Safety and efficacy of iron supplementation after myocardial infarction in mice with moderate blood loss anaemia

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Abstract

Aims  Iron deficiency is frequently observed in patients with acute coronary syndrome and associates with poor prognosis after acute myocardial infarction (AMI). Anaemia is linked to dysregulation of iron metabolism, red blood cell dysfunction, and increased reactive oxygen species generation. Iron supplementation in chronic heart failure is safe and improves cardiac exercise capacity. Increases in iron during ischaemia or immediately after reperfusion are associated with detrimental effects on left ventricular (LV) function. The safety and applicability of iron during or immediately after reperfusion of AMI in anaemia are not known. We aimed to study the safety and efficacy of iron supplementation within 1 h or deferred to 24 h after reperfusion of AMI by analysing LV function and infarct size.

Methods and results  In a mouse model of moderate blood loss anaemia (n = 6–8 mice/group), the effects of iron supplementation (20 mg iron as ferric carboxymaltose per kg body weight) within 1 h and deferred to 24 h after ischaemia/reperfusion were assessed. Cardiac function was analysed in vivo by echocardiography at baseline (Day 3) with and without anaemia, after AMI (24 h), and after administration of intravenous iron. Anaemia was characterized by iron deficiency and a trend towards increased haemolysis, which was supported by increased plasma free-haemoglobin [sham vs. anaemia (n = 8/group); P < 0.05]. Anaemia increased heart rate, LV end-diastolic volume, stroke volume, and cardiac output, while LV end-systolic volume remained unchanged at baseline. Superimposition of AMI deteriorated global LV function, whereas infarct sizes remained unaffected [sham vs. anaemia (n = 6/group); P = 0.9]. Deferred iron supplementation 24 h after ischaemia/reperfusion resulted in reversal of end-systolic volume increase and reduced infarct size [% of area at risk: sham vs. anaemia + iron after 24 h; (n = 6/group); 48 ± 7 vs. 38 ± 7; P < 0.05], whereas administration within 1 h after reperfusion was neutral [sham vs. anaemia + iron; (n = 6/group); 48 ± 7 vs. 42 ± 8; P = 0.56]. Moreover, iron application after reperfused AMI showed unaltered mortality compared with sham.

Conclusions  Iron supplementation 24 h after reperfusion of AMI is safe and reversed enlargement of end-systolic volume after AMI resulting in increased stroke volume and cardiac output. This highlights its potential as adjunctive treatment in anaemia with ID after reperfused AMI. Time point of iron application after reperfusion appears critical.

Keywords  Iron deficiency; Anaemia; Acute myocardial infarction; Drug safety

Introduction

In the past two decades, the mortality of ST-elevation myocardial infarction (STEMI) patients has been reduced due to effective early reperfusion therapy, especially primary percutaneous coronary interventions (pPCI).1,2 Anaemia is associated with post-procedural complications with adverse in-hospital outcomes and mortality after pPCI.3–5 In
addition, 30% of the patients with acute myocardial infarction (AMI) present with decreased baseline haemoglobin (Hb) concentrations,6 which is correlated to a 15% to 25% risk of developing congestive heart failure (HF) during and after the event.7–9 Anaemia itself is associated with reduced iron content in blood and thus iron deficiency (ID).

Iron is a fundamental trace element for various cellular processes and is important in the respiratory chain of mitochondria, and essential for cardiomyocyte contractility.10 Experimental animal models reported mitochondrial iron accumulation after cardiac ischaemia/reperfusion (I/R) injury, associated with significantly impaired cardiac contractility by increased reactive oxygen species (ROS) in cardiomyocytes. Both blocking ferroptosis and chelating iron were cardioprotective in a mouse model of myocardial I/R injury.11,12 Application of the iron chelator deferoxamine (DFO) before and during coronary occlusion did not prevent the loss of systolic wall function that occurred during ischaemia, but significantly enhanced the recovery of segmental shortening.13 Scavenging of iron through DFO administered at the first 15 min of post-ischaemic reflow resulted in greater recovery of myocardial function energy metabolism, and decreased infarct size (IS) in canines14 pointing towards potential deleterious side effects of iron during ischaemia and early reperfusion. In STEMI patients, intravenous (i.v.) iron-oxide administration 4 days after AMI improved infarct healing thereby demonstrating beneficial effects on global left ventricular (LV) remodelling by decreased end-systolic volume (ESV).15 Adjunctive DFO treatment of STEMI patients prior, during, and after pPCI demonstrated significant reduction of indexes of oxidative stress without limiting IS.16 Furthermore, in patients undergoing coronary artery bypass grafting, i.v. DFO infusion reduced ROS production and protected the myocardium against reperfusion injury, especially in patients with reduced LV ejection fraction.17

These results indicate that the time point of iron treatment during AMI and after reperfusion is critical: while iron application prior or during ischaemia, and immediately prior to reperfusion appears detrimental, it seems beneficiary in chronic ischaemic heart disease,18 while its effects early within 1 h or deferred to 24 h after reperfusion are not clear. Trials in chronic heart failure (CHF) patients with LV ejection fraction < 40% demonstrated positive effects of iron supplementation.19,20 In CHF patients with absolute or functional ID with or without anaemia, i.v. administration of iron improved symptoms and exercise capacity. However, the safety and effectiveness of iron supplementation in STEMI and after pPCI of anaemia is not known.21 Therefore, the present study aimed to determine the safety and efficacy of iron supplementation within 1 h or deferred to 24 h after reperfusion in a model of moderate blood loss anaemia with ID and superimposed AMI.

Materials and methods

Study approval

All experiments were performed using protocols approved by LANUV (Landesamt für Natur, Umwelt- und Verbraucherschutz Nordrhein-Westfalen, AZ: 84-02.04.2018.A234). The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Furthermore, every experiment was based on the guidelines of the Federation of European Laboratory Animal Science Associations.

Induction of moderate blood loss anaemia

All experiments were performed with C57BL/6j mice obtained from the Janvier Labs (Saint-Berthevin, France). Anaemia was induced in healthy 10- to 12-week-old male mice by repetitive collection of blood from the facial vein as previously described.22 The amount of every blood draw was less than 15% of the total circulating blood volume, which was calculated per kg BW of each mouse. To compensate for the blood loss, mice were injected with an equal volume of saline (0.9%) one-half intraperitoneally and the second half subcutaneously. To analyse the complete blood count, blood (30–40 μL) was collected from the facial vein and placed in an ethylenediaminetetraacetic acid-coated microcentrifuge tubes. The complete blood count was analysed using Scil Vet abc™ haematology analyser following the manufacturer’s instructions (scilVet, Germany).

Chemicals

Ferinject® (50 mg iron/mL) was purchased from Vifor Pharma AG (Deutschland) and diluted 1:10 (v/v) in saline (0.9%). All mice were injected with 20 mg iron per kg BW Ferinject® through a tail vein in a volume of 100 μL. For each time point, control and anaemic mice came from the same cohort and were processed simultaneously to minimize cohort-to-cohort variability, yielding two to three evaluable samples per group per time point.

Experimental groups

Mice were randomly allocated to four experimental groups (Figure 1, Groups A–D). AMI was performed as described previously.24 Pre-AMI transthoracic echocardiography was performed in sham mice (Group A) and anaemic mice (Groups B–D) after induction of anaemia for 3 days. Iron was not administered in Groups A–B. In anaemic Group C mice, iron (i.v. 20 mg per kg BW) was administered...
immediately after 1 h of AMI, whereas in Group D, it was administered 24 h after AMI. In groups A–B, post-AMI echocardiography was performed after 24 h. In Groups C and D, echocardiographic analysis was performed 24 h after iron application. All mice were sacrificed after post-AMI echocardiography, hearts were isolated for triphenyl tetrazolium chloride (TTC) staining, and IS was determined.

Circulating cell-free-haemoglobin, haptoglobin, erythropoietin, ferritin, and soluble transferrin receptor

To analyse iron and haemolysis after anaemia induction, a separate group of mice were used for the experiments. After 3 days of anaemia induction, mice were sedated with 100 mg/kg of ketamine (Ketanest®) and 10 mg/kg of xylazine (Rompun®), and approximately 1 mL of blood was withdrawn by heart puncture. Blood from anaemic and non-anaemic control mice was collected in heparinized tubes and centrifuged at 3000g for 5 min at 4°C. Plasma was collected and immediately frozen at −80°C until further analysis. Cell-free plasma Hb, haptoglobin, and transferrin were analysed using respective Mouse ELISA Kits according to the manufacturer’s instructions (Abcam; Cambridge, UK). Erythropoietin (Epo), ferritin, and soluble transferrin receptor (sTfR) were analysed in plasma using the respective Mouse ELISA Kits from MyBioSource (San Diego, USA).

In vivo analysis of global left ventricular function and infarct size

Serial echocardiographic measurements of global LV function at baseline (Day 3; pre-AMI), and post-AMI, precisely after i.v. iron supplementation were conducted in all groups (A, B, C, & D). LV function in anaemia was assessed by recording cardiac images using a Vevo 2100 high-resolution ultrasound scanner with an 18–38 MHz linear array microscan transducer (Visual Sonics Inc., Toronto, Canada). LV function was analysed by using B-mode images to determine LV volumes, stroke volume (SV), cardiac output (CO), and heart rate (HR) of maximal and minimal cross-sectional areas.25,26 Area at risk and IS were determined by post-mortem left anterior descending artery re-occlusion, Evans blue infusion and staining with TTC as previously described.26
Statistics

Unless otherwise stated, data are presented as mean ± standard deviation. Multiple comparisons were performed by two-way ANOVA for repeated measures (anaemia vs. sham, time course). Differences among groups were tested by two-way ANOVA with Tukey’s post-hoc test. For comparison of two groups, an unpaired Student’s t-test was used. Investigations of LV function by echocardiography were analysed by paired Student’s t-test. A P value of <0.05 was considered significant. Data were analysed with Prism (GraphPad Software, Version 6.05).

Results

Induction of moderate anaemia

The mouse model of subacute blood loss anaemia resulted in an Hb level of approximately ≤9.0 g/dL as compared with sham mice (Table 1). With induction of anaemia red blood cell (RBC) morphology changed, as evidenced by increased anisocytosis, rouleaux formation, and decreased mean corpuscular volume (MCV), mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, and increased red blood cell distribution width (RDW). Increased levels of MCV and RDW after iron administration are indices of an augmented erythropoiesis, thus representing an accelerated turnover of RBC in anaemia with a raised number of young RBCs, which are characterized by increased RBC sizes and volume (Table 1). We analysed the recovery of Hb levels in a separate group of mice, which showed completely re-established morphology and Hb concentrations within 5 days (data not shown). Intravenous iron was administered within 1 h and after 24 h after reperfusion of AMI (Figure 1). Iron treatment affects blood parameters measured 24 h after reperfusion as demonstrated by increased MCV and RDW (Table 1).

Characterization of iron metabolism and inflammatory response in blood loss anaemia

To investigate the exact effects of anaemia on iron metabolism, we did not select a pharmacological or genetically exhausted mouse model for ID anaemia, but induced blood loss anaemia by repetitive and moderate bleeding of mice. As RBC contains iron, daily blood loss was associated with a loss of cellular iron resulting in ID. In addition, increased plasma cell-free Hb (Figure 2A) reflected mild haemolysis.

Table 1 Blood count with laboratory characteristics of anaemia

| Anaemia | Sham | + | Sham | + | + | + |
|---------|------|---|------|---|---|---|
| Group   | Pre-AMI | Post-AMI |
| Iron (time after I/R) | A | B | A | B | C | D |
| Condition | n = (numbers/group) | (n = 6) | (n = 8) | (n = 6) | (n = 6) | (n = 6) | (n = 6) |
| Haemoglobin (g/dL) | | 14.1 ± 0.5 | 9.0 ± 0.5*** | 13.2 ± 0.3 | 9.3 ± 0.5*** | 9.7 ± 1.9*** | 10.7 ± 1.2* ## |
| Haematocrit (HCT, %) | | 46.0 ± 1.6 | 27.6 ± 1.7*** | 43.2 ± 2.4 | 30.2 ± 2.7*** | 29.2 ± 5.3*** | 33.6 ± 3.2***$ |
| MCV (μm³) | | 50.2 ± 0.4 | 53.4 ± 1.2 | 53.0 ± 1.4## | 56.7 ± 1.6*# | 56.5 ± 4.3**§§ | 61.0 ± 1.7***##$$ |
| MCH (pg) | | 15.4 ± 0.4 | 17.7 ± 0.4*** | 15.9 ± 0.6 | 17.6 ± 1.2## | 18.4 ± 0.8** | 19.5 ± 0.5*** |
| MCHC (g/dL) | | 30.6 ± 0.9 | 33.3 ± 0.6* | 30.6 ± 0.9 | 33.3 ± 0.6* | 33.3 ± 1.3** | 32.1 ± 1.1** |
| RDW (%) | | 13.8 ± 0.3 | 14.8 ± 0.8 | 15.4 ± 0.9 | 16.7 ± 1.3*# | 16.0 ± 0.6**§§ | 20.2 ± 1.3***##$$ |
| RBC (10⁶/μL) | | 9.2 ± 0.3 | 5.2 ± 0.3*** | 7.9 ± 0.5## | 5.3 ± 0.6*** | 5.3 ± 1.4*** | 5.5 ± 0.6*** |
| BC (10³/μL) | | 7.7 ± 2.3 | 7.9 ± 3.9 | 9.6 ± 3.5 | 7.5 ± 3.5 | 5.4 ± 1.2 | 8.6 ± 2.4 |
| Platelets (10³/μL) | | 1450.7 ± 147 | 1759.0 ± 107 | 1318.5 ± 340 | 1397.8 ± 491$ | 1794.0 ± 196 | 1994.0 ± 152* |

Induction of anaemia resulted in changes in haemoglobin and haematocrit levels. Anaemic mice were characterized by thrombocytosis with enhanced platelet counts at baseline. Additional treatment of intravenous iron did not affect normal blood counts. After reperfused AMI, anaemic mice showed increased MCV and RDW as compared with pre-AMI, while MCH and MCHC remained unaffected. Data are mean ± SD from n = 6–8 mice/group. Two-way ANOVA with Tukey’s multiple comparisons test. AMI, acute myocardial infarction; I/R, ischaemia/reperfusion; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cell; RDW, red blood cell distribution width; WBC, white blood cell.

*P < 0.05 vs. sham,
**P < 0.01 vs. sham,
***P < 0.001 vs. sham,
*P < 0.05 vs. pre-AMI,
**P < 0.01 vs. pre-AMI,
***P < 0.001 vs. pre-AMI,
*P < 0.05 vs. anaemia,
**P < 0.01 vs. anaemia,
$P < 0.05 vs. anaemia + iron after 24 h,
$P < 0.01 vs. anaemia + iron after 24 h,
$$P < 0.001 vs. anaemia + iron after 24 h.
Iron supplementation after reperfusion improves left ventricular function after acute myocardial infarction

At baseline, HR, end-diastolic volume (EDV), and SV were increased in moderate anaemia resulting in an increased CO to compensate for reduced oxygen-carrying capacity as compared to sham mice. Additionally, our recent study demonstrated that differences in LV function between anaemic and sham mice persisted after 4 h up until 1 week following perfused AMI. The LV function and LV transformation continued to be reduced even though Hb concentrations were restored (85.0 ± 2.0 on Day 7 post-AMI vs. 89.0 ± 4.0 g/L on Day 0, P < 0.05; n = 5/group). Iron supplementation 24 h after reperfused AMI led to a reversal of ESV enlargement post-AMI with anaemia and was associated with a trend of improved SV and CO (Figure 3). These beneficial effects were seen only modestly with iron application within 1 h after reperfused AMI.

Infarct size in anaemia after iron supplementation in reperfused acute myocardial infarction

Anaemic mice were subjected to induction of AMI followed by treatment with or without a single dose of iron 1 or 24 h after reperfusion. The mean IS after reperfused AMI evaluated by TTC staining was significantly smaller after 24 h of iron administration, whereas early application within 1 h was associated only with a trend of IS reduction (Figure 4). The body weight was comparable in mice of the anaemia and sham groups after surgery [body weights in g; sham (27.25 ± 1.64) vs. anaemia (26.83 ± 0.45); P = 0.893]. Anaemia was associated with increased mortality during ischaemia, whereas iron supplementation within 1 or 24 h post-I/R injury demonstrated no further increase in mortality in respective treatment groups, suggesting that iron supplementation may be safe in the early phase of post-AMI.

Discussion

In summary, this experimental model of reperfused AMI with anaemia shows the following:

- LV compensates the diminished oxygen-carrying capacity in anaemia via increases in HR and EDV consecutively with improved SV and CO.
- This compensation is lost in AMI.
- Reversal of ID in anaemia increases SV post-AMI via a distinct mechanism by limiting enlargement of ESV.
- The time point of iron supplementation after reperfusion is critical.
- Reversal of ID in anaemia post-AMI is effective 24 h after reperfusion and appears to be safe.
- The molecular pathways of this mechanism have to be determined.
- The findings appear to be relevant in planning of future clinical trials aiming to validate the benefit of iron supplementation in patients with anaemia and STEMI, as time points of application after AMI appear to be critical.

Our mouse model of moderate anaemia induced by repetitive blood loss tend to mimic the patterns of anaemia in hospitalized patients with cardiovascular diseases characterized by altered morphology of the erythrocytes, the integrity of the membrane, haemolysis, inflammatory response, and ID. Cardiac function in anaemia was characterized by increased HR, EDV, and consecutively increased CO to compensate for the reduced oxygen-carrying capacity of the blood. Iron supplementation in anaemia 24 h after reperfused AMI had no effects on EDV, but led to a reversal of ESV increase. Reduced ESV led consecutively to improved SV and CO. Early treatment of iron immediately within 1 h demonstrated no additional benefits. In both protocols, iron administration was safe and did not affect mortality. Thus, the present study supports the notion that iron administration at least in ID-associated anaemia appears safe and beneficial when applied 24 h after reperfusion of AMI.

Anaemia and iron deficiency

Anaemia and absolute or relative ID are common entities that coexist in patients with CHF, which are associated with poor clinical status and worse outcomes after AMI. Hb levels of anaemic patients have been shown to correlate with the prognosis and mortality after acute adverse events in hospitals, especially in patients with AMI. Measurement of ferritin, TSAT, sTfR, and the sTfR–ferritin index are more accurate than classical red cell indices to delineate ID in anaemia. In our study, anaemic wildtype mice also exhibited features of iron-restricted erythropoiesis such as increased expression of sTfR and Epo, while TSAT remained unchanged and plasma transferrin showed a tendency to decrease.

It has been recognized that patients with HF show an increased tendency to develop ID, as a consequence of the depletion of iron stores or defective iron absorption.
Figure 2  Blood loss anaemia is associated with iron deficiency and haemolysis. Data are mean ± SD from n = 8 (A–F), n = 7 (G) mice/group. Unpaired t-test; n.s. = not significant; *P < 0.05 vs. sham

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Figure 3  Analysis of LV function in anaemic mice with and without iron supplementation after reperfused AMI. LV function of sham and sham anaemic mice were analysed at baseline (Day 3) and 24 h after reperfused AMI. Anaemic mice were treated with i.v. iron 1 and 24 h after reperfused AMI and underwent echocardiographic analysis. Anaemia was associated with increases in heart rate (HR), end-diastolic volume (EDV), stroke volume (SV), and cardiac output (CO). Superimposition of AMI further impaired global left ventricular (LV) function in anaemia. The increase in end-systolic volume (ESV) associated with AMI is in part rescued by application of iron 24 h post-AMI (C). Data are presented as mean ± SD from $n = 6–8$ mice/group. Multiple comparison with two-way ANOVA with Tukey post-hoc test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. sham; *$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. pre-AMI; and $$P < 0.01$ vs. anaemia. AMI, acute myocardial infarction.
correction of ID with the use of intravenous iron in patients with CHF has been reported with good safety profiles overall.\textsuperscript{19} Despite these beneficial effects of reduced rate of rehospitalization, increased cardiac exercise capacity, and improvement in the quality of life, insufficient data exist whether potential beneficial or harmful effects of iron application given during either ischaemia or immediately after reperfusion in STEMI patients.

Iron administration in the setting of AMI remains controversial because of its potential toxicity, including oxidative stress induction and preventing cardiomyocyte contractility. In iron therapies intended to improve AMI, the dose and time...
point of administration must be taken into the consideration, as higher iron concentrations generate oxidative stress and activate programmed apoptosis.\textsuperscript{32,33} Additionally, lower concentrations of iron promote cell survival through increased inducible nitric oxide synthase activity and increased nitric oxide production.\textsuperscript{34,35} In this study, we applied a dose of i.v. iron (20 mg iron per kg BW), which is equivalent to that evaluated in clinical trials in HF patients.\textsuperscript{19} Superimposition of AMI in anaemic mice impaired LV functional compensation and increased mortality during ischaemia the lower the Hb levels were.\textsuperscript{7,25} These findings are in line with clinical studies that demonstrated increased mortality associated with lower Hb levels (below 90 to 80 g/L).\textsuperscript{36} Consistent with other studies,\textsuperscript{16} we did not observe differences in LV function and IS between non-anaemic and anaemic mice post-AMI. The reason for this observation is partly also explained by a distinct mortality rate in anaemic mice after AMI and resulted decrease in sample size. In our previous study, we showed that AMI resulted in a mortality of 33\% in anaemic mice compared with 2–3\% in the sham group, ratios that were corroborated in the present study. Thus, anaemic mice with larger infarcts may have died earlier thus affecting the comparison of surviving anaemic and non-anaemic mice. Overall, increasing sample size will be considered for future experiments.

Iron administration within 1 h after I/R was neutral with respect to changes in LV function and potential side effects. Administration 24 h after I/R injury showed positive side effects on myocardial function as demonstrated by the enhanced recovery of LV function through reversal of ESV increase and unchanged EDV. While several experimental studies unravelled harmful side effects of iron given during ischaemia and early reperfusion, our data imply that application of iron at least in anaemia with superimposed AMI is safe when deferred with a time delay of 24 h after reperfusion of AMI and early enough to impact on early LV remodelling post-AMI.

Efficacy of iron application to modulate left ventricular function after acute myocardial infarction

Several studies demonstrated that the time point of iron treatment during AMI appears harmful in the acute phase of AMI, in particular during reperfusion, which is caused by mitochondrial iron accumulation and leads to impaired cardiac contractility due to increased ROS within the cardiomyocytes.\textsuperscript{33} Besides, these toxic effects of early iron application during or prior to reperfusion were prevented by the administration of iron chelator DFO 15 min prior to and 15 min throughout of coronary occlusion of reperfusion, which showed a cardioprotective effect through improved myocardial high-energy phosphate metabolism and LV contractility after a period of global ischaemia.\textsuperscript{13} These data support that iron catalysis is involved in the production of oxygen-derived free radicals during I/R injury.\textsuperscript{11,14} In contrast, iron application in chronic ischaemic HF appears beneficial.\textsuperscript{20} No data on the effectiveness of iron supplementation 1 h or after 24 h after reperfusion of AMI in anaemia exist. In the acute phase of reperfusion after AMI deferring, iron application to 24 h appears as an attractive time window to modulate initial LV remodelling.

Conclusions

In conclusion, in anaemia, iron administration 24 h after reperfusion of AMI is associated with a reduced ESV and a trend to decreased IS. These findings highlight the potential of iron treatment in anaemia with ID as an adjunctive treatment in the reperfusion after AMI. We have shown that the time point of iron application is critical, which needs to be proven in clinical studies.

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Conflict of interest

None declared. FF is an employee of Vifor Pharma Management Ltd.

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Data availability statement

The authors declare that all supporting data of this exploratory study are available in the article.
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