced acute anemia. Acute anemia was induced by repetitive mild (150 μl) blood withdrawal (resulting in <20 g/l changes in Hb) from the facial vein daily for 3 consecutive days as described [11]. The loss of plasma volume was replaced with 300 μl of 0.9 % Saline. At the end of the third day, mice showing hemoglobin levels <10 g/dl were considered for experiments.

2.3. Determination of vascular function by flow-mediated dilatation

Flow-mediated dilatation (FMD) responses were assessed using the Vevo 2100 high-resolution ultrasound scanner using a 30–70 MHz linear transducer (Visual Sonics Inc., Toronto, Canada) as previously described [14]. Mice were anesthetized with 1.5–2 % isoflurane in a 40 % oxygen/air mix throughout the experiment. The transducer was placed using a stereotactic holder and adjusted manually to visualise the external iliac artery. A vascular occluder (8 mm diameter, Harvard Apparatus, Boston, MA, USA) was placed around the lower limb [14]. Baseline images of the vessel were first recorded, the cuff was inflated to 200 mmHg, and pressure was kept constant for 5 min (Druckkalibriergerät KAL 84, Halstrup Walcher, Kirchzarten, Germany) then the cuff was released to assess FMD. The upstream diameter of the vessel was determined every 30 s both during inflation and deflation of the cuff. Changes in vessel diameter were quantified as the percentage of baseline (%) = [diameter (max)/diameter (baseline)] × 100.

2.4. Determination of vascular endothelial reactivity ex vivo

Vascular endothelial function was assessed in tissue bath as described before [15]. Briefly, mice were anesthesized by i.p. injection with ketamine (Ketaset®; Zoetis, Parsippany-Troy Hills Township, United States) (100 mg/kg body weight) and xylazine (Rompun®; Serumwerk Bernburg, Bernburg, Germany) (10 mg/kg body weight). The thoracic aorta was dissected free from perivascular adipose tissue, and 2 mm size aortic rings were mounted in a wire-myograph system (Danish Myotechnology, Aarhus, Denmark) with chambers containing Krebs-Henseleit buffer (KHB) (118 mM NaCl, 4.7 mM KCl, 0.8 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5 mM glucose, 110 mM Sodium pyruvate, and 2.5 mM $CaCl_2$). Arterial segments were distended stepwise to 1 gr force or 9.8 mN. All aortic segments were allowed 40 min incubation prior to experiments and stimulated with depolarizing 60 mM KCl Prior to experiment, endothelial integrity was checked by assessing relaxation responses to acetylcholine (ACh; 10 μM) in phenylephrine (PHE; 10 μM) pre-contracted arteries. The aortic segments were pre-contracted and segments which showed less than 80 % relaxation responses were excluded from experiments.

Initially, a concentration-response curve (CRC) for PHE (0.001–10 μM) was constructed. During the contraction induced by 10 μM phenylephrine (PHE), an acetylcholine CRC (1 nM-10 μM) was generated. Next, experiments were repeated in the presence of the cyclooxygenase inhibitor indomethacin (INDO, 10 μM) and in the presence of both INDO and the NOS inhibitor Nω-Nitro-L-arginine methyl ester (L-NAME; 100 μM) to assess the contribution of NOS to arterial relaxation. During contraction of the aortic rings with PHE (10 μM) in the presence of indomethacin (INDO; 10 μM) and L-NAME (100 μM), the relaxing effects of the NO donor sodium nitroprusside (SNP; 10 nM-10 μM) were recorded.

2.5. Assessment of systemic hemodynamics by Millar catheter

To assess hemodynamic parameters, Invasive assessment of hemodynamic parameters was carried out in anemic and non-anemic EC- and RBC-eNOS deficient mice and their respective control mice as described before. Prior to the measurement, mice were given Temgesic (0.05 mg/kg). After anesthesizing the mice with 3.5 % isoflurane in a 40 % oxygen/air mix, the mice were placed on a heating (37 °C) surgical metal plate with the body temperature monitored using rectal probe thought the surgical procedure. A 1.4 F Millar pressure-conductance catheter (SPR-839, Millar Instruments, Houston, TX, USA) was placed in the

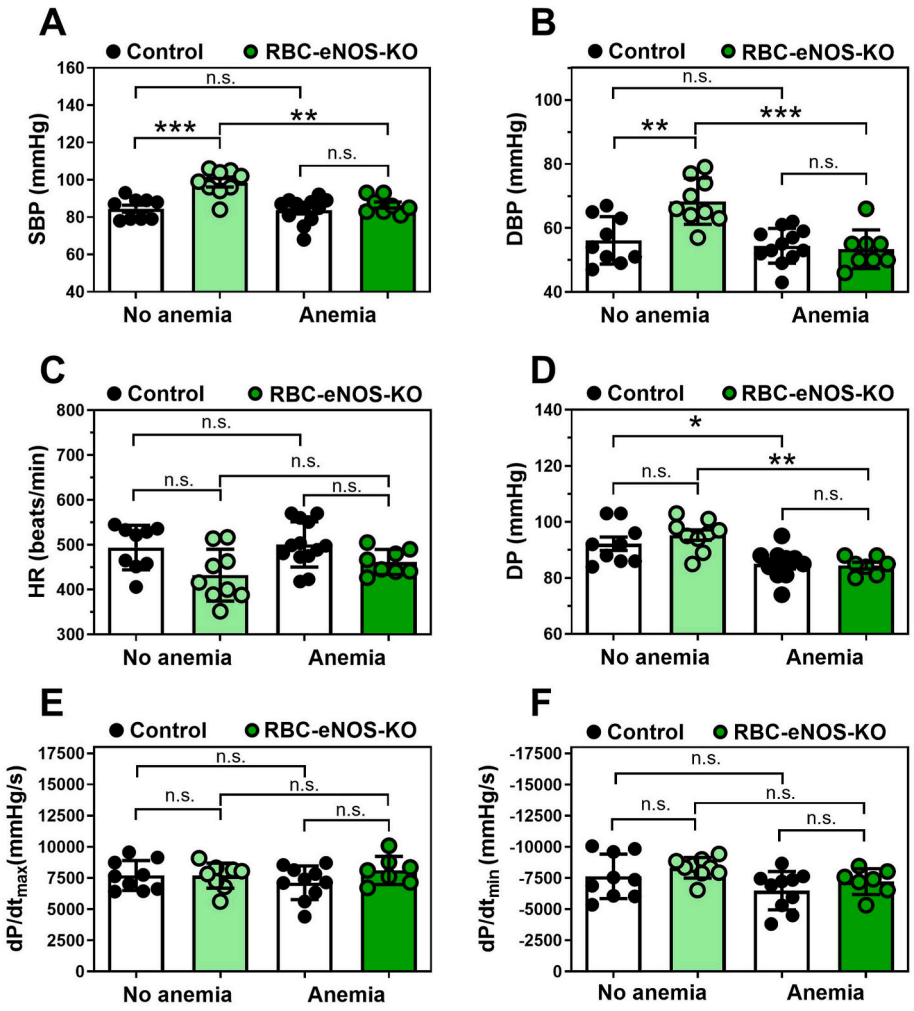

Fig. 1. Acute blood loss anemia mitigates hypertension in RBC-specific eNOS-deficient mice. Blood pressure and heart function were assessed using a Millar catheter in control (white bar) and RBC-eNOS-KO (green bar) mice, with anemia (dark green bar) and without anemia (light green bar). (A) Systolic blood pressure (SBP). (B) Diastolic blood pressure (DBP). (C) Heart rate (HR). (D) Left ventricular developed pressure (DP). (E) Maximal rate of left ventricular pressure increase (dP/dtmax), and (F) minimal rate of left ventricular pressure drop (dP/dtmin). All values are presented as mean ± SEM. Control, n = 9; RBC-eNOS-KO, n = 9; Control with Anemia, n = 8; RBC-eNOS-KO with anemia, n = 12. Multiple groups were compared using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. *. $p \leq 0.05$; **. $p \leq 0.01$; ***. $p \leq 0.001$; n.s. not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

carotid artery. The recorded pressure values were analyzed using IOX software. Systemic vascular resistance (SVR) was calculated according to the following formula: SVR ≅ MAP (mean arterial pressure)/CO (cardiac output). Additionally, end-diastolic developed pressure (LVEDP), developed pressue (DP), maximum rate of pressure increase (dP/dtmax) and maximum rate of pressure decrease (dP/dtmin).

2.6. Induction of acute myocardial infarction (AMI) and LV function analysis

Following the induction of anemia, reperfused AMI was induced in EC-eNOS-KO, RBC-eNOS-KO, and their respective control mice, as previously outlined [16]. Mice were anesthetized with 2 % isoflurane in a 40 % oxygen/air mix, intubated, and placed on a heating (37 °C) surgical metal plate with body temperature monitored using rectal probe thought the surgical procedure. AMI was then induced by occluding the left anterior descending coronary artery for 45 min, followed by 24 h of reperfusion. Buprenorphine (0.5 mg/kg body weight) was administered subcutaneously every 8 h until euthanasia. Left ventricular (LV) function was assessed before and after AMI in mice anesthetized with 2.5 % isoflurane in 100 % oxygen, using transthoracic echocardiography using vevo 2100 (18–38 MHz; Visual Sonics Inc., Toronto, Canada), as previously described [28]. Herat rate (HR), left ventricular end-systolic (ESV) and end-diastolic volumes (EDV), cardiac output (CO), and stroke volume (SV) were measured.

2.7. Triphenyl tetrazolium chloride (TTC) staining

Twenty-four hours after MI, mice were anesthetized (100 mg/kg ketamine and 10 mg/kg xylazine, i.p.) and anticoagulated with heparin (1000 IU, i.p.). Hearts were excised and perfused with cold, oxygenated Krebs–Henseleit buffer (KHB). Evans blue (1 % solution, 1 mL) was injected via the aorta to delineate the area at risk (AAR). Hearts were wrapped in plastic and stored at −20 °C for 1 h, then sectioned into 1 mm slices. Sections were incubated in 1 % TTC at 37 °C for 5 min to differentiate viable and infarcted myocardium. Infarct size, AAR, and

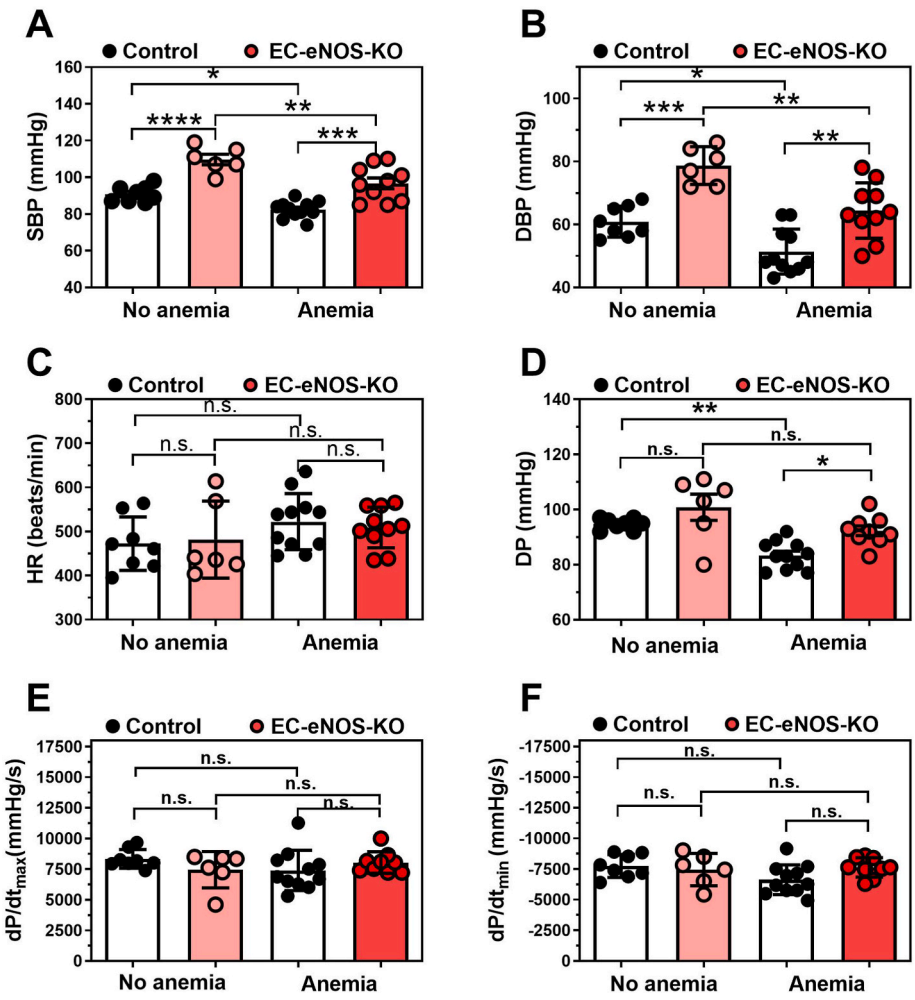

Fig. 2. Endothelial-specific eNOS deficiency leads to high blood pressure irrespective of anemia. Blood pressure and heart function were assessed using a Millar catheter in control (white bar) and EC-eNOS-KO (red bar) mice, with anemia (dark red bar) and without anemia (light red bar). (A) Systolic blood pressure (SBP). (B) Diastolic blood pressure (DBP). (C) Heart rate (HR). (D) Left ventricular developed pressure (DP). (E) Maximal rate of left ventricular pressure increase (dP/dtmax), and (F) minimal rate of left ventricular pressure drop (dP/dtmin). All values are presented as mean ± SEM. Control, n = 8; EC-eNOS-KO, n = 6; Control with anemia, n = 11; EC-eNOS-KO with anemia, n = 10. Multiple groups were compared using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. *. $p \leq 0.05$; ***. $p \leq 0.001$; ****. $p \leq 0.0001$; n.s. not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

non-ischemic LV areas were quantified by computer-assisted planimetry and expressed as a percentage of the AAR.

2.7.1. Statistical analysis

In *ex vivo* vascular function experiments, all CRCs for contractile stimuli are expressed as absolute values. Relaxing responses were expressed as percentage reduction of the level of contraction. Individual CRCs were fitted to a non-linear sigmoid regression curve (Graphpad Prism 10.0). Sensitivity (pEC_{50}), maximal effect (E_{max}) are shown as means ± SEM. In all experiments two groups were compared by unpaired t-tests. One-way or Two-way ANOVA followed by a Bonferroni post-hoc test was used to compare multiple groups.

3. Results

3.1. Hematologic characterization of anemic EC-eNOS-KO/control and RBC-eNOS-KO/control mice

Analysis of blood counts and rheological parameters in the basal state showed no differences between EC-eNOS-KO and RBC-eNOS-KO mice compared to their respective controls (Supplementary Table 1). Anemia induction led to a significant decrease in hemoglobin (Hb) and hematocrit levels across all genotypes (Supplementary Table 1). However, blood counts and rheological parameters in anemic RBC-eNOS-KO mice remained unchanged compared to their respective anemic controls (Supplementary Table 1). In contrast, anemic EC-eNOS-KO mice showed significantly reduced white blood cell counts compared to respective anemic control mice ($P = 0.002$, Supplementary Table 1).

3.2. Dysfunctional RBCs in anemia hamper hypertension in RBC-eNOS-KO mice

To investigate the effects of anemia on systemic hemodynamics, when eNOS is deficient in endothelium and RBC, we assessed the hemodynamic parameters in the mice under anesthesia using invasive pressure catheter. In line with our previous study [6], RBC-eNOS-KO mice show elevated systolic and diastolic BP compared to respective control mice (Fig. 1A and B). However, after acute anemia induction, RBC-eNOS-KO mice show similar systolic (86.5 ± 1.4 mmHg) and diastolic (53.4 ± 1.4 mmHg) BP compared to their eNOS-intact anemic control (83.8 ± 1.8 and 54.4 ± 1.4 mmHg, respectively) group of mice (Fig. 1A and B). As expected, EC-eNOS-KO mice showed significantly elevated systolic (96.7 ± 2.7 mmHg, $P = 0.0001$) and diastolic (64.4 ± 2.5 mmHg, $P = 0.0003$) BP compared to the respective control group

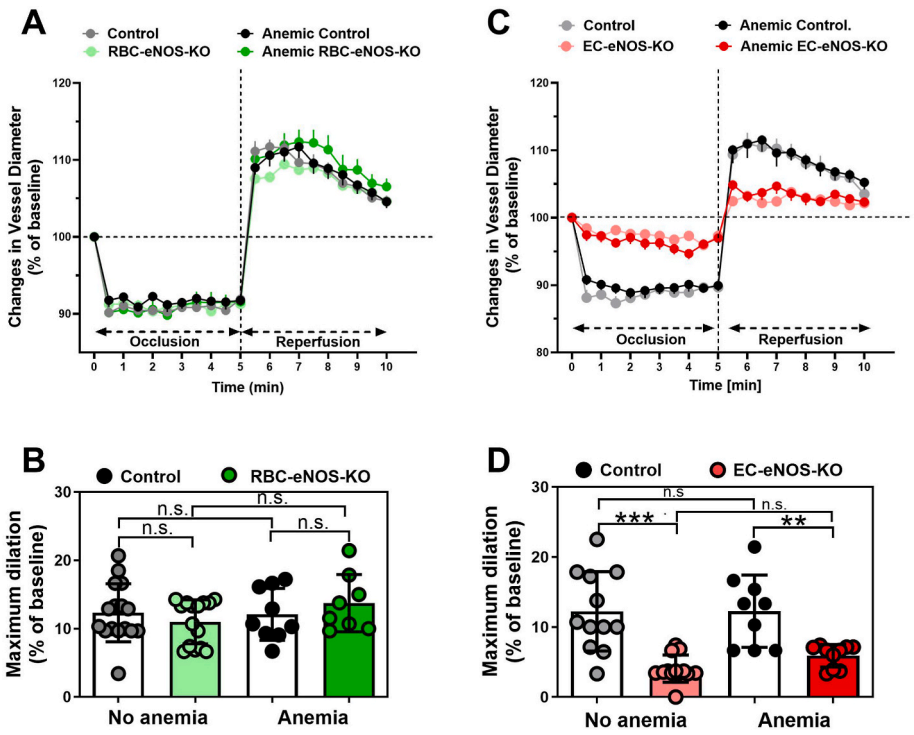

Fig. 3. Anemic RBC-specific eNOS-deficient mice show preserved flow-mediated dilation responses. (A) Changes in vessel diameter (% of baseline diameter) in RBC-eNOS-KO (green circles) mice and respective controls (black circles), with anemia (dark green circles) and without anemia (light green circles). (B) Maximum FMD during the reperfusion phase (6–7 min) averaged and normalized to baseline. (C) Changes in vessel diameter (% of baseline diameter) in EC-eNOS-KO (red circles) and respective controls (black circles), with anemia (dark red circles) and without anemia (light red circles). (D) Maximum FMD during the reperfusion phase (6–7 min) averaged and normalized to baseline. The occlusion and release phases of the cuff (reperfusion) are indicated in the respective panels. All values are presented as mean ± SEM. RBC-eNOS group: Control, n = 16; RBC-eNOS-KO, n = 15; Control with anemia, n = 9; RBC-eNOS-KO with anemia, n = 8. EC-eNOS group: Control, n = 12; EC-eNOS-KO, n = 13; Control with anemia, n = 9; EC-eNOS-KO with anemia, n = 10. A Student's t-test was used to compare two groups. and multiple groups were compared using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. **. $p \leq 0.01$; ***. $p \leq 0.001$; n.s. not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(82.5 ± 1.2 and 51.4 ± 2 mmHg, respectively) of mice (Fig. 2A and B). Under anemic conditions, in EC-eNOS-KO mice showed both systolic (96.7 ± 4.2 mmHg, P = 0.0002) and diastolic (64.4 ± 3.1 mmHg, P = 0.0011) blood pressure remained significantly elevated as compared to respective anemic control (82.4 ± 1.7 and 51.3 ± 2.5 mmHg, respectively) mice (Fig. 2A and B). Heart rate, left ventricular developed pressure (LVDP), myocardial contractility (dP/dt_{max}, dP/dt_{min}) remained comparable in anemic EC-eNOS-KO and RBC-eNOS-KO and their respective anemic control group of mice (Fig. 1C–F, 2C-F). These results conclude that the basal hypertension phenotype in RBC-eNOS-KO is absent after induction of anemia. The hypertension is not affected in EC-eNOS-KO mice with anemia.

3.3. *Endothelial function is preserved in anemic RBC-eNOS-KO mice*

We further investigated the effect of acute anemia on endothelial function *in vivo* by assessing flow-mediated dilation (FMD) responses in EC- and -RBC-eNOS-KO mice. FMD responses were preserved in RBC-eNOS-KO mice compared to respective control mice before and after induction of anemia (Fig. 3A and B). As shown before, EC-eNOS-KO mice showed attenuated maximum FMD responses (4.05 ± 0.6 %, *P* = 0.0001) as compared to respective control mice (12.02 ± 2.0 %) before induction of anemia (Fig. 3C and D). Similarly, after induction of anemia, EC-eNOS-KO mice showed abrogated FMD responses (6.01 ± 0.6 %, *P* = 0.0017) as compared to respective control group (14.7 ± 2.3 %) of mice (Fig. 3C and D).

We also assessed the endothelium-dependent and -independent relaxation response using wire myograph in isolated aortic rings from anemic RBC-eNOS-KO, EC-eNOS-KO mice and respective control groups. The relaxation responses in the presence of indomethacin were comparable in anemic RBC-eNOS-KO mice and control mice (Fig. 4A). As expected, anemic EC-eNOS-KO mice show attenuated relaxation responses (Emax: 12 ± 9 %, *P* = 0.006) compared to respective control (Emax: 65 ± 10 %) group of mice (Fig. 4D). Furthermore, in the presence of a NOS inhibitor (L-NAME, 100 μM), relaxation responses were completely inhibited in all groups (Fig. 4B and E) confirming that the relaxation responses are entirely mediated by NO. In addition, the relaxation responses to exogenous NO donor SNP were similar in all groups irrespective of genotype, suggesting that smooth muscle sensitivity to NO was unchanged in EC-eNOS-KO and RBC-eNOS-KO compared to respective control groups (Fig. 4C and F). As expected, EC-eNOS deficiency leads to abrogated relaxation responses. However, deficiency of eNOS in RBC does not affect endothelial function in anemia.

3.4. *Left ventricle (LV) function is preserved in anemic RBC-eNOS-KO and EC-eNOS-KO mice after AMI*

The effects of anemia on LV function at baseline, 3 days after anemia induction, and 24 h post-AMI was assessed in anemic RBC-eNOS-KO, EC-eNOS-KO and respective control mice using echocardiography. The LV functional parameters end-systolic volume (ESV), end-diastolic volume

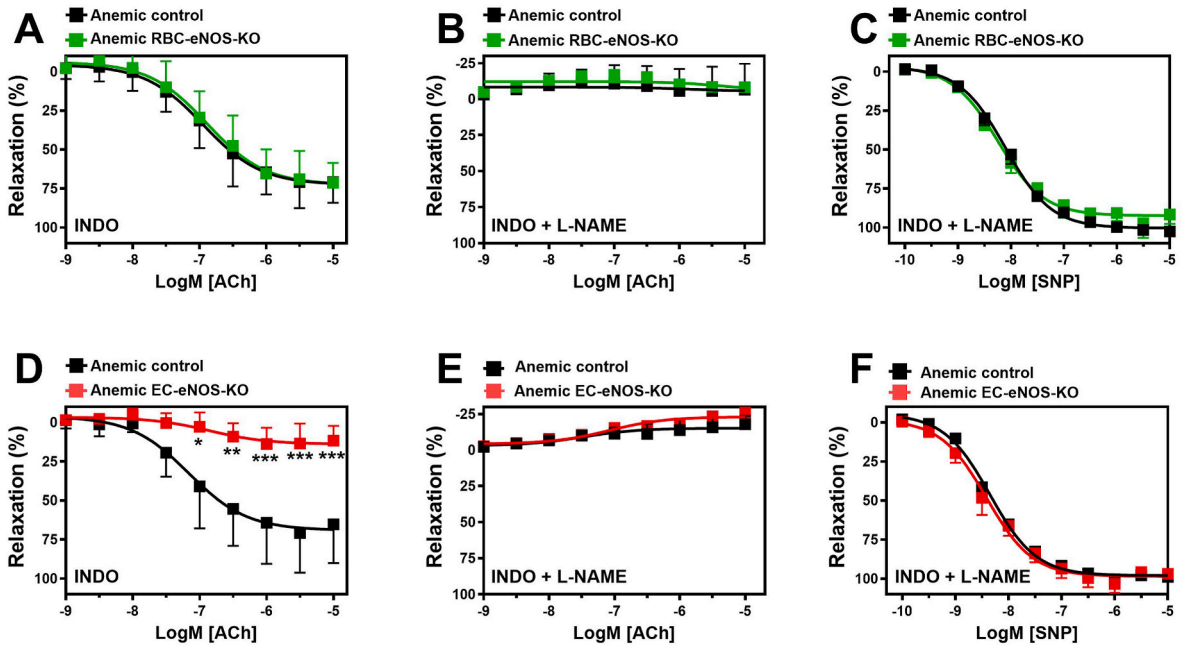

Fig. 4. Anemic RBC-specific eNOS-deficient mice show preserved endothelial-dependent relaxation responses. Aortic rings were isolated from RBC-eNOS-KO (green squares), EC-eNOS-KO (red squares), and respective control (black squares) mice. Aortic segments were pre-contracted with phenylephrine (10 μM) and relaxation responses to acetylcholine (ACh, 1 nM - 10 μM) were measured using a wire myograph. (A, D) Relaxation in the presence of indomethacin (10 μM, COX inhibitor). (B, E) Relaxation in the presence of indomethacin and L-NAME (100 μM, NOS inhibitor). (C, F) Relaxation responses to sodium nitroprusside (SNP, 10 nM - 10 μM) in the presence of indomethacin and L-NAME. All values are presented as mean ± SEM. RBC-eNOS group: Control with anemia, n = 15; RBC-eNOS-KO with anemia, n = 9. EC-eNOS group: Control with anemia, n = 6; EC-eNOS-KO with anemia, n = 6. **. $p \leq 0.01$. CRCs were analyzed by two-way ANOVA and Bonferroni's post hoc test to compare eNOS-deficient mice with their respective control mice. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(EDV), cardiac output (CO), stroke volume (SV), heart rate (HR), ejection fraction (EF) were not significantly different between anemic RBC-eNOS-KO compared to respective anemic control mice (Fig. 5A–E). In addition, the LV parameters are also preserved in anemic EC-eNOS-KO compared to respective anemic control mice (Fig. 6A–E). These results demonstrate that in anemia, eNOS expressed in ECs and RBCs do not modulate LV function after AMI in mice.

3.5. *Anemic RBC-eNOS-KO mice show increased infarct size after AMI*

In our recent study, we showed that EC-eNOS deficiency does not affect the infarct size, whereas RBC-eNOS deficiency leads to significantly increased ($P = 0.0012$) infarct size after AMI [7]. In addition, we also showed that acute anemia does not affect the infarct size after AMI [11]. Here, we further assessed the effect of anemia on infarct size under eNOS deficiency in RBCs and ECs. As shown before [7], infarct size is significantly increased in anemic RBC-eNOS-KO mice compared to anemic control mice (Fig. 7A-C). Yet, anemic EC-eNOS-KO mice show similar infarct size as their respective control mice (Fig. 7D-F).

4. Discussion

In this study, we evaluated the effects of acute anemia on vascular and heart function after AMI in RBC and EC eNOS-KO mice and their respective control littermates. The major findings of this study are as follows: (1) RBC-specific deletion of eNOS increases BP in non-anemic conditions, whereas this increase in BP was abolished in the presence of anemia; (2) Selective deletion of eNOS in the vascular endothelium results in a significant increase in BP, which is preserved in anemia; (3) The eNOS deficiency in the vascular endothelium results in the loss of endothelium-dependent relaxation responses in large arteries; (4) The endothelium-dependent relaxation responses remain preserved in anemic RBC eNOS deficient mice; (5) Endothelium-specific eNOS deletion does not affect LV function or infarct size in anemia; (6) In anemia, ablation of eNOS in RBCs causes greater infarct size after AMI, while LV function remains preserved. The striking finding in this study demonstrates that the blood pressure-increasing effect of RBC-eNOS deficiency was blunted by anemia, while the hypertensive phenotype of EC-eNOS-KO was preserved in anemia.

Anemia is an independent risk factor for CVDs, such as AMI, though the underlying mechanisms are still under investigation. Earlier findings highlighted that acute blood loss anemia is associated with increased reactive oxygen species (ROS) formation and altered membrane integrity of RBCs [11], demonstrating that anemia leads to RBC dysfunction. Moreover, dysfunctional RBCs in anemia impairs the cardioprotective properties due to altered NO bioavailability in STEMI patients [12]. It is well established that RBC-eNOS contributes to blood pressure regulation [6], further demonstrating the role of RBC-eNOS in vascular function regulation. Taken together, all these previous studies emphasize the importance of dissecting the effects of anemia on RBC-eNOS and its associated vascular dysfunction. In this study, we used a well-established and validated genetic mouse model deficient in RBC-eNOS [6], that was subjected to acute blood loss anemia. To further assess the specific role of RBC-eNOS in vascular function, we also included endothelial eNOS-deficient mice for comparison. In line with a previous study, RBC-eNOS-KO mice without anemia showed increased BP, further confirming the role of RBC-eNOS in BP regulation [6]. Interestingly, after inducing acute blood loss anemia, this BP elevation was normalized, suggesting that RBCs in anemia trigger compensatory mechanisms that counteract the typical hypertensive effects of RBC-eNOS deficiency. This likely serves to maintain BP and ensure adequate oxygen delivery when hemoglobin levels are reduced. Some

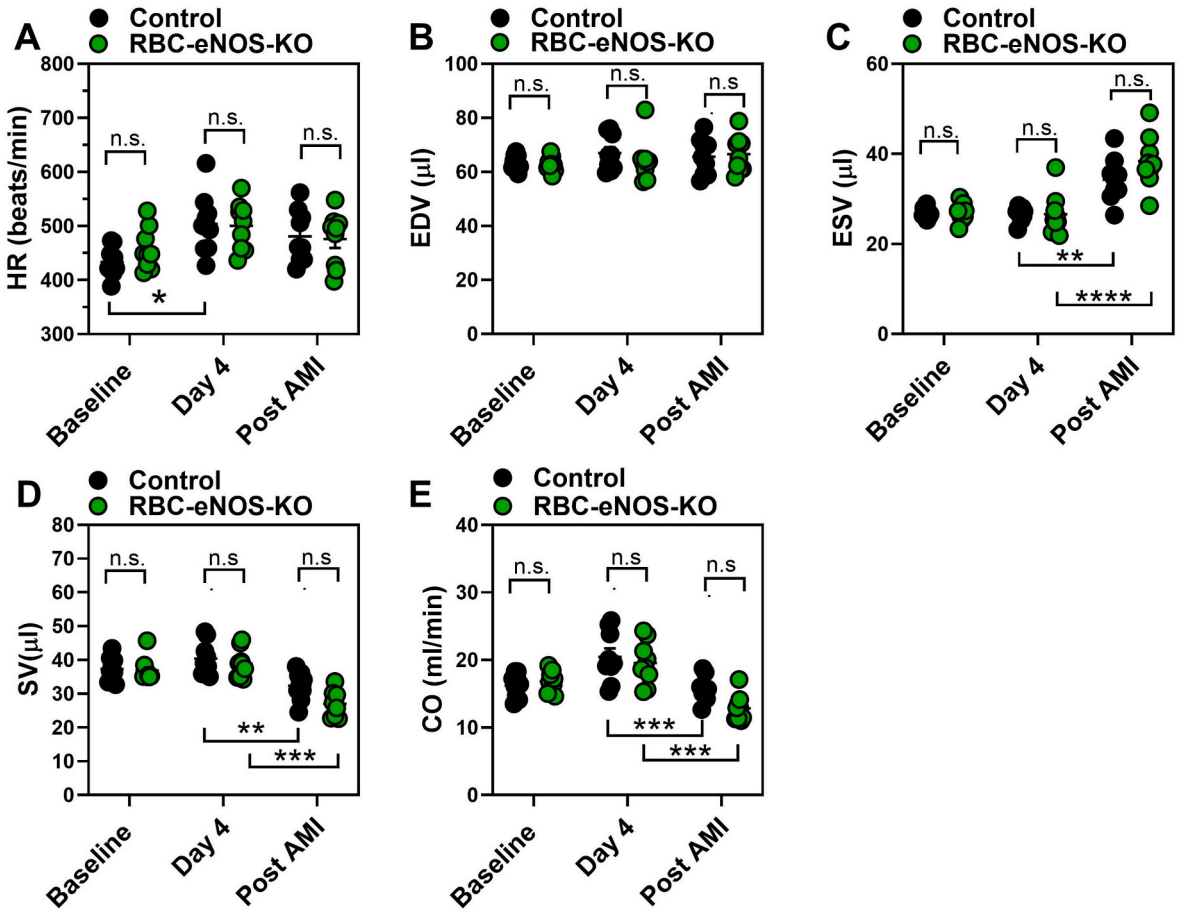

Fig. 5. Left ventricular dysfunction post-AMI is not different in anemic control and RBC-eNOS-deficient mice. Echocardiographic evaluation of control (black circles) and RBC-eNOS-KO (green circles) mice was performed at baseline (basal), 3 days after anemia induction (Day 4), and 24 h after acute myocardial infarction (post-AMI). The following left ventricular parameters were evaluated: A) heart rate (HR), (B) end-diastolic volume (EDV), (C) end-systolic volume (ESV), (D) stroke volume (SV), (E) cardiac output (CO). All values are presented as mean ± SEM. Control, n = 9; RBC-eNOS-KO, n = 9. Multiple groups were compared using two-way analysis of variance (ANOVA) with Tukey's multiple comparison test. **. $p \leq 0.01$; ***. $p \leq 0.001$; ****. $p \leq 0.0001$; n.s. not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

potential mechanisms include the export of NO bioactivity [17] and/or nitrite bioactivation [18] under hypoxic conditions, which merits the further investigation in the future.

Our earlier research demonstrated that RBC-eNOS-KO mice exhibit an increased infarct size after AMI compared to the respective control mice [7]. This phenotype can be partly explained by the fact that RBC-eNOS-KO mice show a reduced capacity to release oxygen at a given oxygen pressure, as demonstrated by increased oxygen affinity and decreased oxygen-binding cooperativity leading to increased infarct size (shown in the supplemental material of Ref [6,7]). Despite the normalized blood pressure in anemic RBC-eNOS-KO mice, the infarct size was still greater compared to anemic control mice after AMI. This might suggest that RBC- eNOS plays a protective role and supports heart function during anemia. The deficiency of RBC-eNOS resulted in a loss of the cardioprotective properties of RBCs, as evidenced by the increased infarct size. Importantly, these effects are independent of EC-eNOS, highlighting the critical role of RBC-derived NO in protecting the heart during anemia.

Consistent with previous studies, [6,19,20], ablation of EC-eNOS led to increased BP, reinforcing the importance of EC-eNOS in regulating BP apart from RBC-eNOS. Inducing acute blood loss anemia resulted in an overall BP decrease in both control and EC-eNOS-deficient anemic mice, likely due to volumetric viscosity changes and/or other increased vasodilatory mechanisms to compensate for reduced oxygen carrying capacity of RBCs [11]. In addition, after the induction of anemia, EC-eNOS-KO mice show a severe reduction in RBCs and other blood cells, such as white blood cells (leukocytes) supporting the notion of hemodilution. Even though the number of RBCs, which plays a crucial role in regulating blood pressure, is reduced following anemia induction, the hypertensive phenotype persists in anemic EC-eNOS-KO mice. This indicates that vascular eNOS is a predominant component in BP regulation apart from RBC-eNOS. Additionally, consistent with previous studies [6], despite increased BP, endothelial eNOS deficiency does not limit infarct size. These effects remain unchanged even upon anemia. These findings are unexpected, as the combination of eNOS deletion and dysfunctional RBCs in anemia was expected to enhance infarct size. The underlying compensatory mechanisms need to be further investigated.

We previously demonstrated that pathological conditions such as anemia are associated with increased oxidative stress and RBC dysfunction [11,12]. Elevated levels of ROS may contribute to eNOS uncoupling within RBCs, thereby impairing their cardioprotective properties [21]. Consistent with previous findings [7], the present study also demonstrated that RBC- eNOS plays an important role in limiting myocardial infarct size after AMI. Therapeutic strategies that prevent eNOS uncoupling, including supplementation with ROS scavengers or folic acid to enhance tetrahydrobiopterin (BH4) bioavailability, may

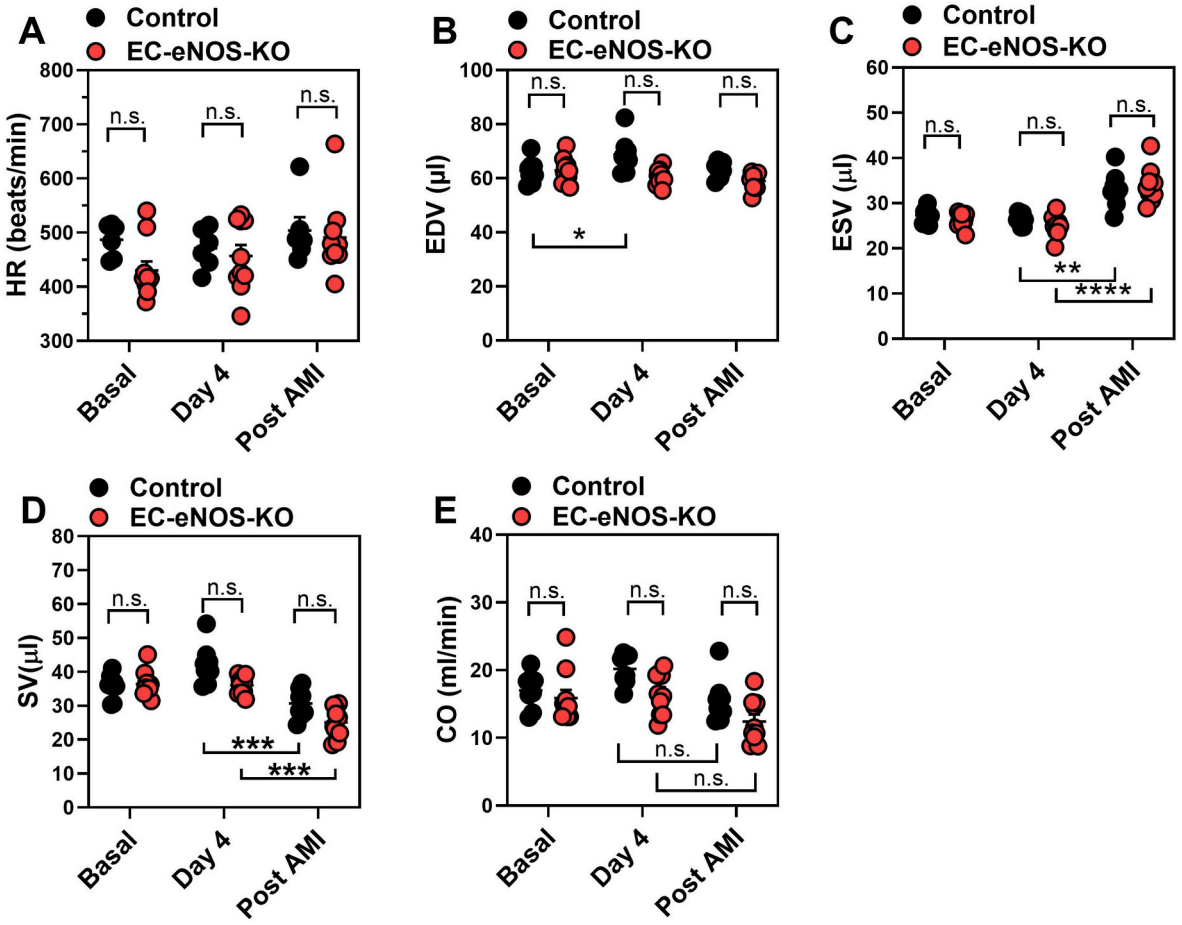

Fig. 6. Left ventricular function is unaltered in anemic control and EC-specific eNOS-deficient mice after myocardial infarction. Echocardiographic evaluation of control (black circles) and EC-eNOS-KO (red circles) mice was performed at baseline (basal), 3 days after anemia induction (Day 4), and 24 h after acute myocardial infarction (post-AMI). The following left ventricular parameters were evaluated: (A) heart rate (HR), (B) end-diastolic volume (EDV), (C) end-systolic volume (ESV), (D) stroke volume (SV), (E) cardiac output (CO). All values are presented as mean ± SEM. Control, n = 8; EC-eNOS-KO, n = 10. Multiple groups were compared using two-way analysis of variance (ANOVA) with Tukey's multiple comparison test. **. $p \leq 0.01$; ***. $p \leq 0.001$; ****. $p \leq 0.0001$; n.s. not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

help restore RBC-eNOS function in disease states. Such interventions could be particularly valuable in anemic patients presenting with myocardial infarction and may ultimately improve outcomes following acute myocardial infarction. These therapeutic interventions may also improve the quality of RBC during blood storage.

5. Limitations

This study has several limitations. First, we used an acute blood loss anemia mouse model, which may not fully replicate the complexity and pathophysiology of chronic anemia in humans. Second, only male mice were used to reduce variability related to hormonal fluctuations during the estrous cycle. However, anemia is commonly observed in young females as well as in older males and females. Therefore, future studies should include both sexes to identify potential sex-specific differences in relation to anemia effects on cardiovascular function.

6. Conclusions

The findings of this study conclude that RBCs in acute blood loss anemia mitigate the increase in BP in red blood cell eNOS deficient mice. RBC-eNOS is crucial for protecting the heart during anemia, and its absence leads to increased infarct size. In contrast, hypertension persists in the absence of endothelial eNOS during anemia, concluding that vascular eNOS is the predominant component regulating BP under anemic conditions. These results suggest that RBC-eNOS could be a novel therapeutic target in patients with anemia following acute myocardial infarction (AMI). Strategies such as L-arginine supplementation may enhance RBC-eNOS activity in anemia, potentially restoring its cardioprotective effects and improving post-AMI outcomes.

CRediT authorship contribution statement

Vithya Yogathasan: Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Patricia Wischmann:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Isabella Solga:** Writing – original draft, Methodology, Investigation. **Lilly Jäger:** Methodology. **Stefanie Becher:** Methodology, Data curation. **Miriam M. Cortese-Krott:** Writing – review & editing, Writing – original draft, Conceptualization. **Norbert Gerdes:** Writing – review & editing, Writing – original draft, Funding acquisition. **Malte Kelm:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition. **Christian Jung:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition. **Ramesh Chennupati:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration,

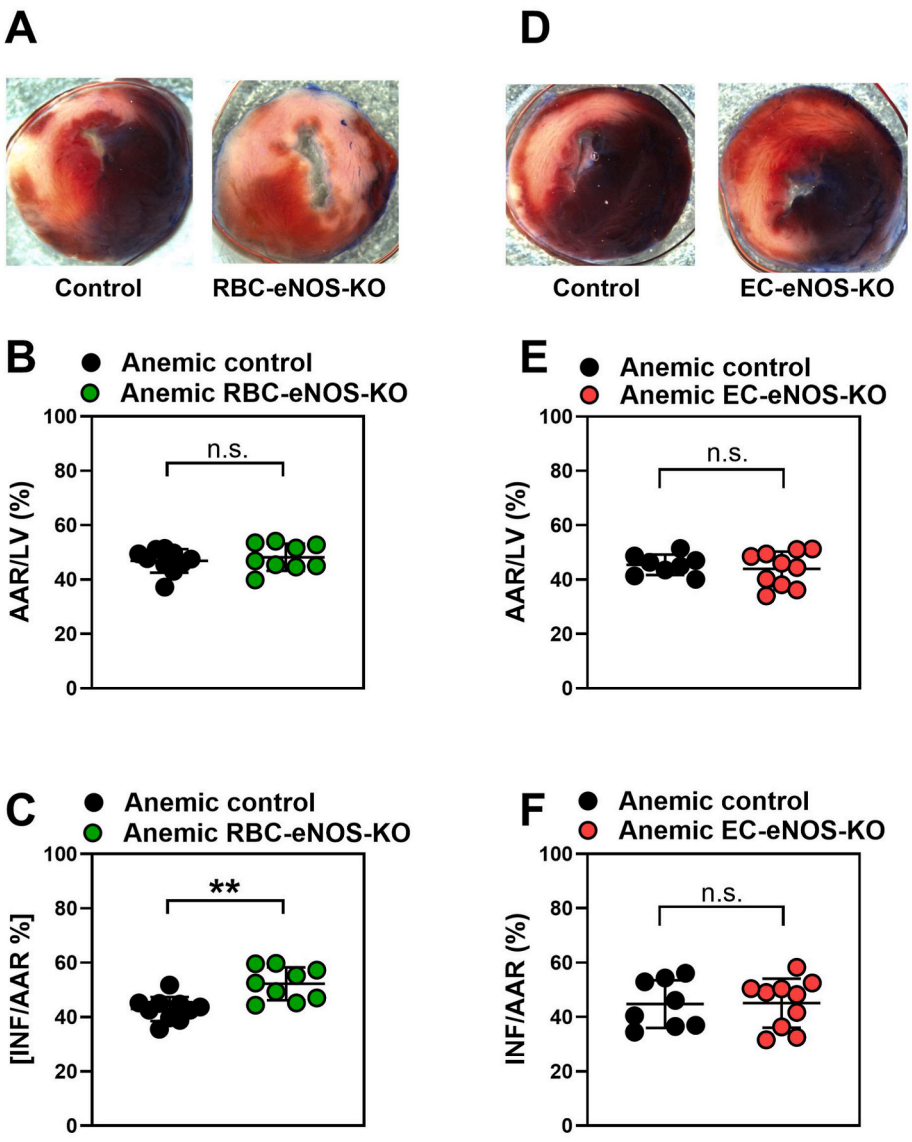

Fig. 7. Anemic RBC-specific eNOS-deficient mice show an increased infarct size as compared to anemic control mice. Acute myocardial infarction (AMI) was induced in RBC-eNOS-KO (green circles), EC-eNOS-KO (red circles), and their respective control mice (black circles). (A, D) Representative heart sections stained with Triphenyl Tetrazolium Chloride (TTC) in the indicated animal groups. The pale/white areas considered infarct and red areas considered viable myocardium. (B, E) Quantification of area-at-risk (AAR) relative to the left ventricle (LV). (C, F) Quantification of infarct area (INF) relative to the area-at-risk (AAR). RBC-eNOS group: Control with anemia, n = 10; RBC-eNOS-KO with anemia, n = 9. EC-eNOS group: Control with anemia, n = 8; EC-eNOS-KO with anemia, n = 10. All values are presented as mean ± SEM. A Student's t-test was used to compare the groups. **. $p \leq 0.01$; n.s. not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Funding

This study was supported by the following grants: Research Commission of the Medical Faculty of Heinrich-Heine University (2021-29 to P.W., 2020-62 to R.C), Düsseldorf, Germany. Deutsche Forschungsgemeinschaft (DFG, German Research Foundation, Germany) - Grant No. 236177352 - CRC1116; projects B06, B09 to M.K., C.J., and N. G. and Grant No. 397484323 - CRC/TRR259; project A05 to N.G. We acknowledge the financial support for purchasing myograph systems from the Susanne-Bunnenberg-Stiftung at the Düsseldorf Heart Center, Germany.

Declaration of competing interest

The authors declare that there are no conflicts of interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2025.08.004.

Data availability

Data will be made available on request.

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