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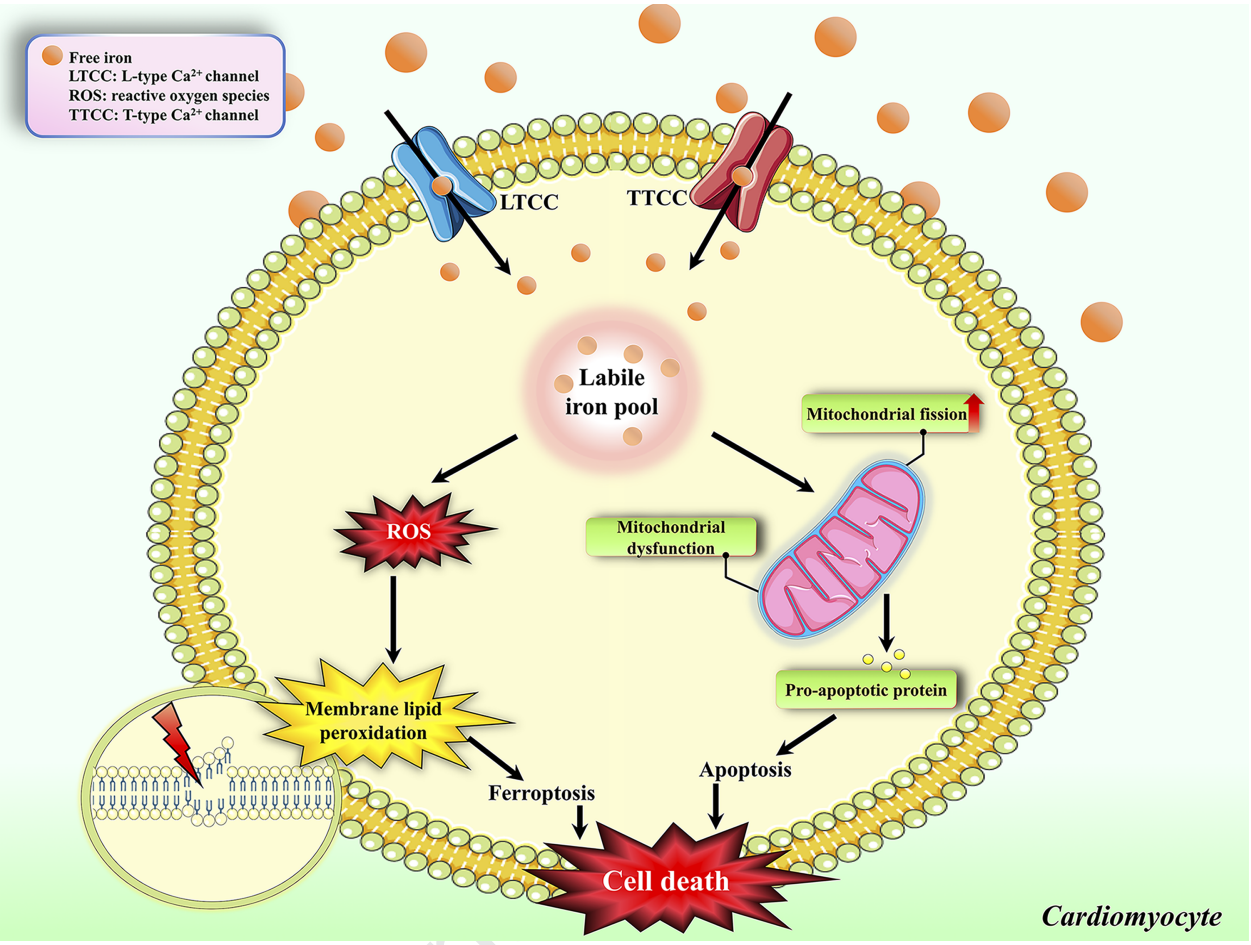
Free iron
LTCC: L-type Ca2+ channel
ROS: reactive oxygen species
TTCC: T-type Ca2+ channel
LTCC
TTCC
Labile iron pool
Mitochondrial fission
ROS
Mitochondrial dysfunction
Pro-apoptotic protein
Membrane lipid peroxidation
Ferroptosis
Apoptosis
Cell death
Cardiomyocyte

The Effects of Iron Overload on Mitochondrial Function, Mitochondrial Dynamics, and Ferroptosis in Cardiomyocytes

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Abstract

Excessive iron accumulation in the heart can lead to iron overload cardiomyopathy (IOC), the leading cause of death in hemochromatosis patients. Current understanding regarding the mechanism by which iron overload causes a deterioration in cardiac performance, mitochondrial dysfunction, and impaired mitochondrial dynamics remains limited. Ferroptosis, a newly identified form of regulated cell death, has recently been revealed influencing the pathophysiological process of IOC. Nevertheless, the direct effect of cardiac iron overload on ferroptotic cell death is incompletely characterized. This review article comprehensively summarizes and discusses the effects of iron overload on cardiac mitochondrial function, cardiac mitochondrial dynamics, ferroptosis of cardiomyocytes, and left ventricular function in *in vitro* and *in vivo* reports. This review also provides relevant consistent and controversial information which can facilitate further mechanistic investigation into iron-induced cardiac dysfunction in the clinical setting in the near future.

Keywords: iron overload; heart; mitochondria; cell death; ferroptosis

Abbreviations:

$^{\bullet}$HO: hydroxyl free radical; Δψm: mitochondrial membrane potential change; ACSL4: acyl-CoA synthethase long chain family member 4; COX: cyclooxygenase; DNA: deoxyribonucleic acid; Drp-1: dynamin-related protein-1; ENPP2: ectonucleotide pyrophosphatase/phosphodiesterase family member 2; Fe^{2+}: ferrous ion; Fe^{3+}: ferric ion; GPX4: glutathione peroxidase-4; GSH: glutathione; HRV: heart rate variability; IOC: iron overload cardiomyopathy; LOX: lipoxygenase; LPA: lysophosphatidic acid; LTCC: L-type Ca^{2+} channel; LVEF: left ventricular ejection fraction; LVFS: left ventricular fractional shortening; MCU: mitochondrial Ca^{2+} uniporter; MDA: malondialdehyde; Mfn-1: mitofusin-1; Mfn-2: mitofusin-2; mPTP: mitochondrial permeability transition pore; mTOR: mechanistic target of rapamycin; Nox4: NADPH oxidase 4; NTBI: non-transferrin-bound iron; $O_2^{\bullet-}$: superoxide anion; PUFA: polyunsaturated fatty acid; ROS: reactive oxygen species; SV: stroke volume; TfR1: transferrin receptor-1; TTCC: T-type Ca^{2+} channel.

1. Introduction

Iron is important in a wide variety of biochemical reactions due to its crucial role as a component of multiple enzymes required for cellular respiration, energy metabolism, and deoxyribonucleic acid (DNA) synthesis and repair [1-3]. Iron is present in various concentrations across major cellular components including cytosol (~6 μM), mitochondria (~16 μM), nuclei (~7 μM), and lysosomes (~16 μM) [4-6]. Nevertheless, excessive iron accumulation in the body, a condition termed iron overload, can cause adverse effects. This condition is an important complication in diseases that disrupt the homeostatic mechanism of systemic iron regulation including primary hemochromatosis and transfusion-dependent anemias [7-9]. Iron overload results in the saturation of plasma transferrin and hence the appearance of circulating non-transferrin bound iron (NTBI), leading to iron deposition in vital organs such as the kidney, liver, and heart [10-13]. Importantly, iron overload cardiomyopathy (IOC), a pathological condition characterized by progressive electromechanical deterioration of the iron-overloaded heart, has been the leading cause of mortality in hemochromatosis patients [14].

At the cellular level, iron overload causes increased production of reactive oxygen species (ROS), resulting in oxidative stress which can induce damage to macromolecules such as DNA, proteins, and membrane lipids [3, 10]. Previous studies have reported that iron overload led to cardiac mitochondrial dysfunction as indicated by decreased mitochondrial respiration, increased mitochondrial ROS level, mitochondrial membrane potential depolarization, and mitochondrial swelling [15, 16]. Iron overload also disturbs mitochondrial dynamics, interfering with the balance between mitochondrial fission and fusion [15]. Mitochondrial fission is regulated by dynamin-related protein-1 (Drp-1), whereas mitochondrial fusion is regulated by mitofusion-1 and -2 (Mfn-1, Mfn-2) [17, 18]. Thus, investigating the changes in these molecular players is crucial to understanding the pathophysiological process of iron-induced cardiac dysfunction.

At tissue and organ levels, regulated cell death plays an important role in myocardial homeostasis and pathologies [19]. Progressive loss of cardiomyocytes has been regarded as a major contributor of the remodeling process that culminates in heart failure [19-23]. Apoptosis, the most extensively studied type of cell death in the heart, is believed to contribute to IOC [20-22]. It has been demonstrated that iron overload can induce apoptosis via mitochondrial dysfunction [15] in which increased mitochondrial oxidative stress triggers cytochrome c release, and activates the caspase-dependent apoptotic pathway [15, 24].

Besides apoptosis, it has also been demonstrated that nonapoptotic iron-dependent cell death, termed ferroptosis, participates in the pathophysiological process of IOC [25-28].

Ferroptosis is a newly identified form of regulated cell death discovered by Dixon *et al* [26]. The distinctive feature that characterizes ferroptotic cell death is iron-dependent lipid peroxidation [25, 26]. The lethal accumulation of lipid peroxides is not only caused by increased lipid peroxidation *per se*, but also the decreased activity of the lipid-reducing enzyme glutathione peroxidase 4 (GPX4) [25, 26, 28]. A redox-active labile iron pool facilitates ferroptosis by catalyzing the production of ROS including the superoxide anion ($O_2^{\bullet-}$) and hydroxyl free radical ($^{\bullet}HO$), thus supplying lipid peroxidation reactions with these potent oxidizing agents [25, 26, 28]. The fact that iron is a key factor in ferroptosis is supported by the ability of iron chelators to attenuate ferroptotic cell death in various experimental models [25, 26, 28]. Since it has been discovered, ferroptosis has been linked to the pathophysiology of many diseases including cardiac ischemia/reperfusion injury, neurodegenerative disorders, and renal failure [25, 27-30]. To date, however, data concerning the alteration of cardiomyocyte ferroptosis due to iron overload itself remain scarce.

There is an increasing amount of information regarding this exciting development and also ongoing attempts to elucidate the detailed pathophysiological mechanism of IOC. This review therefore aims to comprehensively summarizes and discusses *in vitro* and *in vivo* reports regarding the effects of iron overload on mitochondrial function, mitochondrial dynamics, ferroptosis in cardiomyocytes, as well as on cardiac function. General information regarding mitochondrial biology and various types of regulated cell death not specific to cardiac iron overload condition has been extensively reviewed elsewhere [16, 20, 31-36] and will not be included in this review.

2. The *in vitro* evidence pertinent to the effects of iron overload and pharmacological interventions on oxidative stress, cardiac mitochondria, and cardiomyocyte viability

The *in vitro* effects of iron overload on whole-cell and mitochondrial oxidative stress, cardiac mitochondrial function, and cardiomyocyte viability are summarized in Table 1 and Fig. 1. Previous studies have shown that free iron, either the ferric (Fe^{3+}) or ferrous (Fe^{2+}) form, is taken up by cardiomyocytes and then involved in intracellular ROS production through the Fenton reaction [16, 20, 31, 33, 34]. In addition, excessive intracellular free iron can enter the mitochondria and generate mitochondrial oxidative stress. This leads to

impaired cardiac mitochondrial function, as indicated by decreased mitochondrial respiration, mitochondrial membrane potential depolarization, and mitochondrial swelling [16, 20, 31, 33, 34]. Cytochrome c release may also be triggered by mitochondrial membrane depolarization, resulting in activation of caspases and apoptosis [20, 31]. Although both Fe^{3+} and Fe^{2+} could cause the aforementioned cellular derangements, it has been demonstrated that Fe^{2+} has a more potent impact than Fe^{3+} [20, 33].

The effects of pharmacological interventions on cell viability, oxidative stress and mitochondrial function in iron-overloaded cardiomyocytes are summarized in Table 2. Under iron overload conditions, it has been proposed that L-type and T-type Ca^{2+} channels (LTCC and TTCC, respectively) are potential portals for iron entry into cardiomyocytes [11, 37-39]. An *in vitro* study in HL-1 cells, however, demonstrated that LTCC antagonists (verapamil and amlodipine), but not a TTCC antagonist (efonidipine), could attenuate iron uptake into cardiomyocytes [20]. This finding indicates that, in this particular *in vitro* condition, LTCC is more important than TTCC in mediating cardiomyocyte iron uptake [20]. Nevertheless, an LTCC blocker still failed to rescue the cells from apoptosis in this study even though lower levels of intracellular iron were achieved by amlodipine [20], suggesting that blockage of iron influx alone may not be sufficient to cause functional improvement. As previously discussed, the downstream pathophysiological process of cellular iron overload includes excessive ROS production and subsequent mitochondrial damage. As expected, mitochondrial membrane depolarization and apoptosis were reduced in iron-overloaded H9c2 cells treated with an antioxidant, thrombopoietin [31]. In addition, studies that have tried specifically to intervene in cardiac mitochondrial iron uptake also exist [16, 33]. The mitochondrial Ca^{2+} uniporter (MCU) and mitochondrial permeability transition pore (mPTP) have been proposed as pathways for iron entry into mitochondria [33, 40]. According to a study in isolated cardiac mitochondria from wild-type Wistar rats, an MCU blocker (Ru360) reduced mitochondrial iron uptake and exerted greater efficacy, when compared to an mPTP blocker (cyclosporin A), in decreasing mitochondrial ROS and improving mitochondrial function [16, 33]. It was also demonstrated in the same study that similar beneficial effects could be achieved by treatment with an iron chelator (deferoxamine) [33]. To give weight to the findings from the study into wild-type rat mitochondria, it was also found that Ru360 reduced ROS and improved mitochondrial function more efficiently than cyclosporin A in iron-incubated isolated mitochondria from thalassemic mice [16, 33], suggesting that MCU may act as a crucial portal for iron uptake into cardiac mitochondria.

3. The *in vivo* evidence pertinent to the effects of iron overload and pharmacological interventions on oxidative stress, cardiac mitochondria, and cardiac function.

The *in vivo* effects of iron overload on cardiac oxidative stress, cardiac function and cardiac mitochondrial function are summarized in Table 3 and Fig. 1. Chronic iron treatment led to cardiac iron accumulation in various animal models, including wild-type and thalassemic mice, wild-type Wistar rats, and wild-type gerbils [15, 24, 32, 41-45]. The hearts of these iron-overloaded animals consistently exhibited a deterioration in systolic and autonomic functions [15, 24, 32, 43-45]. Both non-invasive (echocardiography) and invasive (cardiac catheterization) measurements revealed impairment of multiple cardiac function parameters. These included a reduction in left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), rates of left ventricular pressure development (dP/dt), stroke volume (SV), stroke work and cardiac output (CO) [15, 24, 32, 43-45] (Table 3). In addition, iron-induced cardiac autonomic dysfunction has been demonstrated by frequency-domain measurement of heart rate variability (HRV) [15, 24, 43, 44]. Cellular and molecular studies have also been carried out using the cardiomyocytes isolated from these animals following *in vivo* iron treatment, and the results are largely consistent with the findings from *in vitro* models discussed in the previous section (Tables 1 and 3). Specifically, in *in vivo* models, increased ROS and malondialdehyde (MDA), a product of membrane lipid peroxidation, have been demonstrated under iron overload conditions [15, 24, 32, 41-44]. In addition, *in vivo* iron overload also exacerbated cardiac mitochondrial dysfunction as indicated by impaired synthesis of cardiac mitochondrial DNA and respiratory chain components, in addition to increased cardiac mitochondrial ROS, mitochondrial membrane depolarization, and mitochondrial swelling [15, 24, 32, 41-45]. To date there is only one study which has investigated cardiac mitochondrial dynamics under conditions of iron overload [15]. It has been reported that chronic iron treatment in wild-type and thalassemic mice led to an increased cardiac Drp-1/Mfn-2 ratio, suggesting a marked imbalance in cardiac mitochondrial dynamics in favor of mitochondrial fission, which may facilitate the development of heart failure [15].

The effects of pharmacological interventions on cardiac oxidative stress, cardiac mitochondrial function and cardiac function in iron-overloaded animal models are summarized in Table 4. There is evidence that treatment with iron chelators (deferiprone, deferoxamine, and deferasirox) could reduce cardiac iron content [46], reduce the formation of ROS, and alleviate cardiac mitochondrial dysfunction, leading to improved cardiac autonomic and systolic function in iron-overloaded animals [15, 24, 32, 43, 44]. Likewise,

iron-overloaded animals treated with a potent antioxidant N-acetylcysteine also showed a similar functional improvement of the heart [24, 43, 44]. Notably, combined treatment of deferiprone with N-acetylcysteine showed greater efficacy than either monotherapy regimen in decreasing cardiac iron concentration and oxidative stress, attenuating mitochondrial dysfunction, and conferring cardioprotection against iron overload [24, 43, 44]. These promising results emphasize the need for future clinical studies to validate the clinical significance of the combined regimen (iron chelator plus N-acetylcysteine) in patients with iron overload condition. Interestingly, in contrast to the previously discussed in *in vitro* experiment which failed to demonstrate the beneficial effect of TTCC blockade [20], cardiac iron deposition and its subsequent detrimental effects were effectively attenuated by treatment with either the LTCC blocker amlodipine or TTCC blocker efonidipine [15, 32]. These discordant findings could be the result of the different experimental models used in the studies (cultured cardiac cell line vs. wild-type/thalassemic mice) as well as different iron administration protocols (short-term incubation vs. long-term iron diet).

4. The evidence showing cardiomyocyte ferroptosis is triggered by excess iron and specific ferroptosis-inducing compounds.

Physiologically, polyunsaturated fatty acids (PUFAs) in the cell membrane are continually acted upon by counterbalancing redox reactions [46]. Lethal levels of lipid peroxidation, the hallmark of ferroptotic cell death, can be reached if the oxidation processes exceed the reduction mechanisms [19, 25, 46]. The membrane lipids can be oxidized either directly by strong oxidizing agents (for example, ROS) or in an enzyme-dependent manner via the actions of various lipoxygenases (LOX) and cyclooxygenases (COX) [19, 25, 46]. On the other hand, GPX4 serves as the major membrane lipid-reducing enzyme [25, 27, 28, 46]. GPX4 action is dependent on glutathione (GSH), a tripeptide antioxidant consisting of glutamate, cysteine, and glycine [25, 27, 28, 46]. Therefore, excessive ROS production, increased LOX and/or COX activity, depletion of GSH or its amino acid constituents, as well as direct inhibition of GPX4, can lead to overwhelming lipid peroxidation and ferroptosis [25, 27, 28, 46]. Accordingly, multiple compounds (mostly synthetic small molecules) such as erastin (inhibitor of system X_c^- which imports cystine, the precursor of cysteine) and Ras-selective lethal small molecule 3 (RSL3, a GPX4 inhibitor) have been regarded as ferroptosis inducers as they cause the aforementioned defects in membrane redox balance maintenance [25, 27, 28, 46]. In this regard, iron is involved in the ferroptotic pathway as it: (1) catalyzes

the reactions for ROS production via the Fenton reaction, and (2) serves as a cofactor for LOX, enabling this enzyme to oxidize PUFAs [25, 27, 28, 46].

Different cell types, or even the same cell type under different conditions, are not equally susceptible to ferroptosis, possibly because of different capacity to control cellular redox status [47, 48]. Thus, decryption of the relevance of ferroptotic cell death in various pathophysiological processes requires disease-specific models. Pertaining to cardiac iron overload, ferroptosis directly induced by iron excess in cardiomyocytes has been reported in one study [36], although this has been shown in some other cell types including HeLa and HT-1080 fibrosarcoma cells, mouse embryonic fibroblasts, and AML12 mouse hepatocytes [47, 48]. By directly incubating isolated mouse cardiomyocytes in ferric citrate (0.1-2 μM), Baba *et al* [36] demonstrated that excess iron could induce cardiac ferroptotic cell death as efficiently as erastin (50 μM) and RSL3 (1 μg/ml) [36]. In several other studies cardiac cell death triggered by specific ferroptosis-inducing compounds has been investigated. These include erastin (8 μM), RSL3 (1 μg/ml), and isopremaline (1 μM) [36, 49]. As previously mentioned, erastin and RSL3 reduce glutathione availability and suppress GPX4 activity, respectively [36, 49]. Isoprenaline interferes with many of the proteins involved in labile iron availability and iron-mediated redox reactions, including GPX4, NADPH oxidase 4 (Nox4) and ferritin heavy chain [49]. Cardiomyocyte death in all of these studies has been confirmed to be of ferroptotic type as it was suppressed by a specific ferroptosis inhibitor, ferrostatin-1 [35, 36, 49], but not by an apoptosis inhibitor Z-vad-fmk [49]. The effects of iron overload, as well as the established ferroptosis-inducing compounds, on cardiomyocyte ferroptosis are summarized in Table 5 and Fig.1.

In addition to the action of ferrostatin-1, ferroptosis in cardiomyocytes could also be prevented by augmented mechanistic target of rapamycin (mTOR) signaling, overexpression of ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (ENPP2), and administration of puerarin (a bioactive compound extracted from a Chinese medicinal plant *Pueraria lobata*) (Table 5) [35, 36, 49]. It has been reported that mTOR regulates iron homeostasis by modulating transferrin receptor 1 (TfR1) stability [50, 51]. As TfR1 is one of the major pathways of iron entry into the cell, it has been hypothesized that ferroptosis could be modified via alterations of mTOR activity. The study by Baba *et al* demonstrated that mTOR transgenic (mTOR-Tg) mice exhibited reduced intracellular iron and ROS levels [36]. Since the iron importer TfR1 and the iron exporter ferroportin were both increased in this mTOR-Tg model, the decreased cardiomyocyte iron burden suggested that mTOR signaling upregulated ferroportin to a greater extent than TfR1 [36].

ENPP2, also known as autotaxin, is a secreted enzyme important for the production of lysophosphatidic acid (LPA), which may act as a signaling molecule in an autocrine/paracrine manner [35]. Adenoviral-transfected cardiomyocytes which overexpressed ENPP2 were less susceptible to ferroptosis. These cells exhibited upregulation of GPX4 and downregulation of acyl-CoA synthethase long chain family member 4 (ACSL4, an enzyme responsible for incorporation of membrane PUFAs). However, LPA signaling is still poorly characterized, and the mechanism by which it modulates the expressions of ferroptosis-related factors is still unknown. Similarly, puerarin increased GPX4 and decreased Nox4 in cardiomyocytes by an unknown signaling pathway, leading to protection against ferroptotic cell death [35].

Conclusion

Iron overload results in impaired cardiac performance by causing a deterioration in cardiac mitochondrial function and interfering with cardiac mitochondrial dynamics. Cardiac ferroptosis has also been investigated in a limited number of studies. However, clarification of the mechanistic link between cardiac ferroptosis and iron overload cardiomyopathy, as well as its relative contribution in comparison to other forms of regulated cell death, requires additional investigation. This insight may lead to the discovery of novel therapeutic targets, more effective pharmacological interventions, and improved clinical outcomes of IOC treatment.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Figure legend

Fig. 1. A summary of the effects of iron overload on mitochondrial function, mitochondrial dynamics, and ferroptosis in cardiomyocytes. Iron overload mediates cardiomyocytes injury via impaired cardiac mitochondrial function, altered cardiac mitochondrial dynamics, and cardiac ferroptosis. Δψm: mitochondrial membrane potential change; ACSL4: acyl-CoA synthetase long-chain family 4; COX: cyclooxygenase; GPX4: glutathione peroxidase 4; GSH: glutathione; ISO: isoprenaline; LIP: labile iron pool; LOX: lipoxygenase; LTCC: L-type Ca^{2+} channel; MCU: mitochondrial Ca^{2+} uniporter; PUFA: polyunsaturated fatty acid; ROS: reactive oxygen species; RSL3: RAS selective lethal 3; system X_c^-: cystine/glutamate antiporter; TTCC: T-type Ca^{2+} channel.

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Table 1. The effects of iron overload on cardiomyocyte viability, oxidative stress and cardiac mitochondrial function: *in vitro* studies

Model	Iron overload induction	Results (vs. control)			Interpretation	Ref.
		Cardiomyocyte iron uptake	Cell viability and whole-cell oxidative stress	Cardiac mitochondrial function		
HL-1 cell	$FeCl_3$ with ascorbic acid (Fe^{2+}), 300-600 μM, 72 hours	↑↑	↑↑ apoptosis (↑active caspase-3, only 600 μM Fe^{2+})	↑ Δψm	Fe^{2+} was more potent than Fe^{3+} in inducing HL-1 cell apoptosis via the mitochondria dysfunction-mediated caspase-3 dependent pathway.	(20)
	$FeCl_3$ (Fe^{3+}), 300-600 μM, 72 hours	↑	↑ apoptosis	-		
H9c2 cell	$FeCl_3$ (Fe^{3+}), 0.0375-0.6 mM, 72 hours	↑	↑ ROS ↑ apoptosis (↑active caspase-3)	↑ Δψm	Fe^{3+} induced oxidative stress-mediated apoptosis in H9c2 cells via mitochondria dysfunction.	(31)
Isolated cardiac mitochondria from male Wistar rats	FAC with ascorbic acid (Fe^{2+}), 286 μM, 5 minutes	-	-	↑↑ iron uptake ↑↑ ROS ↑↑ Δψm ↑ swelling	Fe^{2+} caused more severe cardiac mitochondrial dysfunction than Fe^{3+}.	(33)
	FAC (Fe^{3+}), 286 μM, 5 minutes	-	-	↑ iron uptake ↑ ROS ↑ Δψm ↔ swelling		
Isolated cardiac mitochondria	FAC with ascorbic acid	-	-	↑ ROS ↑ Δψm	Fe^{2+} caused mitochondrial oxidative stress, leading to	(16)

from male C57/BL6 mice (WT and HT mice)	(Fe^{2+}), 1.25-5 µg/ml, 5 minutes			↑ swelling	impaired cardiac mitochondrial function.	
Isolated cardiac mitochondria from male Sprague-Dawley rats	$FeCl_3$ (Fe^{3+}) 0.049 mg/g, s.c., 2 weeks	-	↓ cell viability (↑ LDH)	↑ ROS ↓ RCR ↓ ATP content ↑ α and β subunits of F_1F_0 ATP synthase	Fe^{3+} caused diminished ATP production and mitochondrial dysfunction; with overexpression of F_1 subunit of F_0F_1 ATP synthase as a potential compensatory mechanism.	(34)

Δψm: mitochondrial membrane potential; ATP: adenosine triphosphate; FAC: ferric ammonium citrate; Fe^{2+}: ferrous ion; Fe^{3+}: ferric ion; $FeCl_3$: ferric chloride; HT: heterozygous β^{KO} genotype; LDH: lactate dehydrogenase; RCR: respiratory control ratio; ROS: reactive oxygen species; s.c.: subcutaneous injection; WT: wild-type.

Table 2. The effects of the pharmacological interventions on cardiomyocytes viability, oxidative stress and cardiac mitochondrial function under iron overload condition: *in vitro* studies

Model	Iron overload induction	Intervention	Results (vs. without intervention)			Interpretation	Ref.
			Cardiomyocytes iron uptake	Cell viability/ Oxidative stress	Cardiac mitochondria function		
HL-1 cell	$FeCl_3$ with ascorbic acid (Fe^{2+}), 150-600 µM, 72 hours	**LTCC blockers** Amlo, 0.1-100 µM	↓ iron uptake (only 100 µM in 150-µM Fe^{2+})	↔ apoptosis	-	LTCC blocker, but not TTCC blocker, prevented Fe^{3+} entry into HL-1 cell without improving cardiac apoptosis.	(20)
		Ver, 0.1-100 µM	↔ iron uptake	↔ apoptosis	-		
		TTCC blocker Efo, 0.1-100 µM	↔ iron uptake	↔ apoptosis	-		
	$FeCl_3$ (Fe^{3+}), 150-600 µM, 72 hours	**LTCC blockers** Amlo, 0.1-100 µM	↓ iron uptake	↔ apoptosis	-		
		Ver, 0.1-100 µM	↓ iron uptake	↔ apoptosis	-		
		TTCC blocker Efo, 0.1-100 µM	↔ iron uptake	↔ apoptosis	-		
H9c2 cell	$FeCl_3$ (Fe^{3+}), 0.3 mM, 72 hours	**Antioxidant** TPO, 50 ng/ml	-	↓ ROS ↓ apoptosis ↓ active caspase-3 activity	↓ Δψm	TPO rescued oxidative stress and mitochondria dysfunction-mediated apoptotic pathways under iron-	(31)

						overloaded cardiomyocyte.	
Isolated cardiac mitochondria from male Wistar rats	FAC with ascorbic acid (Fe^{2+}), 286 µM, 5 minutes	**mPTP blocker** CsA, 5 µM	-	-	↓ ROS ↔ Δψm ↔ swelling	Ru360 showed greater improvement in mitochondrial function than the CsA and DFO under iron overloaded condition, suggesting MCU may play a role in iron uptake into cardiac mitochondria.	(33)
		MCU blocker Ru360, 10 µM	-	-	↓↓ ROS ↔ Δψm ↓ swelling ↓ iron uptake		
		Iron chelator DFO, 20 µg/ml	-	-	↓ ROS ↔ Δψm ↓ swelling		
	FAC (Fe^{3+}), 286 µM, 5 minutes	**mPTP blocker** CsA, 5 µM	-	-	↓ ROS ↔ Δψm ↔ swelling		
		MCU blocker Ru360, 10 µM	-	-	↓↓↓ROS ↓ Δψm ↔ swelling ↔ iron uptake		
		Iron chelator DFO, 20 µg/ml	-	-	↓↓ ROS ↓ Δψm		

					↔ swelling		
Isolated cardiac mitochondria from heart of WT and HT mice (adult C57/BL6 mice)	FAC with ascorbic acid (Fe^{2+}), 5 µg/ml, 5 minutes	**mPTP blocker** CsA, 5 µM, 5-10 minutes	-	-	↓ ROS ↔ Δψm ↔ swelling	Ru360 improved mitochondrial function and oxidative status under iron overload condition.	(16)
		MCU blocker Ru360, 10 µM, 5-10 minutes	-	-	↓↓ ROS ↓ Δψm ↓ swelling		

Δψm: mitochondrial membrane potential; Amlo: amlodipine; ATP: adenosine triphosphate; CsA: cyclosporin A; DFO: deferoxamine; Efo: efonidipine; FAC; ferric ammonium citrate; Fe^{2+}: ferrous ion; Fe^{3+}: ferric ion; $FeCl_3$: ferric chloride; HT: heterozygous β^{KO} genotype; LTCC: L-type Ca^{2+} channel; MCU: mitochondrial calcium uniporter; mPTP: mitochondrial permeability transition pore; ROS: reactive oxygen species; Ru360: oxygen-bridged dinuclear ruthenium amine complex; TPO: thrombopoietin; TTCC: T-type Ca^{2+} channel; Ver; verapamil; WT: wild-type.

Table 3. The effects of iron overload on oxidative stress, mitochondrial function/dynamics, and cardiac function: *in vivo* studies

Model	Iron overload induction	Results (vs. control)				Interpretation	Ref.
		CIC	Cardiac oxidative stress	Cardiac mitochondrial function and dynamics	Cardiac function		
Male B6D2F1 mice	Iron dextran, 19 mg/day, i.p., 4 weeks	↑	-	↑ swelling ↓ mtDNA ↓ complex I ↓ complex IV	↓ LVIDd ↓ AWTd ↓ PWTd ↑ RWT ↑ LVEF	Chronic iron overload mediated mtDNA damage and mitochondrial dysfunction by deteriorating mitochondrial respiration chain synthesis, leading to cardiac dysfunction.	(45)
Male B6D2F1 mice	Iron dextran, 20 mg, i.p., 2 hours	↑	↑ GPX activity ↑ MDA ↑ 4-HNE ↑ hexanol	↑ swelling	-	Both acute and chronic iron overload generated oxidative stress which altered cardiac mitochondrial morphology in mice.	(41)
	Iron dextran, 20 mg/day, i.p., 3 weeks	↑↑	↓ GPX activity ↑↑ MDA ↑↑ 4-HNE ↑↑ hexanol	↑ swelling	-		
Female Mongolian gerbil	Iron dextran, 200 mg/kg, i.p., 14-18 weeks	↑	↑ MDA ↓ GPX activity	↑ swelling	-	Iron-mediated lipid peroxidation caused cardiac mitochondrial morphological changes and dysfunction in gerbil mice model.	(42)
Male C57/BL6	Iron diet (0.2%	↑	↑ MDA	↑ ROS	↑ LF/HF ratio	Cardiac iron overload	(32)

mice; WT and HT	ferrocene/kg), 150 days			↑ Δψm ↑ swelling	↓ ESP ↓ P_{max} ↓ dP/dt_{max} ↓ SV ↓ CO ↓ SW	caused cardiac mitochondrial dysfunction, leading to cardiac systolic and autonomic dysfunction.	
Male C57BL/6 mice; WT and HT mice	Iron diet (0.2% ferrocene/kg), 120 days	↑	↑ MDA ↑ cleaved caspase-3	↑ ROS ↑ Δψm ↑ swelling ↑ Mfn-2 ↑ Drp-1/Mfn-2 ↓ complex IV ↓ complex V	↑ LF/HF ratio ↑ MAP ↓ LVEF ↓ LVFS	Iron overload caused cardiac dysfunction via mitochondrial dysfunction, mitochondrial dynamic dysregulation, mitochondrial biogenesis alteration, and apoptosis.	(15)
Male C57/BL6 mice; WT and HT	Iron diet (0.2% ferrocene/kg), 90 days	↑	↑ MDA ↑ cleaved caspase-3	-	↑ LH/HF ratio	Cardiac iron overload caused cardiac oxidative stress and apoptosis, leading to cardiac autonomic dysfunction.	(24)
Male Wistar rats	Iron diet (0.2% ferrocene/kg), 120 days	↑	↑ MDA	↑ ROS ↑ Δψm ↑ swelling	↑ LF/HF ratio ↓ LVEF ↓ LVFS	Cardiac iron overload caused oxidative stress and impaired mitochondrial functions, leading to cardiac systolic and autonomic dysfunction in rat model.	(43, 44)

Δψm: mitochondrial membrane potential; 4-HNE: 4-hydroxynonenal; AWTd: anterior wall thickness; CIC: cardiac iron concentration; CO: cardiac output; DNA: deoxyribonucleic acid; Drp-1: dynamin-related protein 1; EDP: end diastolic pressure; ESP: end systolic pressure; GPX: glutathione peroxidase; HT: heterozygous β^{KO} genotype; i.p.: intraperitoneal injection; LF/HF ratio: a ratio of low frequency to high frequency; LVEF: left ventricular ejection fraction; LVFS: left ventricular fractional shortening; LVIDd: left ventricular internal diameter end diastolic;

LVIDs: left ventricular internal diameter end systolic; MAP: mean atrial pressure; MDA: malondialdehyde; Mfn2: mitofusin-2; PGC-1α: peroxisome proliferator-activated receptor gamma coactivator-1 alpha; PWTd: posterior wall thickness; ROS: reactive oxygen species; RWT; Relative wall thickness; SV: stroke volume; SW: stroke work; WT: wild-type.

Table 4. The effects of the pharmacological interventions on oxidative stress, mitochondrial function/dynamics, and cardiac function under iron overload condition: *in vivo* studies

Model	Iron overload induction	Intervention	Results (vs. without intervention)				Interpretation	Ref.
			CIC	Cardiac oxidative stress	Cardiac mitochondrial function and dynamics	Cardiac function		
Male C57/BL6 mice; WT and HT	Iron diet (0.2% ferrocene/kg), 150 days	**Iron chelators** - DFO, 42 mg/kg/day, s.c., 30 days - DFX, 30 mg/kg/day, oral gavage, 30 days - DFP, 75 mg/kg/day, oral gavage, 30 days **Dual TTCC and LTCC blocker** Efo, 4 mg/kg/day, s.c., 30 days **LTCC blocker** Amlo, 5 mg/kg/day, oral gavage, 30 days	↓	↓ MDA	↓ ROS ↓ Δψm ↓ swelling	↓ LF/HF ratio ↑ ESP ↑ P_{max} ↑ dP/dt_{max} ↑ SV ↑ CO ↑ SW	Dual TTCC and LTCC blockers, LTCC blocker and iron chelators showed similar efficacy in decreasing CIC and improving mitochondrial and cardiac functions in iron overloaded thalassemic mice.	(32)

Male C57BL/6 mice; WT and HT mice	Iron diet (0.2% ferrocene/kg), 120 days	**Iron chelator** DFP, 75 mg/kg/day, oral gavage, 30 days **TTCC blocker** Efo, 4 mg/kg/day, oral gavage, 30 days	↓	↓ MDA	↓ ROS ↓ Δψm ↓ swelling	↓ LF/HF ratio ↑ LVEF ↑ LVFS	Efonidipine provided cardioprotective effects similar to deferiprone by reducing CIC and restoring cardiac and mitochondrial dysfunctions.	(15)
Male C57/BL6 mice; WT and HT	Iron diet (0.2% ferrocene/kg), 120 days	**Iron chelator** DFP, 75 mg/kg/day, oral gavage, 30 days **Antioxidant** NAC, 100 mg/kg/day, oral gavage, 30 days Combined DFP (75 mg/kg/day) plus NAC (100 mg/kg/day), oral gavage, 30 days	↓ ↓↓	↓ MDA ↓ MDA ↓ cleaved caspase-3	- -	↓ LF/HF ratio ↓ LF/HF ratio	Compared to monotherapy, combined DFP plus NAC treatment exerted greater effects in reducing CIC, oxidative stress, and cardiac apoptosis, leading to improved cardiac autonomic function in iron overload condition.	(24)
Male Wistar rats	Iron-diet, (0.2% ferrocene/kg), 120 days	**Iron chelators** - DFP, 75 mg/kg/day, oral gavage,	↓	↓ MDA	↓ ROS ↓ Δψm ↓ swelling	↓ LF/HF ratio ↑ LVEF	Compared to monotherapy, combined DFP plus NAC treatment	(43, 44)

		60 days - DFO, 25 mg/kg/day, s.c., 60 days - DFX, 20 mg/kg/day, oral gavage, 60 days **Antioxidant** NAC, oral gavage, 100mg/kg/day, 60 days Combined DFP (75 mg/kg/day) plus NAC (100 mg/kg/day), oral gavage, 60 days	↓↓	↓↓ MDA	↓↓ ROS ↓↓ Δψm ↓ swelling	↑ LVFS ↓↓ LF/HF ratio ↑↑ LVEF ↑↑ LVFS	exerted greater effects in reducing CIC and oxidative stress, leading to improved cardiac mitochondrial function as well as cardiac autonomic and systolic function in iron overload condition.	

Δψm: mitochondrial membrane potential; Am: amlodipine; AWTd: anterior wall thickness; CIC: cardiac iron concentration; CO: cardiac output; DFP: deferiprone; DFO: deferoxamine; DFP: deferiprone; DFX: deferasirox; EDP: end diastolic pressure; ESP: end systolic pressure; GPX: glutathione peroxidase; HT: heterozygous β^{KO} genotype; LF/HF ratio: a ratio of low frequency to high frequency; LTCC: L-type Ca^{2+} channel; LVEF: left ventricular ejection fraction; LVFS: left ventricular fractional shortening; LVIDd; left ventricular internal diameter end diastolic; LVIDs; left ventricular internal diameter end systolic; MAP: mean atrial pressure; MDA: malondialdehyde; NAC: n-acetyl cysteine; PWTd: posterior wall thickness; ROS: reactive oxygen species; RWT; Relative wall thickness; s.c.: subcutaneous injection; SV: stroke volume; SW: stroke work; TTCC: T-type Ca^{2+} channel; WT: wild-type.

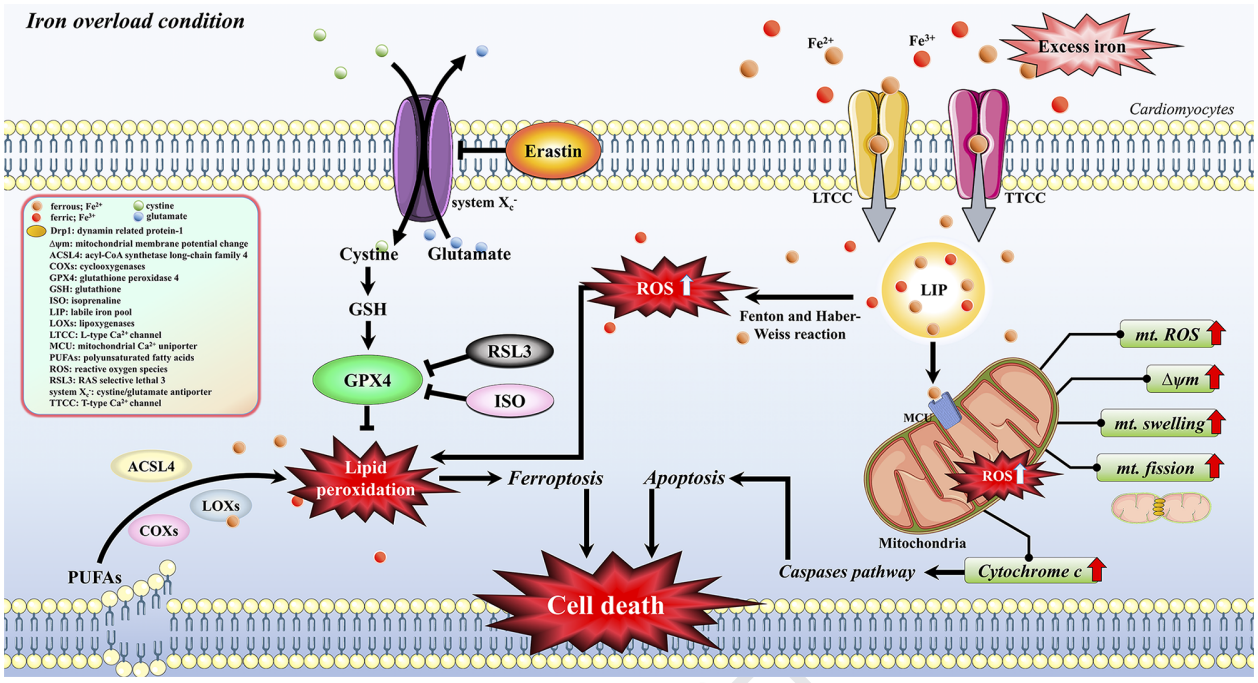
Tale 5. Direct induction of ferroptosis by intracellular iron accumulation and other ferroptosis-inducing molecules in cardiomyocytes

Model	Ferroptosis induction	Results (vs. control)				Interpretation	Ref.
		Without ferrostatin-1		With ferrostatin-1 (doses as specified in parentheses)			
		Cell viability	Oxidative stress/ ferroptosis marker	Cell viability	Oxidative stress/ ferroptosis marker		
Isolated cardiomyocytes from WT mice	Erastin, 50 µM, 24 hours	↓	↑ ROS	↔ (10 µM, 24 h)	↔ (10 µM, 24 h)	Ferroptosis induced by Fe^{3+}, erastin, and RSL3 in cardiomyocytes could be prevented by ferrostatin-1 and mTOR signaling.	(36)
	RSL 3, 1 µg/ml, 24 hours	↓	↑ ROS	↔ (10 µM, 24 h)	-		
	FAC (Fe^{3+}), 0.1-2 mM, 24 hours	↓	↑ ROS	↔ (10 µM, 24 h)	-		
Isolated cardiomyocytes from mTOR transgenic (mTOR-Tg) mice	-	-	↑ TfR1 ↑ ferroportin	-	-		
	Erastin, 50 µM, 24 hours	↑	↓ ROS	-	-		
	RSL 3, 1 µg/ml, 24 hours	↑	↓ ROS	-	-		

	FAC (Fe^{3+}), 0.1-2 mM, 24 hours	↑	↓ ROS ↑ TfR1 ↑ ferroportin	-	-		
Isolated cardiomyocytes from mTOR knockout (mTOR-KO) mice	Erastin, 50 μM, 24 hours	↓	↑ ROS	-	-		
	RSL3, 1 μg/ml, 24 hours	↓	↑ ROS	-	-		
	FAC (Fe^{3+}), 0.1-2 mM, 24 hours	↓	↑ ROS	-	-		
H9c2 cell	Erastin, 1-10 μM, 24 hours	↓	↑ ROS	↔ (1 μM, 24 h)	↔ (1 μM, 24 h)	ENPP2 protected cardiomyocytes from erastin-induced ferroptosis by promoting LPA production, GPX4 expression, and Akt survival signaling as well as suppressing ACSL4 and Nrf2 expression.	(35)
	ENPP2 transfection with erastin, 2.5-5 μM, 4 hours	↔	↔ ROS ↑ GPX4 ↔ p-MAPK/MAPK ↑ p-Akt/Akt ↓ ACSL4 ↓ Nrf2	↔ (1 μM, 24 h)	-		

H9c2 cells	Erastin, 2-8 µM, 24 hours	↓	↑ lipid peroxidation (↑ TBARSs)	↔ (10 µM, 24 h; comparable effect was also achieved with puerarin, 40 µM, 24 h)	-	Erastin and ISO induced ferroptosis via increasing lipid peroxidation and decreasing anti-oxidative proteins.	(49)
	ISO, 0.01-1 µM, 48 hours	↓	↑ lipid peroxidation (↑ TBARSs) ↑ Nox4 ↓ GPX4 ↓ FTH1	↔ (10 µM, 24 h; comparable effect was also achieved with puerarin, 40 µM, 24 h)	↔ Nox4 ↔ GPX4 ↔ FTH1 (10 µM, 24 h; comparable effect was also achieved with puerarin, 40 µM, 24 h)		

ACSL4; acyl-coA synthethase long chain family member 4; Akt: protein kinase B; ENPP2: ectonucleotide pyrophosphatase/phosphodiesterase family member 2; FAC: ferric ammonium citrate; FTH1: ferritin heavy chain 1; GPX4: glutathione peroxidase 4; ISO: isoprenaline; LPA; lysophosphatidic acid; LPAR-1: lysophosphatidic acid receptor 1; MAPK: mitogen-activated protein kinase; mTOR: mechanistic target of rapamycin; Nox4: NADPH oxidase 4; Nrf2: nuclear factor erythroid 2-related factor 2; : phospho-protein kinase B; p-MAPK: phospho-mitogen-activated protein kinase; ROS: reactive oxygen species; RSL3: RAS selective lethal 3; TBARS: thiobarbituric acid reactive substances.



Highlights:

- Excess iron alters mitochondrial function and dynamics in cardiomyocytes.
- Iron overload promotes cardiomyocyte ferroptosis and apoptosis.
- Iron-induced mitochondrial abnormalities and cell death impair cardiac function.