Original Paper

DMT1-Mutant Erythrocytes have Shortened Life Span, Accelerated Glycolysis and Increased Oxidative Stress

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Key Words

DMT1 • Eryptosis • Oxidative stress

Abstract

Background/Aims: Deficiency of the divalent metal transporter 1 (DMT1) leads to hypochromic microcytic anemia. We have previously shown that DMT1 deficiency impairs erythroid differentiation and induces apoptosis of erythroid cells. Here we analyzed metabolic processes and survival of mature erythrocytes in order to address potential involvement of erythrocyte defect in the pathophysiology of the disease. **Methods:** FACS analysis was used to determine the half-life of erythrocytes (CFSE fluorescence), phosphatidylserine exposure (Annexin V binding), cytosolic Ca^{2+} (Fluo3/AM fluorescence) and reactive oxygen species (ROS; DCF fluorescence). Enzyme activities were determined by standard biochemical methods. The concentration of ATP and ADP was measured on HPLC-MS/MS. **Results:** We observed an accelerated clearance of CFSE-labeled DMT1-mutant erythrocytes from circulating blood when compared to wild-type erythrocytes. *In vitro*, DMT1-mutant erythrocytes showed significantly increased Annexin V binding after exposure to hyperosmotic shock and glucose depletion. Despite exaggerated anti-oxidative defense, higher ROS levels were present in DMT1-mutant erythrocytes. Accelerated anaerobic glycolysis and reduced ATP/ADP ratio detected in DMT1-mutant erythrocytes indicate enhanced demand for ATP. **Conclusions:** We propose that DMT1 deficiency negatively affects metabolism and life span of mature erythrocytes; two other aspects of defective erythropoiesis which contribute to the pathophysiology of the disease.

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Introduction

Divalent metal transporter 1 (DMT1; also known as NRAMP2 and SLC11A2) is an intestinal and endosomal iron (Fe^{2+}) transporter [1]. Mutations inactivating DMT1 are associated with a severe defect in erythroid iron utilization and cause moderate to severe hypochromic microcytic anemia in human patients [2-6] and two rodent models, *mk/mk* mice [7] and Belgrade rats [8]. In addition, DMT1 knockout animals (*Slc11a2*^{-/-}) die in the first week of life due to iron deficient erythropoiesis [1].

It has been shown that nutritional iron deficiency [9] and certain types of congenital hypochromic anemia such as thalassemia and sickle cell disease [10] are associated with shortened life span of erythrocytes. The accelerated clearance of erythrocytes can be attributed to excessive hemolysis and/or induction of programmed cell death of erythrocytes, called eryptosis [11]. This suicidal death of erythrocytes is characterized by cell shrinkage, cell membrane blebbing and cell membrane phospholipid scrambling. Eryptosis is triggered by oxidative stress, hyperosmotic shock and glucose depletion likely via activation of Ca^{2+} channels leading to an increase in the concentration of cytosolic Ca^{2+} [12, 13].

We have previously shown that DMT1 deficiency leads to an impaired erythroid differentiation hallmarked by accumulation of immature forms of erythroblast, accelerated apoptosis of erythroid precursors and diminished survival of erythroid progenitors [14]. In the presented study we aimed to explore whether eryptosis contributes to the pathophysiology of anemia associated with mutations in DMT1.

Materials and Methods

Animals

Heterozygous mice bearing the G185R DMT1 mutation (*mk/+*, strain 129S6/SvEvTac) [1] were provided by M. Fleming (Children's Hospital, Boston, USA). Mice were fed a standard diet; 10- to 16-week-old *mk/mk* mice and their wild-type (wt) littermates were included in the study. Experiments were performed in accordance with the Palacky University Institutional Animal Care and Use Committee. Where indicated, erythrocytes from tail vein or heart puncture were depleted from reticulocytes on Percoll (GE Healthcare Life Sciences, United Kingdom, $\rho=1.130\pm0.005$ g/ml) using density gradient centrifugation [15].

Half-life of fluorescently labeled erythrocytes

Erythrocytes of wt and DMT1-mutant mice were isolated from blood by low speed centrifugation followed by two washes with Ringer solution according to Qadri et al. [16]. Separated erythrocytes were stained *in vitro* with the carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Invitrogen, CA, USA) as previously described [17]. The efficiency of CFSE-labeling (>99%) was determined by flow cytometry (FACS Calibur, BD Biosciences, New Jersey, USA). Fluorescently labeled erythrocytes (1×10^9 in 100 μl of PBS) were injected into the tail veins of wt mice. The intensity of erythrocyte fluorescence was measured using the tail vein blood taken on selected days following the injection. The percentage of CFSE-positive erythrocytes determined two hours post-injection was considered as 100%.

Assessment of hemolysis

The *in vitro* hemolysis test was performed as previously described [18]. Erythrocytes (0.4% hematocrit) were incubated at 37°C for 24 hours; thereafter the absorbance at 540 nm was read in the supernatant (spectrophotometer Infinite 200 Nanoquant; Tecan, Switzerland). In the cryohemolysis test, the erythrocytes were resuspended in pre-heated hypertonic sucrose solution (700 mM in 0.9% w/v sodium chloride solution, pH 7.4), incubated 10 min at 37°C and transferred on ice [19]. After additional 10 min the absorbance at 540 nm was read in the supernatant. 100% hemolysate in both hemolysis tests was prepared by mixing the erythrocyte solution with distilled water. Bilirubin levels [μM] in the plasma were measured according to Cruickshank [20] and calculated using the formula: (absorbance at 453 - absorbance at 578) x dilution.

Erythrocyte Annexin V binding in vitro

Percoll-separated erythrocytes were *in vitro* exposed to hyperosmotic shock (700 mOsm) and glucose depletion, respectively [9]. Annexin V binding to erythrocytes was analyzed 5 and 7 hours later using Annexin V/FITC kit (BD Biosciences, New Jersey, USA). Fluorescence intensity was measured by FACS Calibur.

Determination of intracellular Ca^{2+}

Erythrocytes separated on Percoll (2×10^7 /ml) were loaded with 2 μ l of a 2 mM Fluo3/AM (Calbiochem, Bad Soden, Germany) for 15 min at 37°C [21]. Thereafter, 2 μ l of Fluo3/AM were added for additional 25 min. To obtain a positive control, labeled erythrocytes were exposed to 1.0 μ M ionomycin for 10 min [21]. Ca^{2+} -dependent fluorescence intensity was measured by FACS Calibur.

Determination of reactive oxygen species (ROS)

Percoll-separated erythrocytes (2×10^7 /ml) were incubated with 0.4 mM 2',7'-dichlorofluorescein diacetate (DCF; Sigma-Aldrich, Germany) for 15 min at 37°C according to Amer et al. [22]. For a positive control, wt erythrocytes were exposed to 2 mM H_2O_2 for 10 min before DCF-labelling. DCF-dependent intensity of fluorescence was measured by FACS Calibur.

Enzyme assay

The activity of enzymes involved in anaerobic glycolysis, oxidative-defense and nucleotide metabolism was determined according to the methods recommended by the International Committee for Standardization in Haematology [23], with the use of leukocytes- and platelets-free erythrocyte lysates as we previously described [24, 25]. The measurements were adopted for mouse samples. Briefly, the enzyme reaction was pre-incubated for 10 min at 37°C, thereafter substrate and erythrocyte lysate were added. Absorbance was measured at 340 nm in 1 min intervals for 20 min at 37°C (spectrophotometer Infinite 200 Nanoquant) and specific enzyme activity was calculated using the Lambert-Beer law. All chemicals and purified enzymes were purchased from Sigma-Aldrich.

Real-time PCR for hypoxia regulated genes

Reticulocytes were separated from peripheral blood as previously described [26]. Total reticulocyte RNA was isolated using TRI Reagent (Sigma-Aldrich), reverse-transcribed using SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, NY, USA) according to manufacturer's instructions and used for quantitative real-time Polymerase Chain Reaction (qPCR). Transcript levels of *Foxo3A* [27], *Runx1*, *Tfrc* and *Hk1* [28] were measured by real-time PCR using gene specific Taqman probes: (Mm01185722_m1, Mm01213404_m1, Mm00441941_m1 and Mm00439344_m1, respectively). The data were normalized to the expression of beta-actin (Actb; 4352341E) and to mRNA levels of wt reticulocytes. The statistical significance of relative expression changes in target mRNA levels was analyzed using the REST© 2009 software [29].

Measurement of ATP/ADP

ATP and ADP were assayed by reverse phase HPLC-MS/MS with ionization atmospheric pressure (HPLC Dionex Ultimate 3000 MS, Thermo Scientific, USA; LC/MS/MS System MDS API 3200, Applied Biosystems, USA) in freshly prepared erythrocyte lysates extracted with 5% trichloroacetic acid (Sigma-Aldrich).

Statistical analyses

The Student's t-test was used to determine the statistical significance of the results. *P* values were calculated using the Origin 6.1 software (OriginLab Corporation, Norhampton, MA, USA). *P* values less than 0.05 were considered to be statistically significant.

Results*Accelerated clearance of DMT1-mutant erythrocytes in vivo*

Homozygous DMT1-mutant (*mk/mk*) mice exhibit severe hypochromic microcytic anemia (mean corpuscular volume, MCV 37.2 ± 1.8 fl vs. 54.3 ± 0.8 fl in wt mice), reticulocytosis and splenomegaly [14]. We first focused on the *in vivo* erythrocyte survival. Erythrocytes

Fig. 1. Clearance of normal and DMT1-mutant erythrocytes from circulating blood. The percentage of CFSE-labeled DMT1-mutant erythrocytes is lower when compared to wt erythrocytes at indicated time-points post injection. Values are given as % of CFSE-labeled erythrocytes determined two hours after the injection into the wt mice (means $\pm$ SDs, n = 4 each). Some SDs are smaller than the respective symbol. h: hours, d: days, **P<0.01, *P<0.05.

Table 1. Determination of hemolysis and bilirubin levels (values indicate means $\pm$ SDs, n=3-5 for wt mice, n=3-9 for DMT1-mutant mice)

	Hemolysis <i>in vitro</i> [%]	Cryohemolysis [%]	Bilirubin [μ M]
wt	9.9 $\pm$ 0.5	21.8 $\pm$ 3.9	9.2 $\pm$ 4.8
DMT1-mutant	10.5 $\pm$ 1.4	17.6 $\pm$ 3.8	9.0 $\pm$ 3.6

from DMT1-mutant mice and wt controls were *in vitro* labeled with CFSE fluorescent dye and injected into the wt mice. Blood was taken from injected mice, and percentage of circulating CFSE-positive erythrocytes was calculated in the respective time periods. The labeling of wt and DMT1-mutant erythrocytes was comparable, as determined by CFSE fluorescence at 2 hours after the injection (not shown). As shown in Fig. 1, we observed an accelerated clearance of CFSE-labeled DMT1-mutant erythrocytes from circulation when compared to wt erythrocytes; the difference in the percentage of CFSE-positive erythrocytes was significant at day 10 (P=0.016), 14 (P=0.006), 19 (P=0.011) and 30 (P=0.038). This result indicates faster removal of DMT1-mutant erythrocytes from circulating blood *in vivo*.

Exclusion of the erythrocyte membrane integrity defect

We next assessed the membrane integrity by the *in vitro* hemolysis assays. In the first test the hemolysis was determined after 24 hour incubation of erythrocytes in isotonic Ringer solution at 37°C. The percentage of hemolysis was 9.9 $\pm$ 0.5 for wt erythrocytes and 10.5 $\pm$ 1.4 for DMT1-mutant erythrocytes (Table 1). Neither the second test, the cryohemolysis test, revealed any differences between the DMT1-mutant mice and their wt littermates; the hemolysis was 17.6 $\pm$ 3.8% and 21.8 $\pm$ 3.9% respectively (Table 1). In agreement, also the plasma bilirubin levels were comparable (wt mice: 9.2 $\pm$ 4.8 μ M; DMT1-mutant mice: 9.0 $\pm$ 3.6 μ M) (Table 1). These analyses excluded hemolysis as the cause of shortened lifespan of erythrocytes.

Increased destruction of DMT1-mutant erythrocytes in stress conditions

To evaluate possible involvement of eryptosis in the accelerated clearance of DMT1-mutant erythrocytes, we assessed the phosphatidylserine exposure on erythrocyte membrane. Erythrocytes were maintained *in vitro* in Ringer solution and Annexin V binding to exposed phosphatidylserines was determined using FACS. Under physiological conditions (5 hours in isotonic Ringer solution), DMT1-mutant erythrocytes showed significantly enhanced Annexin V binding when compared to wt erythrocytes (3.1 $\pm$ 0.9% vs. 10.0 $\pm$ 6.1%, P=0.021, n=6 each). A short exposure of erythrocytes to hyperosmotic shock (700 mM Ringer) increased the percentage of Annexin V-positive cells for DMT1-mutant mice (to 25.4 $\pm$ 7.6% at 5 hours and to 43.5 $\pm$ 10.8% at 7 hours) while the proportion of Annexin V-positive wt erythrocytes remained almost unchanged (Fig. 2A). More dramatic effect on phosphatidylserine exposure was obtained under glucose-depleted conditions, where

Fig. 2. Annexin V binding after exposure to stress conditions *in vitro*. DMT1-mutant erythrocytes showed increased Annexin V binding after 5 and 7 hour (h) exposure to hyperosmotic shock (Panel A) and glucose depletion (Panel B) *in vitro* when compared to wt erythrocytes. Original histograms of representative measurements are also shown (right). Values are given as mean $\pm$ SDs (n = 4, each). ***P<0.001, **P<0.01.

Fig. 3. Intracellular Ca^{2+} content. The intensity of fluorescence, which reflects the intracellular Ca^{2+} concentration, is higher in DMT1-mutant erythrocytes when compared to wt erythrocytes. Ionomycin treated erythrocytes served as positive controls for increased Ca^{2+} content. Representative histograms of Ca^{2+} -dependent fluorescence are also shown (bottom). Values are given as mean $\pm$ SDs (n = 3 - 5). ***P<0.001, **P<0.01, *P<0.05.

significant increase in Annexin V positivity was observed for both wt ($10.2 \pm 5.7\%$) and DMT1-mutant ($35.8 \pm 6.9\%$) erythrocytes and the proportion further increased with prolonged incubation (Fig. 2B). These results confirmed increased phosphatidylserine exposure on the membrane of DMT1-mutant erythrocytes and their enhanced susceptibility to stress. As cell shrinkage is another characteristic feature of eryptosis, reduction in cell volume (detected by lower forward scatter, FS) has become obvious after 7 hour incubation under

Fig. 4. Determination of reactive oxygen species (ROS). DMT1-mutant erythrocytes have increased intracellular ROS content when compared to wt erythrocytes as determined by increased intensity of DCF-dependent fluorescence. Wt erythrocytes exposed to 2 mM H_2O_2 served as a positive control for high ROS levels. Representative histograms of DCF-dependent fluorescence are also shown (bottom). Values are given as mean $\pm$ SDs ($n = 3$, each). *** $P < 0.001$, * $P < 0.05$.

both stress conditions when compared to isotonic Ringer solution. The mean FS value of wt erythrocytes has reduced from 422 ± 13 to 353 ± 11 for hyperosmotic shock and to 404 ± 3 for glucose depletion; mean FS value of DMT1-mutant erythrocytes has reduced from 235 ± 6 to 216 ± 3 for hyperosmotic shock and to 218 ± 10 for glucose depletion.

Elevated levels of cytosolic Ca^{2+} in DMT1-mutant erythrocytes

Previous study suggested that G185R mutation in DMT1 leads to a gain of constitutive Ca^{2+} permeability [30]. As it is known that eryptosis is triggered by cytosolic Ca^{2+} activity [11] we assessed the concentration of Ca^{2+} within erythrocytes. For this purpose, erythrocytes were loaded with a Ca^{2+} -sensitive fluorescence dye Fluo3/AM and the intensity of fluorescence was measured by FACS [21]. Ionomycin-treated wt erythrocytes were used as positive controls. As it is shown in Fig. 3, the mean intensity of fluorescence was significantly higher for DMT1-mutant erythrocytes (28.1 ± 2.8) when compared to wt erythrocytes (16.0 ± 1.7) confirming higher content of intracellular Ca^{2+} in DMT1-mutant erythrocytes.

DMT1-mutant erythrocytes have increased ROS and hyperactivated anti-oxidative defense

Next we tested whether the observed increased destruction of DMT1-mutant erythrocytes was associated with ROS accumulation. Both wt and DMT1-mutant erythrocytes were *in vitro* incubated with DCF fluorescence dye, thereafter DCF-dependent intensity of fluorescence, which is proportional to the concentration of ROS, was determined on FACS. The measurements revealed higher ROS levels in DMT1-mutant erythrocytes when compared to the erythrocytes of wt littermates (Fig. 4). Erythrocytes of wt mice exposed to H_2O_2 served as a positive control [22].

Then we assessed whether and how increased ROS influence the anti-oxidative defense of DMT1-mutant erythrocytes. The activity of antioxidant enzymes was measured in erythrocyte lysate free of leukocytes and platelets according to recommended methods [23], as we previously described [24]. DMT1-mutant erythrocytes showed significantly higher activity of the following enzymes: glutathione peroxidase (GPx, 1.6-times, $P = 0.023$), catalase (CAT, 1.9-times, $P = 0.018$) and methemoglobin reductase (MhHbR, 1.9-times, $P = 0.00002$) (Fig. 5A) than wt controls. In agreement, we also detected 2.4-times higher activity of glutathione reductase (GR) (Fig. 5A) and 1.8-higher activity of glucose-6-phosphate dehydrogenase (G6PD) (Fig. 5B) in DMT1-mutant erythrocytes than in wt erythrocytes, suggesting elevated levels of the oxidized form of glutathione (GSSG) that needs to be catalyzed to its reduced form (GSH). In addition, the expression levels of Foxo3A, a transcription factor responsible for

Fig. 5. Assessment of anti-oxidative defense and hypoxia regulated genes. A-B) The activity of antioxidant enzymes is higher in DMT1-mutant erythrocytes than in wt erythrocytes; glutathione peroxidase (GPx), catalase (CAT), methemoglobin reductase (MtHbR), glutathione reductase (GR) (Panel A) and glucose-6-phosphate dehydrogenase (G6PD) (Panel B). Enzyme activity is expressed in IU/g hemoglobin (Hb). C) Real-time PCR analysis revealed increased expression of *Foxo3A*, *Runx1*, *Tfrc* and *Hk1* in DMT1-mutant reticulocytes when compared to wt reticulocytes. The data were normalized to the expression of beta-actin and to mRNA levels of wt reticulocytes (black bars). Values indicate means $\pm$ SDs, $n = 3$ for wt mice, $n = 3$ for DMT1-mutant mice; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, NS – not significant.

the upregulation of the anti-oxidative defense enzymes [27], were found to be significantly elevated in DMT1-mutant reticulocytes when compared to the wt controls, along with other selected markers of augmented hypoxia sensing (Fig. 5C) [28]. These data suggest that ROS exaggerate anti-oxidative defense in DMT1-mutant erythrocytes but the buffering capacity of this defense is insufficient to eliminate ROS effectively. In addition, the results also indicate that tissue hypoxia may limit the extent of anti-oxidative capacity in DMT1-mutant red blood cells.

Increased need for ATP in DMT1-mutant erythrocytes

Another essential metabolic pathway in erythrocytes is anaerobic glycolysis responsible for the production of ATP [31]. The enzyme assays of selected enzymes of this pathway

Fig. 6. Analysis of anaerobic glycolysis in erythrocytes. DMT1-mutant erythrocytes showed accelerated anaerobic glycolysis when compared to wt erythrocytes as documented by increased activity of the following enzymes involved in Embden-Meyerhof pathway: hexokinase (HK), pyruvate kinase (PK) (Panel A) and glucose-phosphate isomerase (GPI) (Panel B). Enzyme activity is expressed in IU/g hemoglobin (Hb). Values indicate means $\pm$ SDs, $n = 5-7$ for wt mice, $n = 3$ for DMT1-mutant mice; *** $P < 0.001$.

Table 2. Concentration of metabolites (values indicate means $\pm$ SDs, $n = 14$ for wt mice, $n = 13$ for DMT1 mutant mice; *** $P < 0.001$)

	ATP [$\mu\text{mol/l}$]	ADP [$\mu\text{mol/l}$]	ATP/ADP
wt	2.04 $\pm$ 0.52	0.72 $\pm$ 0.20	2.83 $\pm$ 0.56
DMT1-mutant	1.76 $\pm$ 0.60	1.03 $\pm$ 0.37	1.70 $\pm$ 0.49***

revealed that in comparison with wt erythrocytes the DMT1-mutant erythrocytes have increased activities of hexokinase (HK, 2.5-times), pyruvate kinase (PK, 2.4-times) (Fig. 6A) and glucose-phosphate isomerase (GPI, 3.2-times) (Fig. 6B). Using HPLC-MS/MS we subsequently determined the concentration of ATP and ADP and calculated the ATP/ADP ratio - an indicator of cellular energy status. As shown in Table 2, DMT1-mutant erythrocytes had elevated levels of ADP and reduced levels of ATP and consequently reduced the ATP/ADP ratio than wt controls. This result is in agreement with accelerated anaerobic glycolysis and suggests increased need for ATP in DMT1-mutant erythrocytes.

Discussion

DMT1 deficiency leads to severe repression of numerous processes in developing erythroid cells, namely to defective differentiation, reduced survival of erythroid progenitors and increased apoptosis of erythroblasts [14]. On the level of reticulocytes, DMT1 deficiency leads to reduced rate of heme synthesis [32]. Our earlier data also revealed block of terminal hemoglobinization in the DMT1-mutant burst forming unit-erythroid (BFU-E) colonies that was associated with the activation of heme-regulated inhibitor (HRI) kinase, which specifically phosphorylates the α subunit of the eukaryotic initiation factor 2 (eIF2 α) and attenuates globin translation [33]. Therefore, it was reasonable to address a possible detrimental effect of DMT1 deficiency on life span and metabolism of mature erythrocytes, the last stage of DMT1-mutant erythroid differentiation that has not been analyzed yet.

A specific role of iron deficiency in the programmed cell death of erythrocytes (eryptosis) has been proposed in an earlier study [9]. Eryptosis is initiated by an increase in red cell cytosolic calcium; iron-depleted erythrocytes, when exposed to stress conditions, were reported to activate Ca^{2+} -permeable cation channel allowing Ca^{2+} entry [9, 11, 12]. Increased concentration of Ca^{2+} within erythrocytes was shown to stimulate Ca^{2+} -sensitive cell membrane scrambling with subsequent phosphatidylserine exposure, leading to removal of such erythrocytes from circulation [11, 12, 34]. Indeed, our experiments revealed markers of eryptosis and accelerated clearance of DMT1-mutant erythrocytes from circulating blood. However, it is important to note, that studies by Xu et al. [30] predicted that increased Ca^{2+} entry into DMT1-mutant cells is an intrinsic feature of the G185R mutation. Although we cannot estimate the contribution of G185R mutation and the iron-depleted erythroid environment *per se*, we observed significantly elevated levels of cytosolic Ca^{2+} in DMT1-mutant erythrocytes as a possible cause of events resulting in suicidal cell death of DMT1-mutant red blood cells. The possible involvement of a membrane defect in the *in vivo* accelerated clearance of erythrocytes was excluded by normal results of tests used to determine hemolysis and by unaltered bilirubin levels when compared to wt mice.

Iron deficiency of erythroid cells in DMT1-mutant mice, along with increase in cytosolic Ca^{2+} and membrane damage are all consistent with augmented state of cellular oxidative stress [35]. Therefore, we hypothesize that oxidative stress contributes to shortened life span of DMT1-deficient erythrocytes. Our data revealed increased ROS and hyperactivated anti-oxidative defense in DMT1-mutant red blood cells, which, however, was insufficient to fully buffer elevated toxic radicals. It seems likely that the contributing factor, causing ROS generation that exceeds anti-oxidant capacity of the DMT1-mutant erythrocyte defense system, is tissue hypoxia.

In DMT1-mutant mice, anemia causes decreased tissue oxygenation, which in turn leads to increased HIF activity as demonstrated by increased expression of HIF-targets (hypoxia signature genes) in the DMT1-mutant reticulocytes. In hypoxia, ROS production in erythrocytes often exceeds anti-oxidant system's buffering ability, creating hypoxia-induced oxidative stress of red blood cells [36]. Thus, we propose that hypoxia limits the anti-oxidative capacity of DMT1-mutant erythrocytes and renders them vulnerable to oxidative injury caused primarily by iron deficiency.

Eryptosis is associated with decreased cytosolic ATP concentration [37]. Oxidative injury of red blood cells, together with deformation of erythrocyte membrane, leads to ATP release from erythrocytes [38]. These events likely contributed to reduced ATP/ADP ratio in our DMT1-mutant erythrocytes. As a consequence, selected enzymes of the Embden-Meyerhof glycolytic pathway were found to be upregulated, consistently with higher demand for ATP in DMT1-mutant erythrocytes.

In conclusion our study revealed that DMT1-deficiency negatively affects erythrocyte metabolism and reduces their capacity to cope with stress. Increased content of cytosolic calcium, insufficient anti-oxidative defense and reduced levels of ATP shorten the life span of mature DMT1-mutant erythrocytes. We propose that this erythrocyte defect is a contributing factor in the pathophysiology of anemia caused by DMT1 mutations.

Disclosure Statement

The authors declare no conflicts of interest.

Acknowledgments

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