Suicidal erythrocyte death, eryptosis, as a novel mechanism in heart failure-associated anaemia

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Aims 
Suicidal death of erythrocytes (eryptosis) is characterized by cell shrinkage and exposure of phosphatidylserine (PS) residues at the cell surface. Excessive eryptosis may lead to anaemia. We aimed to study the role of eryptosis in heart failure (HF)-associated anaemia.

Methods and results 
We measured eryptosis in rodent models of HF. Typical measures of eryptosis including PS-exposure, increased intracellular Ca\(^{2+}\) levels, and decreased cell volume were determined by flow cytometry. Transgenic REN2 rats displayed mild anaemia which was associated with a two-fold increase in erythrocyte PS-exposure when compared with Sprague Dawley (SD) control rats (\(P < 0.01\)). Upon stimulation with eryptotic triggers such as oxidative stress, hyperosmotic shock and energy depletion, eryptosis was more prominent in REN2 as shown by increased PS-exposure, cytosolic Ca\(^{2+}\) influx, and cell shrinkage (\(P < 0.05\) vs. SD). Increasing cytosolic Ca\(^{2+}\) levels resulted in a stronger increase in PS-exposure in REN2 erythrocytes (\(P < 0.01\) vs. SD). Accordingly, inhibition of Ca\(^{2+}\) entry blunted the increased PS-exposure upon oxidative stress. The REN2 rats had significantly higher reticulocytes (REN2: 10.6 ± 2.3%; SD: 5.4 ± 0.1%; \(P < 0.05\)) and erythrocyte turnover was increased, indicated by increased clearance of eryptotic erythrocytes. Eryptosis was also increased in a rat model of hypertensive cardiac remodelling (uninephrectomized rats implanted with deoxycorticosterone acetate pellets), in mice after transverse aortic constriction, as well as in a small proof-of-concept study in human HF patients.

Conclusion 
Eryptosis is increased during HF development and could contribute to HF-associated anaemia. Eryptosis may therefore become a novel target for therapy in HF-associated anaemia.

Keywords 
Anaemia • Eryptosis • Heart failure • Calcium • Renin

1. Introduction

Anaemia is common in heart failure (HF) patients and has consistently been associated with impaired prognosis.\(^1\) – \(^3\) Although several factors have been implicated in HF-associated anaemia, the exact pathophysiology remains unclear.\(^1\) – \(^6\) Identification of novel and potentially reversible aetiologies for anaemia in HF is therefore needed.

Eryptosis refers to suicidal erythrocyte death and is defined by erythrocyte shrinkage, membrane blebbing, activation of proteases, and breakdown of phosphatidylserine (PS) asymmetry as well as all typical features of apoptosis in nucleated cells (despite the absence of nuclei in erythrocytes).\(^7,8\) From an evolutionary perspective, eryptosis may be designed to prevent spontaneous haemolysis of aged and defective erythrocytes. However, excessive eryptosis could cause anaemia. Indeed, in several diseases that are associated with anaemia, eryptosis has been shown to occur in, for example, sepsis,\(^9\) malaria,\(^10\) iron deficiency,\(^11\) Wilson’s disease,\(^12\) and haemolytic-ic uremic syndrome.\(^13\) HF is associated with oxidative stress, energy depletion, and osmotic imbalance, which are all established triggers for eryptosis.\(^14\) – \(^16\) Furthermore, eryptosis may be a target for therapy. In vitro studies have demonstrated that eryptosis may be attenuated by anti-oxidants, such as thymol.\(^17,18\)

We hypothesized herein that a previously overlooked cause for anaemia in HF may be eryptosis and anti-eryptotic treatment with thymol may be useful in counteracting anaemia. In the present study, we tested our hypothesis by measuring indices of eryptosis in transgenic REN2 rats with severe cardiac dysfunction and HF-associated anaemia,\(^19\) and confirmed this in a rat model of
hypertensive cardiac remodelling (uninephrectomized rats treated with deoxycorticosterone acetate, UNX + DOCA salt) and in mice after transverse aortic constriction (TAC). Finally, we measured eryptosis in acute HF patients. Our results provide evidence for a role of eryptosis in HF-associated anaemia.

2. Methods

2.1 Animal models

Initial animal experiments were performed in 8–14-week-old male homozygous TGR (mREN2)27 rats (n = 11). Age- and gender-matched Sprague Dawley (SD) rats (n = 11) served as controls. REN2 rats overexpress the murine Ren-2 gene that causes hypertension and progressive HF.

We studied two other models of pathological cardiac remodelling with progression towards HF. First, 8–10-week-old male SD rats (n = 8) were subjected to uninephrectomy (UNX, left kidney) and implanted with a deoxycorticosterone acetate or DOCA pellet (Innovative Research of America, FL, USA; 15 mg/week release) and supplemented with 0.9% NaCl drinking water), or implanted with placebo pellet (normal tap water) for 6 weeks. These rats develop severe hypertension leading to hypertensive cardiac remodelling (uninephrectomized rats treated with deoxycorticosterone acetate or DOCA pellet and supplemented with 0.9% NaCl drinking water, or implanted with placebo pellet (normal tap water) for 6 weeks. These rats develop severe hypertension leading to the heart failure phenotype.

Secondly, 8–12-week-old male mice (n = 6) were subjected to TAC or sham for 6 weeks procedure as previously described.

At indicated timepoints, blood was drawn from the tail vein and anticogulated with EDTA for haematology analysis, or with heparin for the eryptosis assays. Echocardiography measurements were performed prior to sacrifice (Vivid 7, GE Healthcare, Belgium). At sacrifice, haemodynamic measurements were performed with a micropipette pressure transducer (Millar Instr., Inc., Houston, TX, USA) as described previously.

2.2 Ethics

The protocols describing the animal experiments were approved by the Animal Ethical Committee of the University of Groningen, the Netherlands, and conforms with the Guide for the Care and Use of Laboratory Animals published by the Directive 2010/63/EU of the European Parliament. Human blood samples that were studied by H.M. were anonymous and could not be connected to the identity of the subjects. As such, this procedure and investigation conforms with the principles outlined in the Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/).

2.3 Blood parameters and reticulocyte count

For blood count, red blood cell number (RBC), haematocrit (HCT), haemoglobin concentration (HGB), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC), EDTA blood was analysed using an electronic haematology particle counter (scil VET abc, Weinheim, Germany). Relative reticulocyte numbers were determined using Retic-COUNT reagent (BD, Heidelberg, Germany) according to the manufacturer's instructions.

2.4 Human erythrocytes

Heparin-anticoagulated blood was drawn from patients (n = 5) admitted due to acute decompensated HF at the University Medical Center Groningen for standard clinical work-up including a full blood count. A 100 μL aliquot of heparinized whole blood, left over after standard blood work, was recovered from routine blood samples. The investigator (H.M.) was completely blinded to patients' data. As a result, outcomes of the eryptosis study cannot be traced back to individual patients. Control human blood was taken from healthy volunteers (n = 5).

2.5 Chemicals and culture

For the in vitro experiments on eryptosis, erythrocytes were washed two times with Ringer solution containing 125 mM NaCl, 5 mM KCl, 1 mM MgSO4, 32 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid, 5 mM glucose, 1 mM CaCl2, pH 7.4 and incubations were carried out at 37°C at a haematocrit of 0.4% in a total volume of 500 μL.

Erythrocytes were exposed to established triggers of eryptosis, including 30 min of oxidative stress (addition of 200 μM tert-butyl hydroperoxide [t-BOOH], Sigma Aldrich), 2 h of hyperosmotic shock (addition of 550 mM sucrose on top of isotonic Ringer), and 12 h of energy depletion (reduced glucose conditions). In addition, the Ca2⁺ sensitivity of annexin-V binding was tested by exposing erythrocytes to the Ca2⁺ ionophore ionomycin for 30 min (10 μM, Sigma Aldrich). To define the role of Ca2⁺ entry for the triggering of oxidative stress-induced eryptosis, erythrocytes were treated for 30 min with 200 μM t-BOOH after an hour pre-treatment with or without amiloride (1 mM, Sigma Aldrich), an inhibitor of the non-specific cation conductance in erythrocytes.

2.6 PS exposure and forward scatter

Flow cytometry was conducted as described previously. Briefly, erythrocytes were washed once with Ringer solution containing 5 mM CaCl2. Cells were then stained with annexin-V Fluos and forward scatter and annexin-V intensity were measured by flow cytometric analysis (FACSCalibur; BD), using an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

2.7 Measurement of intracellular calcium

For intracellular Ca2⁺ measurements, erythrocytes were loaded with Fluo-3A/M (Calbiochem) in Ringer solution containing 5 mM CaCl2 and 2 μM Fluo-3A/M for 20 min. Ca2⁺-dependent Fluo-3-A/M intensity was determined by flow cytometry as described earlier for measurement of annexin-V.

2.8 Measurement of in vivo clearance of labelled erythrocytes

The in vivo clearance of labelled erythrocytes was described previously. Briefly, erythrocytes were exposed to oxidative stress (500 μM t-BOOH and incubation for 30 min at 37°C). After incubation, erythrocytes were washed twice in PBS and then fluorescence-labelled with 5 μM carboxyfluorescein diacetate, succinimidyl ester (CFSE, Molecular Probes) for 30 min at 37°C under light protection. Cells were washed again (twice) in PBS containing 1% FCS by centrifuging at 400 g for 5 min. The pellet was then re-suspended in fresh, pre-warmed Ringer solution. One millilitre of fluorescence-labelled erythrocytes was injected intravenously into the same rats tail vein. After 5, 15, 30, and 60 min, blood was taken from the rats, and CFSE-dependent fluorescence intensity of the erythrocytes was measured in the fluorescence channel FL-1. The percentage of CFSE-positive erythrocytes was expressed as per cent of the total erythrocyte number.

2.9 Fluorescence microscopy

For the detection of annexin-V binding and CFSE-dependent fluorescence of erythrocytes in splenic tissue, the rats were sacrificed by overdose of isoflurane. The spleens of REN2 and of SD rats were removed, weighed, and mechanically homogenized in 1 mL cold PBS. The suspension was then centrifuged at 500 g for 10 min at 4°C. The cell pellet was resuspended in 200 μL cold PBS. Five microlitres of annexin-V APC (BD, Heidelberg, Germany) was added, and incubation was carried out for 20 min at 37°C protected from light. The suspension was then transferred onto a glass slide and mounted with Vectashield® mounting medium (Vector laboratory Inc.). Images were taken with a Leica Fluorescence Microscope (Leica Microsystems CMS GmbH, Germany).
2.10 Anti-eryptotic treatment with thymol
Since oxidative stress is a key contributory factor in the development of eryptosis, a pilot proof-of-concept study was performed to prevent anaemia in experimental HF by using a potent antioxidant, thymol. REN2 rats were treated with 50 mg/kg per day thymol (Sigma Aldrich) in the chow for 6 weeks.

2.11 Statistical analyses
Data are expressed as means ± SEM. Comparisons between groups were made using non-parametric tests (Mann–Whitney U); a P-value of <0.05 (alpha) was considered to denote statistically significant difference. Kruskal–Wallis test was used for multiple groups with post-hoc Bonferroni corrections; a P-value of <0.01 (alpha/5 = 0.002) was considered to denote statistically significant difference.

3. Results

3.1 Heart failure, anaemia, and increased eryptosis in REN2 rats
As shown by our group, REN2 rats exhibited severe hypertension and cardiac dysfunction.19 In our study, haemodynamic data at sacrifice confirmed typical signs of HF in REN2 rats (Table 1). Left-ventricular weight (adjusted for tibia length) and left-ventricular end-diastolic pressure (LVEDP) were significantly increased, while % fractional shortening (FS) was decreased in REN2 compared with SD rats. Other typical signs of HF, for example, lung weight and liver weight (both adjusted for body weight) and expression of atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) mRNA were also significantly higher in REN2 rats. As expected, REN2 rats also showed significantly higher plasma creatinine concentration (Table 1).

Full blood count revealed that REN2 rats have significantly decreased HGB, HCT, MCV, MCH, and MCHC (Figure 1A) which is in line with a previous study.19 Haematocrit progressively decreased during HF development in REN2 rats (Figure 1B). Reticulocyte percentages were, however, two-fold higher in REN2 compared with SD rats (Figure 1C), suggestive of enhanced erythrocyte turnover. To investigate whether eryptosis contributed to the observed anaemia in REN2 rats, annexin-V binding of erythrocytes of REN2 and SD rats were determined. As shown in Figure 1D, the percentage of annexin-V positive erythrocytes was almost two-fold higher in REN2 when compared with SD rats.

3.2 Erythrocytes from REN2 rats are more susceptible to eryptosis in vitro
To assess whether cells from REN2 rats were more susceptible to eryptosis, erythrocytes were subjected to established inducers of eryptosis in vitro, including oxidative stress, glucose deprivation, and hyperosmotic shock. The percentage of PS-exposing erythrocytes was significantly higher in the REN2 group when compared with the SD group, after treatment with the oxidative stress inducer t-BOOH (Figure 2A and B). Also Ca$^2+$ influx, as determined by Fluo-3-A/M intensity, was higher in the REN2 group (Figure 2C and D) and additionally more cell shrinkage was observed in REN2 rats (Figure 2E and F), suggesting that these cells are more sensitive to oxidative stress. Similar results, although less pronounced, were observed after glucose deprivation and osmotic stress (Figure 3A–D), but no difference in cell shrinkage was observed between the REN2 and SD after these treatments (data not shown).

3.3 Role of Ca$^{2+}$ entry in increased eryptosis in REN2 rats
Another series of experiments were performed to determine if the increased susceptibility of REN2 erythrocytes to eryptosis could be explained by an increased sensitivity to Ca$^{2+}$. First, when cytosolic Ca$^{2+}$ levels were increased with ionomycin, the percentage of annexin-V positive erythrocytes was significantly higher in REN2 vs. SD rats (Figure 4A). Secondly, blocking calcium entry with amiloride, a non-specific inhibitor of cation conductance of erythrocytes, blunted the increase in PS-exposure following oxidative stress in both REN2 and SD rats, but did not normalize this difference (Figure 4B). In these experiments, the spleens from REN2 rats were also dissected and the percentages were calculated as a ratio to the number of initially labelled erythrocytes (REN2; 74% and SD; 70%) and erythrocytes were then labelled with CFSE and subsequently re-injected into the same rats. As shown in Figure 5A, the percentage of circulating CFSE-positive erythrocytes was significantly lower in REN2 when compared with SD rats at 30 min after injection. The percentages were calculated as a ratio to the number of initially labelled erythrocytes (REN2; 5.35% and SD; 4.94%) present in the circulation 5 min after injection, which was considered as 100% CFSE labelled erythrocytes (Figure 5A).

Cleared erythrocytes are mostly retained in the spleen where they are degraded by macrophages. To investigate the fate of the cleared erythrocytes, the spleens of the REN2 and SD rats were analysed. The spleen weight of REN2 group was significantly higher than SD group (Figure 5B and C). Furthermore, we analysed erythrocytes from spleens of REN2 and SD rats by fluorescence microscopy 24 h after injection of labelled erythrocytes. Analysis revealed that the number of CFSE-positive PS-exposing erythrocytes was higher in the spleens from REN2 than from SD control rats (Figure 5D).

3.4 Increased in vivo clearance of oxidized erythrocytes in REN2 rats
Arguably, the increased eryptosis observed in REN2 rats could be attributable to defective clearance of damaged erythrocytes rather than an increased susceptibility to eryptosis. Therefore, systemic clearance of in vitro oxidized and CFSE labelled erythrocytes was studied as well. Briefly, erythrocytes were drawn and treated with high concentration (500 μM) of t-BOOH to reach same levels of PS-exposure in REN2 and SD erythrocytes. PS-exposures were measured (REN2: 74% and SD: 70%) and erythrocytes were then labelled with CFSE and subsequently re-injected into the same rats. As shown in Figure 5A, the percentage of circulating CFSE-positive erythrocytes was significantly lower in REN2 when compared with SD rats at 30 min after injection. The percentages were calculated as a ratio to the number of initially labelled erythrocytes (REN2; 5.35% and SD; 4.94%) present in the circulation 5 min after injection, which was considered as 100% CFSE labelled erythrocytes (Figure 5A).

3.5 Thymol prevents anaemia in REN2 rats
To investigate whether thymol prevents HF-associated anaemia, a pilot intervention study was performed by treating REN2 rats with thymol. As shown in Figure 5E, the decrease in haematocrit in REN2 rats was significantly attenuated in thymol treated REN2 rats compared with control REN2 rats after 6 weeks treatment with thymol. Thus, thymol appears to exert an inhibitory effect on anaemia suggesting that eryptosis might be targeted in vivo.

3.6 Cardiac remodelling, anaemia, and increased eryptosis in UNX + DOCA salt-treated hypertensive rats
To confirm our findings in REN2 rats, additional experiments were performed in UNX + DOCA salt rats and sham-operated rats. Haemodynamic data at sacrifice confirmed typical signs of...
Figure 1 Anaemia and enhanced eryptosis in REN2 rats. (A) Means ± SEM (n = 11) of erythrocyte count (RBC), haemoglobin concentration (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) of 12-week-old REN2 (black bar) and SD (fine bar) rats. (B) Means ± SEM (n = 8) of haematocrit (HCT) of REN2 and SD rats at different ages (8, 10, and 12 weeks). (C) Mean ± SEM (n = 11) of reticulocyte percentages in blood from REN2 (black bar) and SD (fine bar) groups. (D) Means ± SEM (n = 11) of the percentage of annexin V-binding erythrocytes in freshly drawn blood (spontaneous) of REN2 (black bar) and SD (fine bar) rats. *P < 0.05, **P < 0.01, ***P < 0.001, SD vs. REN2, respectively. #P < 0.05, HCT at 8 vs. 12 weeks in REN2 rats.

Table 1 Animal characteristics

<table>
<thead>
<tr>
<th></th>
<th>SD (n = 11)</th>
<th>REN2 (n = 11)</th>
<th>Sham (n = 8)</th>
<th>UNIX + DOCA (n = 8)</th>
<th>Sham (n = 6)</th>
<th>TAC (n = 6)</th>
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<tr>
<td>BW (g)</td>
<td>367 ± 6</td>
<td>309 ± 7***</td>
<td>492 ± 17</td>
<td>456 ± 16</td>
<td>30.1 ± 0.7</td>
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<tr>
<td>LV-W/TL (mg/mm)</td>
<td>21.9 ± 0.3</td>
<td>28.8 ± 1.0***</td>
<td>23.9 ± 0.6</td>
<td>32.2 ± 1.2***</td>
<td>7.6 ± 0.2</td>
<td>12.5 ± 0.8**</td>
</tr>
<tr>
<td>FS (%)</td>
<td>43.4 ± 1.4</td>
<td>36.0 ± 3.3***</td>
<td>45.6 ± 0.3</td>
<td>519 ± 6.7</td>
<td>37.4 ± 1.5</td>
<td>22.7 ± 3.7**</td>
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<tr>
<td>LVEDP (mmHg)</td>
<td>5.3 ± 0.8</td>
<td>9.7 ± 1**</td>
<td>9.9 ± 1.6</td>
<td>13.4 ± 1.5</td>
<td>11.5 ± 2.5</td>
<td>21.2 ± 4.4</td>
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<tr>
<td>Lung weight/BW (wet, mg/g)</td>
<td>4.4 ± 0.2</td>
<td>5.6 ± 0.3**</td>
<td>3.3 ± 0.1</td>
<td>3.7 ± 0.1</td>
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<td>NA</td>
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<tr>
<td>Liver weight/BW (wet, mg/g)</td>
<td>32.6 ± 0.4</td>
<td>43.5 ± 1.1***</td>
<td>29.5 ± 0.5</td>
<td>30.8 ± 1.2</td>
<td>54.2 ± 2.2</td>
<td>53.7 ± 1.8</td>
</tr>
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<td>ANP mRNA expression (fold change)</td>
<td>1.0 ± 0.2</td>
<td>11.2 ± 1.4***</td>
<td>1.0 ± 0.01</td>
<td>13.7 ± 0.2***</td>
<td>1.0 ± 0.01</td>
<td>12.2 ± 0.3**</td>
</tr>
<tr>
<td>BNP mRNA expression (fold change)</td>
<td>1.0 ± 0.08</td>
<td>2.0 ± 0.1***</td>
<td>1.0 ± 0.06</td>
<td>2.6 ± 0.2**</td>
<td>1.0 ± 0.03</td>
<td>2.5 ± 0.3**</td>
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<td>EPO plasma concentration (pg/mL)</td>
<td>12.7 ± 2.5</td>
<td>9.9 ± 3.8</td>
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<td>NA</td>
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<tr>
<td>Creatinine plasma concentration (µmol/L)</td>
<td>26.5 ± 1.5</td>
<td>43.6 ± 4.0*</td>
<td>NA</td>
<td>NA</td>
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BW, body weight; LV-W, left-ventricular weight; TL, tibia length; FS, fractional shortening; LVEDP, left-ventricular end-diastolic pressure; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; EPO, erythropoietin. NA, not available.

*p < 0.05.

**p < 0.01.

***p < 0.001.
pathological remodelling in UNX + DOCA rats (Table 1). Left-ventricular weight (adjusted for tibia length), ANP and BNP mRNA expression were all significantly increased in remodelled hearts compared with control hearts.

The analysis of peripheral blood revealed anaemia in UNX + DOCA rats as evidenced by a significantly decreased RBC, HGB, and HCT (Figure 6A). To assess if eryptosis may account for the observed anaemia of UNX + DOCA rats, the percentage of PS-exposing erythrocytes was determined immediately after retrieval. As shown in Figure 6B, PS-exposure of freshly drawn erythrocytes was indeed significantly more pronounced in UNX + DOCA rats than in sham-operated rats. Further experiments were performed to investigate whether erythrocytes from UNX + DOCA rats are also more susceptible to eryptosis due to energy depletion and oxidative stress in vitro. The increase of PS-exposing cells upon energy depletion and oxidative stress was significantly higher in UNX + DOCA rats than in sham-operated rats (Figure 6B). In addition, Ca\(^{2+}\) influx was significantly higher in UNX + DOCA treated rats in both conditions (Figure 6C).

**Figure 2** Enhanced phosphatidylserine exposure at the surface of erythrocytes from REN2 rats upon oxidative stress. (A) Original histogram of annexin V-binding reflecting PS-exposure in a representative experiment of erythrocytes from REN2 rats and SD rats treated with tert-butylhydroperoxide (t-BOOH). (B) Means ± SEM (n = 8) of the percentage of annexin V-binding of REN2 (black bar) and SD (fine bar) erythrocytes incubated in the absence (control) or in presence (t-BOOH) of t-BOOH. (C) Original histogram of fluo3 fluorescence reflecting Ca\(^{2+}\) influx in a representative experiment of erythrocytes from REN2 rats and SD rats after 30 min incubation in the presence of 200 μM t-BOOH. (D) Means ± SEM (n = 8) of fluo3 fluorescence reflecting Ca\(^{2+}\) influx of REN2 (black bar) and SD (fine bar) erythrocytes incubated in the absence (control) or in the presence (t-BOOH) of t-BOOH. (E) Original histogram of forward scatter reflecting cell size of representative experiment of erythrocytes from REN2 rats and SD rats after 30 min incubation in presence of 200 μM t-BOOH. (F) Means ± SEM (n = 8) of forward scatter of REN2 (black bar) and SD (fine bar) erythrocytes incubated in the absence (control) or in the presence (t-BOOH) of t-BOOH. **P < 0.01, SD vs. REN2, respectively, under control condition. *P < 0.05, **P < 0.01, SD vs. REN2, in the presence of t-BOOH.
3.7 Erythrocytes from pressure overload-induced cardiac remodelling are more susceptible to oxidative stress-induced eryptosis

An additional mouse model was included to assess whether eryptosis is present in murine cardiac remodelling. Similarly, haemodynamic data at sacrifice confirmed typical signs of cardiac remodelling in TAC mice (Table 1). Left-ventricular weight (adjusted for tibia length), % fractional shortening (FS), ANP and BNP mRNA expression were all significantly increased in remodelled hearts compared with control hearts.

The full blood count was similar in TAC and sham-operated mice (data not shown). Similarly, the percentage of PS-exposing cells from freshly drawn erythrocytes was also similar between the groups (Figure 6D). To test if erythrocytes from TAC mice are more prone to eryptosis upon energy depletion and oxidative stress, erythrocytes from TAC and sham mice were exposed to energy depletion and oxidative stress. As shown in Figure 6D, number of PS-exposing erythrocytes from TAC mice was significantly higher compared with sham-operated mice in response to oxidative stress. Similarly, upon oxidative stress, the increase in intracellular Ca^{2+} influx was significantly more pronounced in erythrocytes from TAC-operated mice compared with sham-operated mice (Figure 6E).

3.8 Eryptosis in acute HF patients

We analysed blood from five patients with HF (average age 78 years; four females, one male), and five controls (average age 30 years; four females, one male). As shown in Figure 6F, annexin-V binding for PS-exposure of freshly drawn erythrocytes was significantly increased in acute HF compared with healthy controls and when subjected to oxidative stress.

4. Discussion

The present study shows for the first time that eryptosis (suicidal erythrocyte death) is present in HF and may contribute to HF-induced anaemia. We demonstrate that spontaneous eryptosis was increased in patients and in rats with HF. In rats, the increase in eryptosis was associated with anaemia. Erythrocytes from rats as well as mice and humans with HF were all more susceptible to eryptosis-induction in vitro.

We hypothesized that eryptosis may contribute to HF-induced anaemia for several reasons. First, the association of anaemia with HF has previously been described in numerous studies and is
Eryptosis in heart failure-associated anaemia

Figure 4 Erythrocytes Ca\(^{2+}\) sensitivity of REN2 and SD rats. (A) Means ± SEM (n = 6) of the percentage of annexin V-binding of REN2 (black bar) and SD (fine bar) erythrocytes incubated for 30 min with or without 10 μM ionomycin. "\*\*P < 0.05. "\*P < 0.01. SD vs. REN2, respectively. (B) Means ± SEM (n = 6) of the percentage of annexin V-binding of REN2 (black bar) and SD (fine bar) erythrocytes incubated in the absence (ringer) or in the presence (t-BOOH) of t-BOOH with or without 1 mM amiloride. Comparisons were made by Kruskal–Wallis test with post-hoc Bonferroni correction. "\*\*P < 0.001, SD vs. REN2, without amiloride in the absence of t-BOOH. "\*\*P < 0.01, "\*\*\*P < 0.001, SD vs. REN2, without or with amiloride in the absence of t-BOOH. "\*P < 0.05, between the REN2 rats with or without amiloride in the presence of t-BOOH.

established.\(^1\)–\(^3\) Furthermore, eryptosis and HF share many pathophysiological pathways. It has been demonstrated that such pathways including oxidative stress, energy depletion, and a dysregulated osmotic balance are all present in HF\(^14\)–\(^16\) and these pathways are the main triggers of eryptosis.\(^25,26\) Furthermore, other organs may be affected by eryptotic events, such as endothelial cells to which eryptotic erythrocytes adhere, and which may lead to impaired microcirculation and ultimately contribute to organ failure.\(^27\)

We previously described that REN2 rats develop bone marrow dysfunction and HF-associated anaemia.\(^19\) Therefore, when we aimed to study eryptosis as a contributory mechanism of HF-associated anaemia, we extensively studied eryptosis signalling in this experimental HF model. The REN2 rat displays severe hypertensive and end-organ damage, including HF and nephropathy, which is caused by overexpression of murine ren-2 gene.\(^20,28\) The onset and progression of HF, from compensated left-ventricular (LV) hypertrophy towards LV dilatation and finally overt HF, strongly mimics the sequelae in human HF development. Various stress pathways typical for HF are activated in the REN2 rat model, including increased sympathetic tone, LV hypertrophy, myocardial fibrosis, and stress-related pathways,\(^28,29\) therefore deeming anaemia in the REN2 rats similar to human HF-associated anaemia. In accordance with this, we further found that REN2 rats display a higher reticulocyte count, increased spleen weights, and increased clearance of stressed erythrocytes from the circulation, suggesting that accelerated eryptosis results in an increased turnover of erythrocytes.

In our study, several main triggers for eryptosis were studied. We observed that oxidative stress leads to increased cytosolic Ca\(^{2+}\) activity in REN2 rats, UNX + DOCA rats, and TAC mice which was associated with enhanced scrambling of cell-membrane phospholipids. The difference in cell shrinkage was observed only in erythrocytes in REN2 rats. The increased eryptosis of rodent models of cardiac remodelling is at least, in part, due to increased cytosolic Ca\(^{2+}\) activity.

Hyperosmotic shock and energy depletion are also known triggers of eryptosis.\(^25,26\) but the difference in eryptosis between REN2 and control animals was less pronounced, although significant. Thus, although an imbalance in osmolarity and impaired energy metabolism are known to occur in HF,\(^15,16\) oxidative stress possibly best explains the difference in eryptosis between HF and control animals. In acute HF patients, oxidative stress also enhanced the difference between acute HF patients and control patients in the scrambling of cell-membrane phospholipids by increased Ca\(^{2+}\) activity. Oxidative stress has been implicated in the pathogenesis in HF.\(^30\) Collectively, this suggests that erythrocytes may become more sensitive to oxidative stress in HF, and ongoing oxidative stress in HF may accelerate eryptosis and contribute to anaemia.

Eryptotic triggers activate Ca\(^{2+}\)-permeable cation channels with subsequent Ca\(^{2+}\) entry. Ca\(^{2+}\) activates Ca\(^{2+}\)-sensitive K\(^{+}\) channels leading to cell shrinkage. Furthermore, the entry of Ca\(^{2+}\) stimulates phospholipids scrambling of the cell membrane with subsequent PS-exposure at the cell surface, which are hallmarks of eryptosis.\(^26\) Altered intracellular Ca\(^{2+}\) handling has important pathological consequences in failing cardiomyocytes.\(^31,32\) In this study, we investigated if altered Ca\(^{2+}\) handling would also affect erythrocytes. Erythrocytes from HF models are more susceptible to the (eryptotic) effects of increased cytosolic Ca\(^{2+}\) entry with or without eryptotic stimulators. The ionomycin effect on PS-exposing cells was also accelerated in REN2 erythrocytes suggesting that Ca\(^{2+}\) sensitivity is increased in REN2 erythrocytes. Therefore, the present study provides evidence that HF may lead to both enhanced Ca\(^{2+}\) entry and enhanced Ca\(^{2+}\) sensitivity, supporting previous studies that highlighted the importance of cellular Ca\(^{2+}\) influx in eryptosis.\(^8\)–\(^13,26\) Overall, this impacts not only the function and survival of erythrocytes, but nucleated cells as well.\(^33\)

Erythrocytes exposing PS at their surface bind to PS receptors on macrophages, which engulf PS-exposing cells.\(^34\) It is known that
PS-exposing erythrocytes are rapidly cleared from the circulation and trapped in the spleen, therefore, only a small increase in circulating PS-exposing cells is observed. Interestingly, the clearance of eryptotic erythrocytes similarly or slightly increased in REN2 rats compared with control SD rats. Therefore, the increase in eryptotic cells cannot be explained by the impaired clearance. Although speculative, the somewhat faster clearance in REN2 animals could reflect increased activity of clearance mechanisms. Our study was, however, not focused on the efficiency of phagocytosis and future studies are required to address this issue. We also observed splenomegaly in REN2 rats and other studies have observed splenomegaly in the setting of eryptosis activation in mice. The splenomegaly may in turn contribute to enhanced erythrocyte clearance. In support of this, CFSE-annexin-V-labelled erythrocytes were cleared faster from the circulation in REN2 rats, and remained present for a longer time in their spleens.

Since the REN2 rat is a monogenetic model of HF, we corroborated our findings by studying additional models of adverse cardiac remodelling with progression towards HF, i.e. the UNX + DOCA salt rat model and murine model of pressure overload (TAC), as well as in human subjects with acute HF. The UNX + DOCA salt rats also displayed anaemia and eryptosis and showed more susceptibility to eryptosis-inducing agents in vitro. We also observed enhanced susceptibility to eryptosis upon oxidative stress in TAC mice. Finally, human subjects with HF have increased eryptosis. From these collective data, we postulate that eryptosis is a newly detected contributor to HF-induced anaemia.

4.1 Limitations

There are limitations that must be acknowledged. The aetiology of anaemia in HF is multifactorial and complex, and therefore it is difficult to dissect what exact contribution eryptosis makes to the anaemic phenotype. We have previously excluded other common causes of anaemia in REN2 rats, for instance deficiency for iron, vitamin B12, and folic acid. The increased plasma creatinine in REN2 rats was associated with renal dysfunction, however, it was not accompanied by decreased EPO production. There is ample evidence that cardio-renal interaction is a key player in HF-associated anaemia. Renal failure is a known risk factor for eryptosis. We did observe renal dysfunction in our REN2 rats was associated with increased plasma creatinine, however, it was not accompanied by decreased EPO production. There is ample evidence that cardio-renal interaction is a key player in HF-associated anaemia. Renal failure is a known risk factor for eryptosis. We did observe renal dysfunction in our REN2 rats was associated with increased plasma creatinine, however, it was not accompanied by decreased EPO production. There is ample evidence that cardio-renal interaction is a key player in HF-associated anaemia. Renal failure is a known risk factor for eryptosis. We did observe renal dysfunction in our REN2 rats was associated with increased plasma creatinine, however, it was not accompanied by decreased EPO production. There is ample evidence that cardio-renal interaction is a key player in HF-associated anaemia.
our findings were corroborated in two pathophysiologically distinct HF models that were not driven by renin overexpression. Finally, we only studied a small number of patients and have no clinical characteristics of these patients. The human data should therefore be considered as preliminary, however, given the consistency of eryptotic tendency between rodents and humans, we consider these data invaluable.

4.2 Clinical implications

Intervention studies targeted against eryptosis may reveal to what extent eryptosis contributes to the anaemic burden. Our pilot study with thymol suggests this may be a feasible approach although the mechanisms underlying this observation were not studied. Thymol is a natural antioxidant which inhibits eryptosis and may influence generation of reactive oxygen species and decrease the cytosolic Ca$^{2+}$ activity, however, the exact mechanisms of action of thymol are unknown. Collectively, our data strongly suggests a role for eryptosis in HF-associated anaemia. Since anaemia is an established predictor of mortality in HF, correction of anaemia may improve prognosis, and we propose that targeting eryptosis should be taken into consideration.

4.3 Conclusion

In conclusion, the present study identifies, for the first time, that eryptosis contributes to HF-associated anaemia. Erythrocytes from rats and humans with HF have higher baseline levels of eryptosis, and failing erythrocytes are substantially more vulnerable to eryptotic stimuli. Our proof-of-concept study suggests that inhibition of eryptosis may be beneficial for the treatment of HF-associated anaemia.

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