Kardiologia Polska 2017; 75, 3: 267–275; DOI: 10.5603/KP.a2016.0155 ISSN 0022–9032

ARTYKUŁ ORYGINALNY / *ORIGINAL ARTICLE*

Both iron excess and iron depletion impair viability of rat H9C2 cardiomyocytes and L6G8C5 myocytes

Monika Kasztura[1], Magdalena Dzięgała[1], Kamil Kobak[1], Jacek Bania[2], Grzegorz Mazur[3], Waldemar Banasiak[4], Piotr Ponikowski[4, 5], Ewa Anita Jankowska[1, 4]

[1]Laboratory for Applied Research on the Cardiovascular System, Department of Heart Diseases, Wroclaw Medical University, Wroclaw, Poland
[2]Department of Food Hygiene and Consumer Health Protection, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland
[3]Department of Internal and Occupational Diseases and Hypertension, Wroclaw Medical University, Wroclaw, Poland
[4]Cardiology Department, Centre for Heart Diseases, Military Hospital, Wroclaw, Poland
[5]Department of Heart Diseases, Wroclaw Medical University, Wroclaw, Poland

Abstract

Background: Iron is presumed to play an important role in the functioning of cardiomyocytes and skeletal myocytes. There is scarcity of direct data characterising the cells functioning when exposed to iron depletion or iron overload in a cellular environment. There is some clinical evidence demonstrating that iron deficiency has serious negative prognostic consequences in heart failure (HF) patients and its correction brought clinical benefit.

Aim: The viability of the cells upon unfavourable iron concentration in the cell culture medium and the presence of the molecular system of proteins involved in intracellular iron metabolism in these cells have been studied.

Methods: H9C2 rat adult cardiomyocytes and L6G8C5 rat adult skeletal myocytes were cultured for 24 h in optimal vs. reduced vs. increased iron concentrations. Intracellular iron content was measured by flame atomic absorption spectroscopy (FAAS). We analysed the mRNA expression of: ferritin heavy and light chains (*FTH* and *FTL*; iron storage proteins), myoglobin (*MB*, oxygen storage protein) ferroportin type 1 (*FPN1*; iron exporter), transferrin receptor type 1 (*TfR1*; iron importer), hepcidin (*HAMP*; iron metabolism regulator) using qPCR, the level of respective proteins using Western Blot (WB), and the viability of the cells using flow cytometry and cell viability tetrazolium reduction assay (MTS).

Results: Cardiomyocytes exposed to gradually reduced iron concentrations in the medium demonstrated a decrease in the mRNA expression of *FTH, FTL, FPN1, MB,* and *HAMP* (all $R = -0.75$, $p < 0.05$), indicating depleted iron status in the cells. As a consequence, the expression of *TfR1* ($R = 0.7$, $p < 0.05$) was increased, reflecting a facilitated entrance of iron to the cells. The inverse changes occurred in H9C2 cells exposed to increased iron concentrations in the medium in comparison to control cells. The same pattern of changes in the mRNA expressions was observed in myocytes, and there was a strong correlation between analogous genes in both cell lines (all $R > 0.9$, $p < 0.0001$). WB analysis revealed the analogous pattern of changes in protein expression as an mRNA profile. Both iron depletion and iron excess impaired viability of cardiomyocytes and skeletal myocytes.

Conclusions: Both rat cardiomyocytes and myocyte cells contain the set of genes involved in the intracellular iron metabolism, and both types of investigated cells respond to changing iron concentrations in the cultured environment. Both iron deficiency (ID) and iron overload is detrimental for the cells. This data may explain the beneficial effects of iron supplementation in patients with ID in HF.

Key words: iron deficiency, iron excess, iron metabolism genes, cardiomyocyte, skeletal myocyte

Kardiol Pol 2017; 75, 3: 267–275

Address for correspondence:
Monika Kasztura, PhD, Laboratory for Applied Research on the Cardiovascular System, Department of Heart Diseases, Wroclaw Medical University, ul. Weigla 5, 53–114 Wrocław, Poland, e-mail: monikakasztura@gmail.com

Received: 07.12.2015 **Accepted:** 21.06.2016 **Available as AoP:** 14.10.2016

INTRODUCTION

Iron is an essential micronutrient for the functioning of all living cells [1–3], being critical for the cells of high energy demand, such as cardiomyocytes and myocytes [4, 5]. Moreover, it constitutes the main component of cofactors not only for abundant globins responsible for oxygen transport and storage (haemoglobin, myoglobin), but also for numerous enzymes of the oxidative metabolism and mitochondrial functioning [2, 4, 6]. The particular importance of optimal iron status is well reflected by the fact that both intracellular iron excess and depletion impair the functioning of mitochondria, leading to excessive oxidative stress and deranged energy metabolism [6, 7].

What is more, recent evidence suggests a critical role of optimal iron status for patients with various cardiovascular disorders, including coronary artery disease (CAD) [8] and heart failure (HF) [9, 10]. The vast majority of data refers to the systemic iron metabolism, which can be assessed by iron biomarkers, e.g. ferritin, transferrin saturation (Tsat), hepcidin, or soluble transferrin receptor (sTfR) [3, 9, 11–14], all measured in peripheral blood. However, there is a scarcity of direct data characterising the intrinsic defects of intracellular mechanisms orchestrating iron metabolism and iron transfer within compartments of cells when exposed to iron depletion or iron overload in cellular environment.

There is some clinical evidence demonstrating that not only iron excess and related oxidative stress, but also iron deficiency impairs the functioning of tissues such as myocardium and skeletal muscles in patients with HF [10, 15, 16]. Obviously, the direct *in vivo* insight into these two human tissues can be methodologically difficult. For that reason, we applied the model of two rat cell lines: cardiomyocytes (H9C2) and skeletal myocytes (L6G8C5 [L6]) to investigate the viability of the high energy demand cell lines and to study the association between the cellular responses and differential iron status.

METHODS

Experimental schedule

Cell culture conditions. We cultured two cell lines: rat cardiomyocytes H9C2 (ATCC CRL1446) and rat skeletal myocytes L6G8C5 (Sigma-Aldrich) with the addition of deferoxamine (DFO) or ammonium ferric citrate (AFC) (both from Sigma-Aldrich) to change iron concentration in the cultured medium. DFO is a selective iron chelator commonly used in cell culture studies. It has been reported that the addition of DFO into the culture medium reduces iron concentration both in the cellular environment and inside the cell because DFO can be taken up by fluid phase endocytosis [17].

H9C2 and L6G8C5 (L6) cells were grown in Dulbecco's Modified Eagle Medium (DMEM, from Sigma-Aldrich) with the addition of 10% foetal bovine serum (FBS, from Invitrogen), 2 mmol/L glutamine, 10^4 × diluted 10,000 U/mL penicillin and 10 mg/mL streptomycin (from Sigma-Aldrich). For passages, cells were washed with phosphate buffered saline (PBS) (without ions Ca^{2+} and Mg^{2+}) and released by trypsinisation (trypsin from Sigma-Aldrich). The cells were cultured according to the manufacturer's guidelines.

Modulation of iron concentration in the cellular environment. H9C2 and L6 cells were seeded at the density of 0.3×10^6 per well in a volume of 2 mL, in a six-well plate. Subsequently, they were cultured under differential iron availability in the growth medium for 24 h: a) in the optimal iron concentration (standard iron concentration in DMEM with 10% FBS); b) in the reduced iron concentration (iron chelation using 50 μM and 100 μM of DFO); and c) in the increased iron concentration (supplementation with 100 μM and 200 μM of AFC). Compounds were added from 1000× concentrated stock after being diluted in the culture medium.

Assessed parameters

Iron content in the cells. The direct measurement of intracellular iron content was performed by means of FAAS assay [18]. Briefly: 2×10^6 of the cells cultured as reported above were dissociated by trypsinisation, pelleted by centrifugation, and washed five times with PBS in order to remove the contamination with free iron ions from medium. Pellet was dissolved in 250 μL of RIPA buffer for 30 min on ice and sonicated to disaggregate cellular structures. The protein concentration in the cell lysate was determined using the Lowry method. Intracellular iron content was measured in 250 μL of liquid containing 1 mg of protein lysate using an atomic absorption spectrometer SOLAAR M6; (ThermoElemental, UK) equipped with a deuterium lamp for background radiation correction by direct calibration against aqueous standards. An air-acetylene flame was used (gas flow: 0.9 L/min). The calibration solutions were prepared after successive dilutions of the stock standard solutions (iron standard for AAS TraceCERT; 1000 ppm, Fluka). For Fe the concentration varied from 0.5 to 2 ppm. All solutions were diluted to 1:5 ratio with deionised water. The determination was made under the following conditions: burner 100 mm, wavelength: λ = 253.7 nm, background correction: Quadline bandpass: 0.2 nm, lamp current: 75%. The method was verified by determination of reference material: Seronorm™ Trace Elements Serum level I: Fe = 1.39 mg/L, level II: Fe = 1.91 mg/L.

Expression of genes and proteins involved in intracellular iron metabolism. The following parameters reflecting cellular condition and iron status assessed in the states of different iron availability in culture medium of H9C2 and L6 lines were measured by quantitative polimerase chain reaction (PCR) and Western blotting:

- ferritin light and heavy chain (FTL and FTH; iron storage protein);
- ferroportin type 1 (FPN1; cellular iron exporter);
- transferrin receptor type 1 (TfR1; cellular iron importer);
- hepcidin (HAMP; iron metabolism regulator protein);
- myoglobin (MB; oxygen storage protein).

Cell viability by flow cytometry and MTS test. The application of FACS assay enabled the determination of change in cell size and granulation as the markers of cell condition and viability in the course of iron-derangement experiments. The smallest and the most granulated cells were treated as dead, which was confirmed by iodide propidium treatment.

Cell viability tetrazolium reduction assay (MTS) was a colorimetric method used for determining the number of viable and metabolically active cells in proliferation and cytotoxicity assays.

Experimental techniques

Quantitative real time-PCR. Total RNA was prepared from H9C2 or L6 cells harvested on six-well tissue culture plates using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Poland) following the manufacturer's instructions. The protocol included an on-column DNAse digestion to remove the genomic DNA. First-strand cDNA was synthesised using a SuperScript III First-Strand Synthesis System with oligo(dT)20 primer (Invitrogen, Poland).

Based on the genomic and cDNA sequences the primers for rat *HAMP* (For: GCAACAGACGAGACAGACTAC; Rev: GCAACAGAGACCACAGGAG), *FTL* (For: CCGCACAGACCCTCACCTC; Rev: AGAGATACTCGCCCAGAGATGC), *FTH* (For: GCCAAATACTTTCTCCATCAATCTC; Rev: CCGCTCTCCCAGTCATCAC), *FPN1* (For: TCGGTCTTTGGTCCTTTGATTTG; Rev: GGCTGACTTTCATCTGTAACTTCC), *MB* (For: CTCATCAGTCTATTTAAGGCTCACC; Rev: GATCTCAGCAGCATGTTGTCC), *TfR1* (For: GAGACTACTTCCGTGCTACTTC; Rev: TGGAGATACATAGGGTGACAGG) were designed with Molecular Beacon Software (Bio-Rad). The primers spanned exon junctions to prevent the amplification of genomic DNA.

All samples were performed in triplicate. The specificity of PCR was determined by melt-curve analysis for each reaction. The amplification efficiency was estimated by running serial dilutions of a template. Successive dilutions were plotted against the appropriate threshold cycle values to generate a standard curve. The slope calculated from the standard curve was used to determine the amplification efficiency (E) according to the formula: E = 10–1/slope.

Western blotting. 2×10^6 of H9C2 or L6 cells from each series of experiments were homogenised on ice in RIPA buffer for 60 min, followed by sonication. Protein concentration was estimated by Lowry's method. In order to determine FTL and FTH, FPN, MB, and TfR1 protein levels 50 μg of appropriate protein lysates (about 15 μL) were added on 12% SDS/polyacrylamide gel. For hepcidin determination 50 μg of protein lysate was loaded on Tricine gel. Proteins were electro-transferred on PVDF membranes (Millipore) in Towbin buffer (25 mM Tris, 192 mM glycine) supplemented with 10% methanol. The membranes were blocked with 10% skimmed milk or with bovine serum albumin for 1 h and incubated with primary antibodies (anti-ferritin light and heavy chain, anti-ferroportin, anti-myoglobin and anti-actin C11 [Santa Cruz], anti-transferrin receptor 1 [Hycult Biotech], anti-hepcidin [Ebioscience BD Biosciences, Oxford, United Kingdom]) overnight. After washing, the membranes were incubated with secondary horseradish-conjugated anti-goat (Sigma-Adrich) and anti-mouse (Sigma-Aldrich) antibodies and developed with ECL detection system (Pierce, Rockford, IL, USA)

Flow cytometry. Viability and size of cells were measured using flow cytometer (FACS Calibur, Becton-Dickinson). After incubation with DFO or AFC for 24 h the medium was removed from the cultures and cells were washed once with PBS and once with medium before trypsinisation. The cells were collected and washed with PBS by centrifugation at 300 × g for 5 min. The cell pellets were resuspended with PBS (1–2 mL) and screened (~1×10^4 cells) using a flow cytometer. The forward light scatter (FS) and side light scatter (SS) were recorded for each cell to determine the relative size and density, respectively. The viability of the cells was determined before and after treatment with iodide propidium and compared to control cells (H9C2 or L6G8C5 cells incubated without DFO or AFC).

MTS test. MTS tests were performed according to the manufacturer's instruction (Promega). Briefly, 2×10^5 of H9C2 or L6 cells were seeded on a 96-well plate and treated for 24 h with DFO, AFC, or PBS (as a control). 20 μL of CellTiter 96® AQueous One Solution Reagent was added to each well and absorbance at 490 nm was measured after two hours of incubation at 37°C.

Statistical analysis

Data are presented as the mean ± standard deviation values unless otherwise indicated. Spearman's rank correlation coefficient (R) reflects the relationship between the mRNA expression of respective genes and three different states of iron concentration (control vs. medium with 100 μM AFC vs. 200 μM AFC, or control vs. medium with 50 μM DFO vs. 100 μM DFO). A p-value < 0.05 is considered statistically significant. All molecular assessments are performed in triplicate.

RESULTS

Changes in intracellular iron due to the addition of DFO or AFC to culture media

The intracellular content of iron in both cell lines cultured in the standard DMEM medium was, on average, 0.88 mg/L. The direct measurement of intracellular iron proved that the addition of 50 μM and 100 μM of DFO into the medium caused a decrement of intracellular iron concentration in the range of 0.46 mg/L and 0.44 mg/L for L6 cells and 0.54 mg/L and 0.52 mg/L for H9C2 cells, respectively. In contrast, a supplementation of medium with 100 μM and 200 μM of AFC increased intracellular iron concentration on average to 4.14 mg/L and 4.87 mg/L for L6 cells and 6.2 mg/L and 9.48 mg/L for H9C2 cells, respectively (Fig. 1).

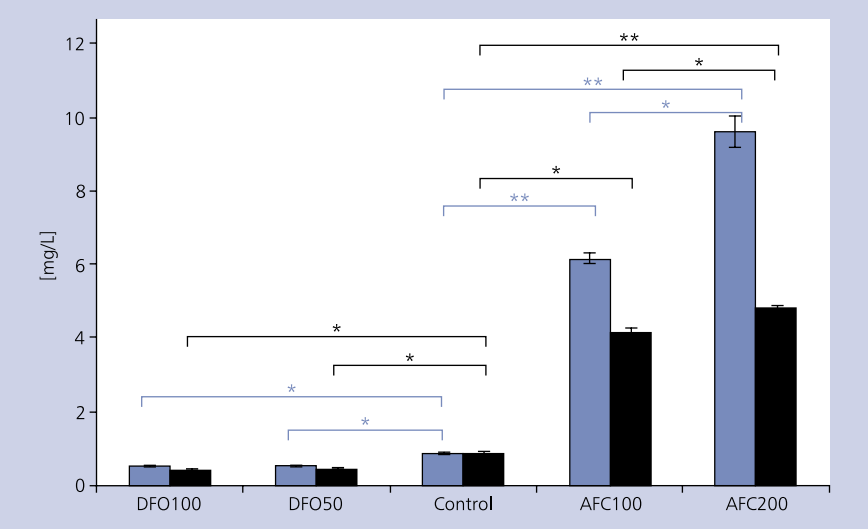

Figure 1. Intracellular iron concentration in L6G8C5 (in black) and H9C2 (in blue) cells exposed to different availability of iron. Control cells (Control) contain neither ammonium ferric citrate (AFC) nor deferoxamine (DFO); *p < 0.05; **p < 0.01

Expression of genes involved in iron metabolism in cultured cells in different iron conditions

H9C2 cell line exposed to an iron-deficient environment demonstrated a decrease in expression of genes involved in iron storage *FTH* ($R = -0.91$, $p < 0.0001$), *FTL* ($R = -0.91$, $p < 0.001$), iron efflux from the cell *FPN1* ($R = -0.96$, $p < 0.0001$), oxygen storage *MB* ($R = -0.75$, $p < 0.05$), and iron regulator *HAMP* ($R = -0.75$, $p < 0.05$), as compared to control conditions. On the other hand, the expression of *TfR1* was increased in the case of DFO treatment ($R = 0.70$, $p < 0.05$), which reflected iron demand, and thus facilitated entrance of iron to the cells. The opposite changes were observed upon exposition of H9C2 cell line to AFC. An increase in expression of such genes was noted: *FTH* ($R = 0.95$, $p < 0.005$), *FTL* ($R = 0.95$, $p < 0.005$), *FPN1* ($R = 0.99$, $p < 0.0001$), *MB* ($R = 0.91$, $p < 0.005$), *HAMP* ($R = 0.94$, $p < 0.0001$) as compared to control conditions. On the other hand, the expression of *TfR1* was decreased upon AFC treatment ($R = -0.83$, $p < 0.05$).

The same pattern of changes was observed in the case of L6G8C5 cells cultured with DFO addition into the medium: *FTH* ($R = -0.96$, $p < 0.0001$), *FTL* ($R = -0.91$, $p < 0.001$), *FPN1* ($R = -0.91$, $p < 0.001$), *MB* ($R = -0.91$, $p < 0.001$), *HAMP* ($R = -0.86$, $p < 0.05$), and *TfR1* ($R = 0.85$, $p < 0.005$), and with the addition of iron salt into the medium: *FTH* ($R = 0.91$, $p < 0.0001$), *FTL* ($R = 0.88$, $p < 0.001$), *FPN1* ($R = 0.96$, $p < 0.001$), *MB* ($R = 0.87$, $p < 0.001$), *HAMP* ($R = 0.95$, $p < 0.05$), and *TfR1* ($R = -0.95$, $p < 0.005$). The relative values of the differential mRNA expression of the studied genes upon changing iron availability are presented in Figure 2.

The expression changes of the aforementioned genes were correlated within each cell line (Table 1). Furthermore, there were correlations between changes in expression of analogous genes in H9C2 and L6 cells (all $R > 0.90$, $p < 0.0001$). The levels of expression of studied genes were similar in both cell lines, apart from *FTL* and *FTH* genes, which were approximately two- and six-fold highly expressed in H9C2 vs. L6. That observation indicates that both cell lines respond similarly to the changing iron environment.

The Western Blot analysis of the proteins encoded by the aforementioned genes revealed an analogous pattern of changes to that seen in the profile of mRNA expression, except for *TfR1*, which was not detected by the used antibodies (Figs. 2, 3).

Viability of cultured cells in different iron conditions

The percentage of living cells in both cell lines cultured in control conditions remained constant when measured at the beginning and after 24 h of the experiment. However, the viability of the cells assessed by flow cytometry was reduced by an average of 20% and 15% in the case of H9C2 cell line and 10% and 8% for L6 cell line, while exposed to 100 μM of DFO or 200 μM of AFC during the 24-h incubation period. Those data are in concordance with the results of the MTS tests, which revealed that the viability of H9C2 cells was reduced by 16% and 7% while exposed to 100 μM of DFO or 200 μM of AFC, respectively, as compared to cells cultured in control conditions. DFO and AFC were also cytotoxic for L6 cells reducing their viability by 14% and 11%, respectively (Fig. 4).

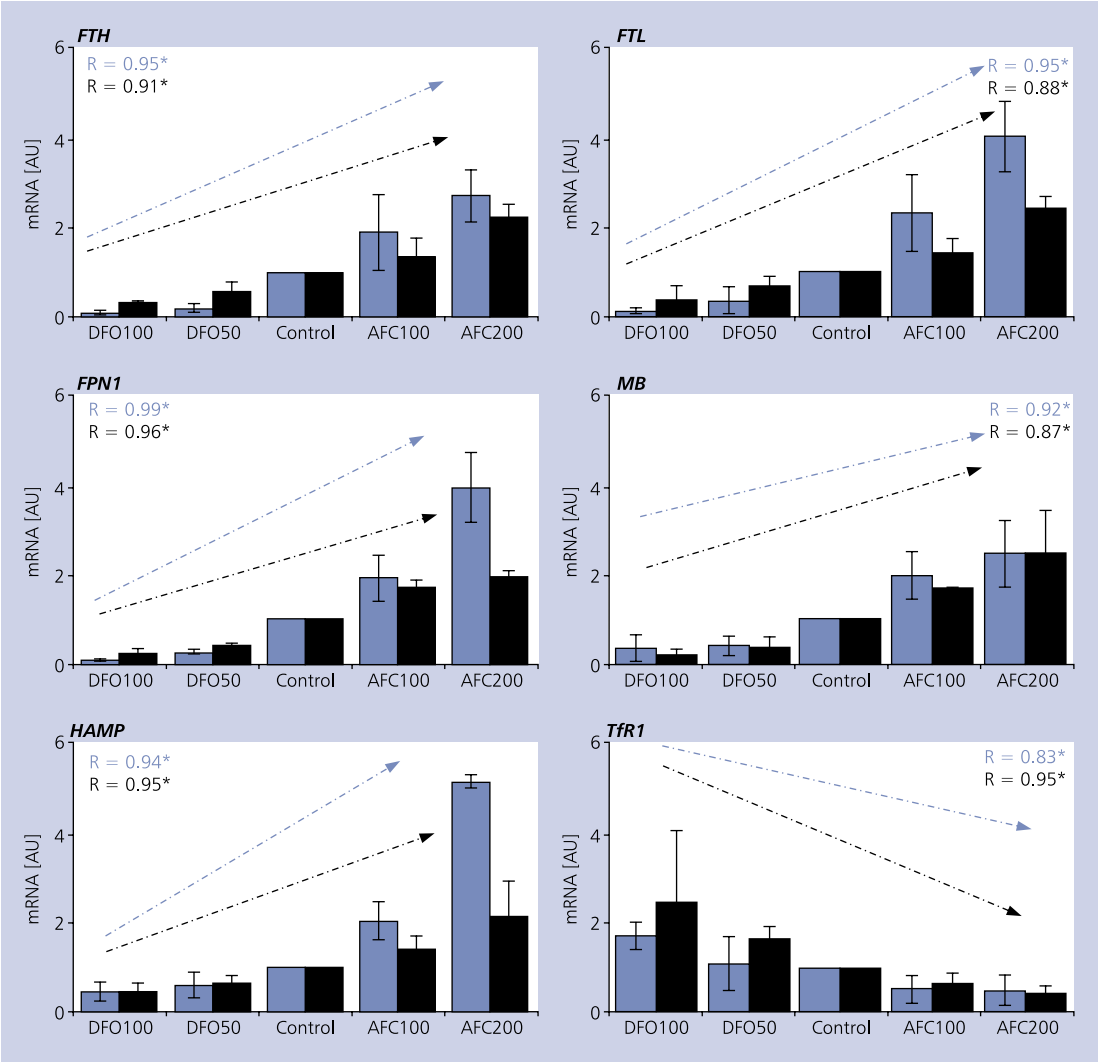

Figure 2. Expression of iron metabolism genes in L6G8C5 and H9C2 cells exposed to different availability of iron. mRNA expression of selected genes by quantitative polymerase chain reaction in L6G8C5 (in black) and H9C2 (in blue) cells cultured in 50 μM DFO, 100 μM DFO, 100 μM AFC, 200 μM AFC, and control medium. The arrows indicate the direction while the R values indicate the strength of the correlation between level of mRNA expression and the whole spectrum of iron concentration; *$p < 0.001$; FTH and FTL — ferritin heavy and light chains; FPN1 — ferroportin type 1; TfR1 — transferrin receptor type 1; MB — myoglobin; HAMP — hepcidin; DFO — deferoxamine; AFC — ammonium ferric citrate

DISCUSSION

Recently formulated so-called 'muscle hypothesis' recognises the significant contribution of structural and functional changes of skeletal muscles to exercise intolerance and related limited everyday functioning and poor outcome in patients with HF [19, 20]. There are also theories highlighting the abnormal energy generation and utilisation, mainly due to deranged oxidative metabolism, within failing myocardium and other peripheral tissues (such as skeletal muscles) as the major element of complex pathophysiology of HF [21–23]. Taking into consideration the critical role of optimal iron status for cellular energetics as well as the fact that iron deficiency has been recently recognised as a common and ominous co-morbidity in patients with systolic HF [10], we have formulated the hypothesis that deranged iron metabolism seen in HF may also affect skeletal muscles and lead to dysfunction of this tissue. Since the clinical studies are not powerful enough to get an insight into the mechanism of the deranged iron metabolism on the cellular level (the situation that is potentially present in iron-deficient non-anaemic patients with HF), there is a need for in-depth molecular analysis to unravel the cellular alterations that may be potentially translated into a clinical picture. The cell culture model of two tissues of great importance to the pathophysiology of HF, namely cardiomyocytes and skeletal myocytes, accounts for a useful research tool for such analysis. Importantly, to date, studies on iron metabolism have

Table 1. Correlations between expression of respective genes of iron metabolism within each cell line cultured upon changing iron availability (e.g. correlation between mRNA expression of transferrin receptor 1 and expression of hepcidin within H9C2 cells). Spearman's rank correlation coefficient (R) is reflecting the interrelationship between the mRNA expression of respective genes (FTH — ferritin heavy chain; FTL — ferritin light chain; FPN1 — ferroportin type 1; TfR1 — transferrin receptor type 1; MB — myoglobin; HAMP — hepcidin) within H9C2 or L6G8C5 in the whole spectrum of iron concentration

	HAMP	*TfR1*	*FNP1*	*FTL*	*FTH*
H9C2					
TfR1	R = –0.64*				
FNP1	R = 0.93****	R = –0.92****			
FTL	R = 0.92****	R = –0.81***	R = 0.97****		
FTH	R = 0.92****	R = –0.77**	R = 0.91****	R = 0.97***	
MB	R = 0.89****	R = –0.81****	R = 0.94****	R = 0.98****	R = 0.95****
L6					
TfR1	R = –0.87****				
FNP1	R = 0.94****	R = –0.88****			
FTL	R = 0.86****	R = –0.73**	R = 0.93****		
FTH	R = 0.88****	R = –0.81***	R = 0.92****	R = 0.95****	
MB	R = 0.89****	R = –0.77**	R = 0.93****	R = 0.85****	R = 0.85****

*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001

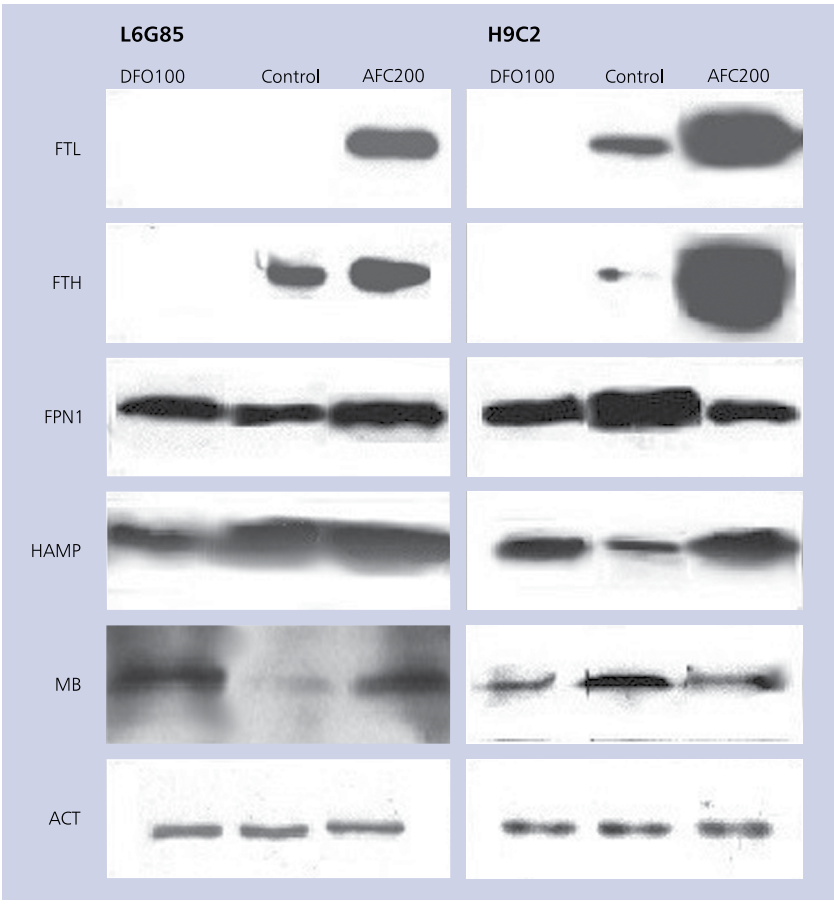

Figure 3. Expression of iron metabolism proteins in L6G8C5 and H9C2 cells exposed to different availability of iron. Extracts of total cellular proteins from L6G8C5 and H9C2 cells were analysed by Western blotting using appropriate antibodies. Actin (ACT) was used as a control of the protein load. Proteins were assayed in three independent experiments; FTH and FTL — ferritin heavy and light chains; FPN1 — ferroportin type 1; TfR1 — transferrin receptor type 1; MB — myoglobin; HAMP — hepcidin; DFO — deferoxamine; AFC — ammonium ferric citrate

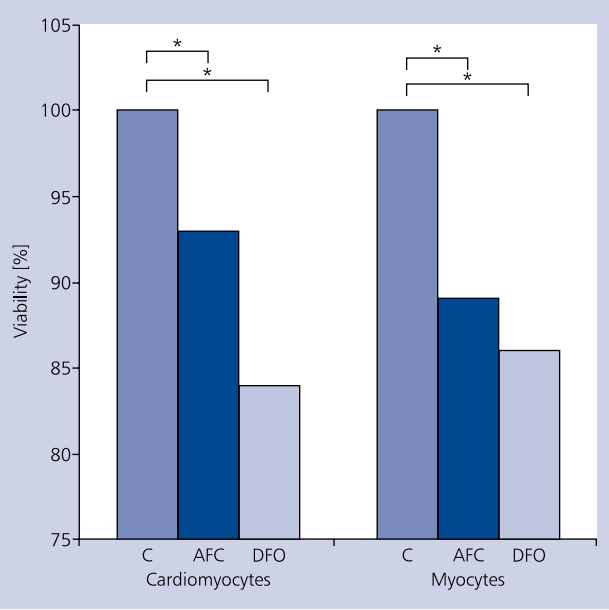

Figure 4. Multicellular tumour spheroids test — viability of H9C2 and L6 cells exposed to 100 μM deferoxamine (DFO) or 200 μM ammonium ferric citrate (AFC) vs. control cells (C) when cultured for 24 h. Control cells contain neither AFC nor DFO; *$p < 0.05$

been performed mainly in the cells involved in systemic iron homeostasis, such as hepatocytes, macrophages, enterocytes, or erythroid precursor cells [24–26]. However, it is worth mentioning that local iron homeostasis may differ from the systemic one [27]; therefore, we aimed to investigate the molecular machinery present in the cells of high energy demand, such as cardiomyocytes and skeletal myocytes.

In our study we confirmed the presence of the molecular machinery involved in iron import, export, storage, and regulation in rat cardiomyocytes H9C2 and myocytes L6, which represent cells of high energy demand. Changes in iron availability (both iron excess and iron depletion) in the culture medium have altered the expression of genes of iron metabolism and diminished the viability of the studied cells.

The presence of the particular elements of molecular machinery involved in iron metabolism such as transferrin receptor, ferroportin, ferritin, and hepcidin in cardiomyocytes had already been reported by others [28], but our study provided further evidence demonstrating alterations in the expression of these particles due to changing iron availability in the culture medium. Indeed, little is known about iron metabolism in skeletal myocytes cultured *in vitro* because the aforementioned genes until now have been identified only in skeletal muscle tissue [29]. Therefore, our study provided the first evidence of the presence of these elements in cultured rat myocyte L6. Furthermore, we showed that the changes in expression of iron turnover genes induced in L6 cells by different iron availability are correlated with the changes of analogous genes in H9C2 cardiomyocytes.

We applied the isolated cell culture model of cardiomyocytes and myocytes in order to investigate the influence of the changing iron availability without any other stimuli. We have demonstrated that both cardiomyocytes and myocytes, when exposed to intracellular iron depletion, demonstrate an increase in expression of transferrin receptor gene and a decrease in expression of ferroportin. These findings are consistent with the previous observations derived from cultures of others type of cells [30] and indicate that environmental iron depletion may induce a potential adaptive reaction of the cells, which enables iron resorption and iron efflux reduction. Interestingly, it has been observed that the low ferroportin level is accompanied by a diminished hepcidin expression, and this result contradicts the well-known pattern of hepcidin-ferroportin interaction present in hepatocytes and macrophages where a decreased hepcidin level is accompanied by an increased expression of ferroportin [30]. Our finding indicates that ferroportin-hepcidin regulatory pathway in cardiomyocytes and myocytes may be influenced by the additional signalling pathways. Based on these findings, it can be concluded that the regulation of iron metabolism may differ between cells of high energy demand such as cardiomyocytes and myocytes, and cells responsible for iron storage such as hepatocytes or macrophages.

Furthermore, we have shown that both iron depletion and iron excess diminish the viability of non-erythroid cells such as skeletal myocytes and cardiomyocytes which is not a common knowledge. This finding is consistent with the study of Walter et al. [7], who demonstrated that both iron overload and iron deficiency were detrimental for the functioning of liver mitochondria [7]. It is well known that iron excess deranges the energy metabolism of tissues and constitutes a bad prognosis in patients with cardiac disorders. However, in this work we proved that not only iron excess, but also iron depletion alters the viability of the studied cells. This result accounts for an important premise about the necessity of iron supplementation in the group of heart-failure patients with iron deficiency.

CONCLUSIONS

In conclusion, both cell lines of rat cardiomyocytes and myocytes have been proven to contain similar machineries for local iron metabolism, which respond to a changing iron environment similarly. The hepcidin-ferroportin regulatory pathway of iron metabolism present in cardiomyocytes and myocytes may differ from the regulatory pathways involved in systemic iron homeostasis, which may be interesting in the context of further studies on local iron metabolism in these tissues.

Acknowledgements

This research was financially supported by the National Science Centre (Krakow, Poland) grant allocated on the basis of the decision number DEC-2012/05/E/NZ5/00590.

Conflict of interest: *Wroclaw Medical University received an unrestricted grant from Vifor Pharma outside the submitted work. Monika Kasztura reports financial support from Vifor Pharma for travel and accommodation for scientific meetings. Ewa Anita Jankowska reports grants and personal fees from Vifor Pharma and FRESENIUS, during the conduct of the study and outside the submitted work. Waldemar Banasiak reports personal fees from Vifor Pharma, outside the submitted work. Piotr Ponikowski reports grants and personal fees from Vifor Pharma, personal fees from AMGEN, during the conduct of the study; grants and personal fees from Vifor Pharma, personal fees from AMGEN, outside the submitted work. Magdalena Dzięgała, Kamil Kobak, Jacek Bania, and Grzegorz Mazur have nothing to disclose. All the other authors report no conflict of interest related to the content of this manuscript.*

References

1. Cairo G, Bernuzzi F, Recalcati S. A precious metal: Iron, an essential nutrient for all cells. Genes Nutr. 2006; 1(1): 25–39, doi: 10.1007/BF02829934, indexed in Pubmed: 18850218.
2. Hower V, Mendes P, Torti FM, et al. A general map of iron metabolism and tissue-specific subnetworks. Mol Biosyst. 2009; 5(5): 422–443, doi: 10.1039/b816714c, indexed in Pubmed: 19381358.
3. Hentze MW, Muckenthaler MU, Galy B, et al. Two to tango: regulation of Mammalian iron metabolism. Cell. 2010; 142(1): 24–38, doi: 10.1016/j.cell.2010.06.028, indexed in Pubmed: 20603012.
4. Beard JL. Iron biology in immune function, muscle metabolism and neuronal functioning. J Nutr. 2001; 131: 568S–579S.
5. Jankowska EA, von Haehling S, Anker SD, et al. Iron deficiency and heart failure: diagnostic dilemmas and therapeutic perspectives. Eur Heart J. 2013; 34(11): 816–829, doi: 10.1093/eurheartj/ehs224, indexed in Pubmed: 23100285.
6. Andrews NC. Disorders of iron metabolism. N Engl J Med. 1999; 341(26): 1986–1995, doi: 10.1056/NEJM199912233412607, indexed in Pubmed: 10607817.
7. Walter PB, Knutson MD, Paler-Martinez A, et al. Iron deficiency and iron excess damage mitochondria and mitochondrial DNA in rats. Proc Natl Acad Sci USA. 2002; 99(4): 2264–2269, doi: 10.1073/pnas.261708798, indexed in Pubmed: 11854522.
8. Ponikowska B, Suchocki T, Paleczny B, et al. Iron status and survival in diabetic patients with coronary artery disease. Diabetes Care. 2013; 36(12): 4147–4156, doi: 10.2337/dc13-0528, indexed in Pubmed: 24130349.
9. Jankowska EA, Malyszko J, Ardehali H, et al. Iron status in patients with chronic heart failure. Eur Heart J. 2013; 34(11): 827–834, doi: 10.1093/eurheartj/ehs377, indexed in Pubmed: 23178646.
10. Jankowska EA, Rozentryt P, Witkowska A, et al. Iron deficiency: an ominous sign in patients with systolic chronic heart failure. Eur Heart J. 2010; 31(15): 1872–1880, doi: 10.1093/eurheartj/ehq158, indexed in Pubmed: 20570952.
11. Wish JB. Assessing iron status: beyond serum ferritin and transferrin saturation. Clin J Am Soc Nephrol. 2006; 1 Suppl 1: S4–S8, doi: 10.2215/CJN.01490506, indexed in Pubmed: 17699374.
12. Franchini M, Montagnana M, Lippi G. Hepcidin and iron metabolism: from laboratory to clinical implications. Clin Chim Acta. 2010; 411(21-22): 1565–1569, doi: 10.1016/j.cca.2010.07.003, indexed in Pubmed: 20620132.
13. Skikne BS. Serum transferrin receptor. Am J Hematol. 2008; 83: 872–885.
14. Koulaouzidis A, Said E, Cottier R, et al. Soluble transferrin receptors and iron deficiency, a step beyond ferritin. A systematic review. J Gastrointestin Liver Dis. 2009; 18(3): 345–352, indexed in Pubmed: 19795030.
15. Jandl JH, Inman JK, Simmons RL, et al. Transfer of iron from serum iron-binding protein to human reticulocytes. J Clin Invest. 1959; 38(1, Part 1): 161–185, doi: 10.1172/JCI103786, indexed in Pubmed: 13620780.
16. O'Meara E, de Denus S. Management of anemia and iron deficiency in heart failure. Curr Treat Options Cardiovasc Med. 2010; 12(6): 532–548, doi: 10.1007/s11936-010-0095-4, indexed in Pubmed: 21063931.
17. Woo KJ, Lee TJ, Park JW, et al. Desferrioxamine, an iron chelator, enhances HIF-1alpha accumulation via cyclooxygenase-2 signaling pathway. Biochem Biophys Res Commun. 2006; 343(1): 8–14, doi: 10.1016/j.bbrc.2006.02.116, indexed in Pubmed: 16527254.
18. Chandio Z, Talpur F, Khan H, et al. Determination of cadmium and zinc in vegetables with online FAAS after simultaneous pre-concentration with 1,5-diphenylthiocarbazone immobilised on naphthalene. Food Additives & Contaminants: Part A. 2013; 30(1): 110–115, doi: 10.1080/19440049.2012.728721.
19. Coats AJ, Clark AL, Piepoli M, et al. Symptoms and quality of life in heart failure: the muscle hypothesis. Br Heart J. 1994; 72(2 Suppl): S36–S39, indexed in Pubmed: 7946756.
20. Clark AL, Poole-Wilson PA, Coats AJ. Exercise limitation in chronic heart failure: central role of the periphery. J Am Coll Cardiol. 1996; 28(5): 1092–1102, doi: 10.1016/S0735-1097(96)00323-3, indexed in Pubmed: 8890800.
21. Ingwall JS. Energy metabolism in heart failure and remodelling. Cardiovasc Res. 2009; 81(3): 412–419, doi: 10.1093/cvr/cvn301, indexed in Pubmed: 18987051.
22. Ventura-Clapier R. Exercise training, energy metabolism, and heart failure. Appl Physiol Nutr Metab. 2009; 34: 336–339, doi: 10.1139/H09-013.
23. Turer AT, Malloy CR, Newgard CB, et al. Energetics and metabolism in the failing heart: important but poorly understood. Curr Opin Clin Nutr Metab Care. 2010; 13(4): 458–465, doi: 10.1097/MCO.0b013e32833a55a5, indexed in Pubmed: 20453645.
24. Ramey G, Deschemin JC, Durel B, et al. Hepcidin targets ferroportin for degradation in hepatocytes. Haematologica. 2010; 95(3): 501–504, doi: 10.3324/haematol.2009.014399, indexed in Pubmed: 19773263.
25. Kuriyama-Matsumura K, Sato H, Yamaguchi M, et al. Regulation of ferritin synthesis and iron regulatory protein 1 by oxygen in mouse peritoneal macrophages. Biochem Biophys Res Commun. 1998; 249(1): 241–246, doi: 10.1006/bbrc.1998.9046, indexed in Pubmed: 9705865.
26. Cianetti L, Segnalini P, Calzolari A, et al. Expression of alternative transcripts of ferroportin-1 during human erythroid differentiation. Haematologica. 2005; 90(12): 1595–1606, indexed in Pubmed: 16330432.
27. Stugiewicz M, Tkaczyszyn M, Kasztura M, et al. The influence of iron deficiency on the functioning of skeletal muscles: experimental evidence and clinical implications. Eur J Heart Fail. 2016; 18(7): 762–773, doi: 10.1002/ejhf.467, indexed in Pubmed: 26800032.
28. Ge XHu, Wang Q, Qian ZM, et al. The iron regulatory hormone hepcidin reduces ferroportin 1 content and iron release in H9C2 cardiomyocytes. J Nutr Biochem. 2009; 20(11): 860–865, doi: 10.1016/j.jnutbio.2008.07.014, indexed in Pubmed: 19027283.
29. Polonifi A, Politou M, Kalotychou V, et al. Iron metabolism gene expression in human skeletal muscle. Blood Cells Mol Dis. 2010; 45(3): 233–237, doi: 10.1016/j.bcmd.2010.07.002, indexed in Pubmed: 20691620.
30. Fein E, Merle U, Ehehalt R, et al. Regulation of hepcidin in HepG2 and RINm5F cells. Peptides. 2007; 28(5): 951–957, doi: 10.1016/j.peptides.2007.01.016, indexed in Pubmed: 17363110.

Cite this article as: Kasztura M, Dzięgała M, Kobak K, et al. Both iron excess and iron depletion impair viability of rat H9C2 cardiomyocytes and L6G8C5 myocytes. Kardiol Pol. 2017; 75(3): 267–275, doi: 10.5603/KP.a2016.0155.

Zarówno nadmiar, jak i niedobór żelaza osłabiają żywotność szczurzych kardiomiocytów H9C2 oraz miocytów L6G8C5

Monika Kasztura[1], Magdalena Dzięgała[1], Kamil Kobak[1], Jacek Bania[2], Grzegorz Mazur[3], Waldemar Banasiak[4], Piotr Ponikowski[4, 5], Ewa Anita Jankowska[1, 4]

[1]Samodzielna Pracownia Badań Układu Krążenia, Katedra Chorób Serca, Uniwersytet Medyczny we Wrocławiu, Wrocław

[2]Katedra Higieny Żywności i Ochrony Zdrowia Konsumenta, Uniwersytet Przyrodniczy we Wrocławiu, Wrocław

[3]Katedra i Klinika Chorób Wewnętrznych, Zawodowych, Nadciśnienia Tętniczego i Onkologii Klinicznej, Uniwersytet Medyczny we Wrocławiu, Wrocław

[4]Ośrodek Chorób Serca, 4. Wojskowy Szpital Kliniczny, Wrocław

[5]Katedra i Klinika Chorób Serca, Uniwersytet Medyczny we Wrocławiu, Wrocław

Streszczenie

Wstęp: Istnieją przesłanki o znaczącym wpływie żelaza na funkcjonowanie kardiomiocytów i komórek mięśni szkieletowych. Niewiele dostępnych danych charakteryzuje te komórki, gdy są hodowane w warunkach niedoboru lub nadmiaru stężenia żelaza. Niedobór żelaza u osób ze schorzeniami sercowo-naczyniowymi upośledza tolerancję wysiłku fizycznego, zwiększa ryzyko hospitalizacji sercowo-naczyniowej i zgonu w obserwacji krótko- i długoterminowej, a dożylna suplementacja żelaza przynosi korzyści kliniczne m.in. chorym z niewydolnością serca.

Cel: Celem pracy było zbadanie żywotności komórek oraz obecności zestawu genów zaangażowanych w wewnątrzkomórkowy metabolizm żelaza w warunkach nadmiaru i niedoboru żelaza w medium hodowlanym.

Metody: Dwie szczurze linie komórkowe: kardiomiocyty H9C2 i miocyty L6G8C5 hodowano przez 24 h w warunkach optymalnych, nadmiaru lub niedoboru żelaza w medium. Wewnątrzkomórkowe stężenie żelaza zostało oznaczone techniką spektroskopii absorpcji atomowej. Przeprowadzono analizę ilościową mRNA następujących genów: łańcucha ciężkiego i lekkiego ferrytyny (*FTH* i *FTL*; magazynowanie żelaza), mioglobiny (MB; magazynowanie tlenu), ferroportyny (*FPN1*; eksporter żelaza), receptora transferynowego typu 1 (*TfR1*; wychwyt żelaza z otoczenia), hepcydyny (*HAMP*; regulator metabolizmu żelaza) z wykorzystaniem qPCR, oraz analizę poziomu odpowiednich białek techniką *Western Blot*. Żywotność komórek zbadano metodą MTS oraz za pomocą cytometrii przepływowej.

Wyniki: Kardiomiocyty H9C2 wystawione na zmniejszające się stopniowo stężenie żelaza w medium charakteryzowały obniżoną ekspresją *FTH*, *FTL*, *FPN1*, *MB* i *HAMP* (wszystkie $R = -0{,}75$, $p < 0{,}05$) oraz wzrostem ekspresji *TfR1* ($R = 0{,}7$, $p < 0{,}05$), co odzwierciedlało ułatwione wnikanie żelaza do komórki. Zmiana ekspresji wymienionych genów była potwierdzeniem zmniejszającej się dostępności żelaza w medium hodowlanym. Odwrotne zmiany ekspresji odnotowano w komórkach H9C2 wystawionych na wzrastające stężenie żelaza w medium w porównaniu z komórkami kontrolnymi. Identyczny wzór zmian charakteryzował miocyty L6G8C5. Zaobserwowano także silną korelację między analogicznymi genami obu linii komórkowych (wszystkie $R > 0{,}9$, $p < 0{,}0001$). Zarówno niedobór, jak i nadmiar żelaza negatywnie wpływał na żywotność obu linii komórkowych.

Wnioski: Obie szczurze linie komórkowe posiadają zestaw genów zaangażowanych w wewnątrzkomórkowy metabolizm żelaza, których odpowiedź na zmieniające się w środowisku stężenie żelaza jest podobna. Żywotność komórek zmniejsza się zarówno w warunkach nadmiaru, jak i niedoboru żelaza.

Słowa kluczowe: niedobór żelaza, nadmiar żelaza, geny metabolizmu żelaza, kardiomiocyty, miocyty

Kardiol Pol 2017; 75, 3: 267–275

Adres do korespondencji:
dr Monika Kasztura, Katedra Chorób Serca, Uniwersytet Medyczny we Wrocławiu, ul. Weigla 5, 53–114 Wrocław, e-mail: monikakasztura@gmail.com
Praca wpłynęła: 07.12.2015 r. **Zaakceptowana do druku:** 21.06.2016 r. **Data publikacji AoP:** 14.10.2016 r.