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Are the health effects of exercise related to changes in iron metabolism?

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Abstract. There are increasing number of evidences that regular exercise reduce risks of several morbidities like cancer, heart attack, type II diabetes, hypertension and slows down ageing process. The mechanism of pro-healthy effects of exercise is far from being completely understood. For example exercise increase insulin sensitivity in type II diabetics and this is related to activation of AMP kinase and increase biogenesis of mitochondria in skeletal muscle. Interestingly, increase in insulin sensitivity was also observed in patients, whose body iron stores has been reduced. The fact that exercise has been reported to influence body iron metabolism made us ponder the question whether many positive effects of exercise can be related to these changes. Iron is essential for most of cellular processes like energy metabolism, cell proliferation, synthesis of DNA and collagen, posttranslational modification of some proteins and many others. However, for our body iron like oxygen when in excess, is toxic. The elevated body iron reserves have been linked with an increased risk of health problems such as cancer, type 2 diabetes, and coronary artery disease. Total body iron accumulates when iron intake begins to exceed its loss. This data are confirmed by studies on animal models of iron-induced cancer, and diabetes. Life-style changes such as exercise, calorie restriction, a diet rich in iron chelators, and phlebotomy are all associated with the reduction of total body iron. These treatments lead to diminished risk of several morbidities. Nevertheless, studies on cell cultures demonstrated that the labile iron pool (LIP) mainly determines its toxicity rather than total amount of accumulated iron. The LIP level is regulated by several signaling pathways and gene expression. Thus, it is expected that even at a high concentration of intracellular iron, its toxicity is not obvious until a cell is able to maintain a low level of LIP. These data suggest that an effective control of body iron stores (diet, exercise, and possibly phlebotomy) would be a wise strategy for disease prevention.

Keywords: Oxidative stress, p66Shc, LIP, JNK, ferritin

1. Introduction

Iron is vital for cell survival and proliferation. Its deficiency may lead to anemia, causing a decrease in physical and mental performance [1, 2]. On the other hand, its excessive accumulation in tissues has been linked to several morbidities such as neurodegeneration, cancer, type 2 diabetes, and coronary artery disease [3, 4]. For example, type 2 diabetes is a common complication of iron overload. More than 50% of hemochromatosis patients develop type 2 diabetes [5].

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The total body iron in an adult is around 3000 to 4000 mg, while the iron requirement for erythropoiesis and other biochemical processes is about 20 mg per day. The human diet provides around 15–20 mg of iron, yet roughly only 5% of this allowance is absorbed in duodeum. Non-heme iron, mainly present in food of plant origin, is transported via a divalent metal ion transporter, DMT1, into enterocytes, and then stored by ferritin. The intestinal uptake of heme occurs via the heme carrier protein-1 (HCP1). Afterwards, the iron ion is liberated by heme oxygenase-1 and eventually stored by ferritin. Liberation of iron from enterocytes into the bloodstream is mediated by the iron transport protein, ferroportin 1 (Fpn1). Ferroportin concentration and activity are controlled by the iron-metabolism-regulating hormone-hepcidin. Hepcidin acts as a negative regulator of iron efflux. When the blood iron concentration rises, hepcidin increases, leading to a drop in intestinal iron absorption [6]. Although iron metabolism processes are effectively controlled by hepcidin, iron is still accumulated in several tissues due to either aging or an iron-rich diet. Diet particular rich in iron contains meat especially red meat but also fish and meat products like sausage etc. Recently published data reports that a high-fat, high-fructose diet (HFHFr) both induce hepatic iron overload via a hepcidin-independent mechanism, and may have a pathogenic effect in the onset of liver steatosis and insulin resistance [7]. The hepatic iron content and oxidative stress significantly increased on the HFHFr diet two weeks earlier than hepatic FFA accumulation and decreased insulin sensitivity [7].

Among several indicators which are used to evaluate iron storage, serum ferritin concentration is being the most common. A blood ferritin level below 25 μg/L is set as a threshold for iron deficiency in athletes according to Australian Institute of Sport, while ferritin levels for iron overload are less precisely defined. Several studies, which are presented in this review indicate that optimal blood ferritin should be below 100 μg/L [8, 9]. Interestingly, these values are observed in menstruating females, and in many physically fit males and females who are considered to be at low risk of several morbidities [10–12].

It is worth noting that most of the accumulated iron is stored intracellularly by ferritin, and does not stimulate Reactive Oxygen Species (ROS) formation. Only labile, redox-active iron is able to stimulate ROS formation through reacting with hydrogen peroxide, lipid hydroperoxides, and oxygen [13]. However, some data suggest that body iron contributes to excess oxidative stress at a normal concentration in humans [14]. Moreover, iron is also reported to aggravate ROS-mediated injuries and the signaling pathways involved [15]. Hence, not only the amount of accumulated iron, but also the mechanisms responsible for the labile iron pool (LIP) growth determine iron toxicity. Mechanisms controlling the level of LIP are multidimensional, and are related to changes in gene expression (e.g. ferritin, Fp1, transferrin receptor) and proteolysis (e.g. ferritin, ferroportin). Nevertheless, the questions why iron is toxic in specific conditions, and how exercise can influence iron toxicity still require more research to be fully answered.

2. Signaling role of iron

Intracellular ROS formation in the presence of free iron is quite distinct. Iron participates in the Fenton reaction, which produces hydroxyl radicals ($^{\cdot}OH$), the most reactive chemical species in biological systems (Equation 1).

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{-}OH + {}^{\cdot}OH \quad (1)$$

Iron-mediated oxidation of specific amino acids in proteins seems to be the main mechanism regulating signaling pathways. Oxidation of some proteins may lead either to their inactivation or in some cases to their activation [16]. It is important to note that iron-mediated oxidation of proteins is side specific [17] and that this process is not well characterized for most of the proteins. For example, with an elevated intracellular iron level, iron-responding protein 2 can undergo site-specific oxidation, which targets this protein for proteolytic degradation. This lead to ferritin biosynthesis and sequestration of free iron [18].

Among amino acids, cysteine has a very high affinity for chelatable iron. Interestingly, hydrogen peroxide (H_2O_2) itself may modify different enzymes by reacting with protein cysteine residues. Still, only certain proteins have cysteine residues that are vulnerable to oxidation by H_2O_2 alone. This is due to a high pKa of sulfydryl groups of most cysteine residues in proteins, around pKa = 8.5. Only the cysteine thiolate anion (Cys-S-) is vulnerable to oxidation by H_2O_2. Iron bound to cysteine sulfhydryl may stimulate hydroxyl radical formation and subsequent oxidation of cysteine. Furthermore, Fe^{2+} and oxygen may give rise to strong oxidants like perferyl and ferryl

ions with reactivities approaching that of.OH [19]. Therefore, protein modification and signaling initiated by ROS may be significantly different depending on the amount of chelatable iron. *In vivo* experiments confirm this. For instance, iron has been shown to play an important role in myocardial ischemic preconditioning, which is a well-established process of cardiomyocyte protection [20]. Short ischemic periods applied to the heart, separated by short periods of perfusion, induced an iron-mediated rise in ferritin protein levels. These rises in ferritin levels have been proven to be an adaptive change protecting the heart against prolonged ischemic insult. Iron chelators completely abolished these changes [20]. Increased ferritin protein synthesis has been suggested to be necessary for chelation of labile iron, which might be responsible for heart reperfusion injury [21]. Consequently, studies show that on the one hand, iron induces adaptive changes and on the other hand, when liberated in large quantity, aggravates tissue damage.

Iron bound by ferritin or transferrin is metabolically inert. Thus, toxicity of iron is dependent on an increase in its labile pool, which may result either from a decreased ferritin protein level or an increased transferrin receptor level [22]. On the other hand, cell ability to produce more ferritin and sequester iron, or diminish iron transport into cells is often a defense against an oxidative challenge. Another way to increase the LIP is to liberate iron from ferritin. Ferritin degradation in lysosomes or by proteasomes also leads to elevation of LIP. Under *in vitro* conditions superoxide anions, and some other reducing compounds like vitamin C, were shown to liberate iron from ferritin [23]. However, there is not enough convincing evidence that such mechanisms function *in vivo*.

3. Stress-activated protein kinases are activated by ROS and increase iron-dependent ROS formation

Stress-activated protein kinases (SAPK) belong to MAP kinases. ROS have been reported to activate SAPK, yet at the same time, some studies demonstrate that activation of SAPK may lead to an enhanced formation of ROS [24]. For instance, the apoptosis signal-regulating kinase (ASK) has been reported to be activated by free radicals, which oxidize thioredoxin – the physiological inhibitor of ASK1 [25]. Active ASK leads to activation of downstream kinases such as the c-Jun NH(2)-terminal kinase (JNK), and the p38 MAP kinase pathways. Moreover, JNK activity is inhibited by bound GST, which can be liberated by ROS [26]. Nevertheless, under some experimental conditions, the down-regulation of JNK substantially diminished ROS formation. The mechanism of this change have not been precisely determined [27]. Recently, we were able to demonstrate that JNK-dependent ROS formation is associated with an increase in LIP [28]. This data are in agreement with data showing that the LIP level determines intracellular ROS formation [29]. Both normal and neoplastic cells, when exposed to diallyl trisulfate (DATS - the anti-neoplastic compound from garlic), hydrogen peroxide, or tumor necrosis factor α (TNFα), show an elevated LIP. All of these treatments are known to activate SAPK. Thus the question arises whether these kinases somehow regulate intracellular LIP level [28, 30, 31].

We observed that cells with knocked-out SEK1 or expressing a plasmid encoding a catalytically inactive mutant of JNKK2 (which are JNK-specific upstream kinases), were resistant to DATS-induced ferritin degradation and ROS formation [32]. Several studies have shown that JNK can regulate the degradation of some proteins, yet the mechanism of this change remains unclear. The Karin group was able to demonstrate that JNK1 mediates ubiquitination and degradation of anti-apoptotic protein c-flip activating ubiquitin ligase E3 Itch [33]. Upon phosphorylation by JNK1, Itch undergoes conformational changes, which allow it to bind the substrate [34].

Following this discovery, we hypothesized that JNK-dependent ferritin degradation is mediated by Itch. In fact, cells transfected with a plasmid encoding a negative mutant of Itch were resistant to DATS-induced ferritin degradation [28]. Moreover, the involvement of the p66Shc protein, phosphorylated at serine 36, in the process of ferritin degradation was demonstrated [30]. Overall, these data suggest that during stress conditions ferritin degradation increases the LIP, and iron-dependent ROS formation is mediated by Itch and p66Shc, which are activated by JNK (Fig. 1).

JNK activation has been observed in muscle and heart post-exercise [35]. Regular training induced some adaptive changes that protected JNK from exercise-induced activation [35]. On the other hand, our preliminary data indicate that single bout of exercise induces ferritin degradation and iron-dependent oxidative stress in rat hearts. More work needs to be done to fully explore the role of JNK in this process. However, it is quite possible that regular training increases ferritin stability by protecting JNK from stress activation, and thus indirectly protects against iron toxicity (Fig. 1).

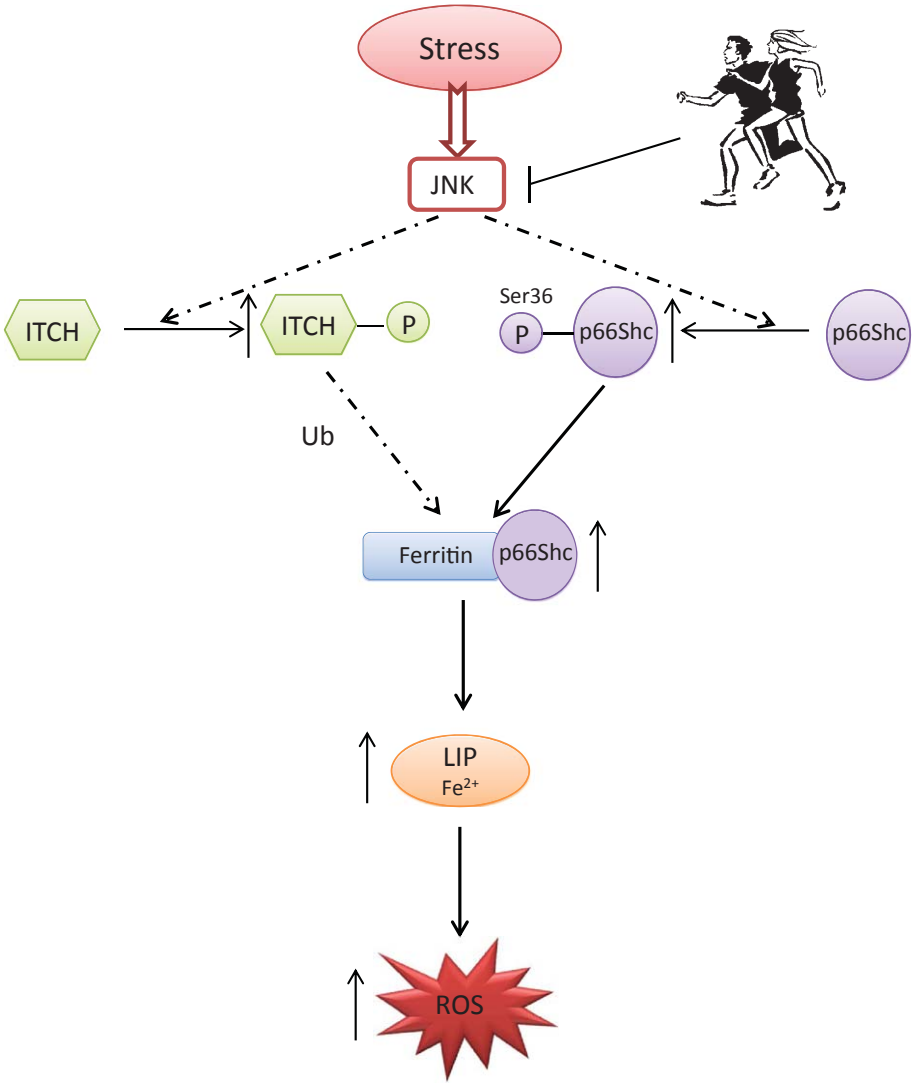

Fig. 1. The hypothetical role of JNK, Itch, and p66Shc in the mechanism of iron-dependent ROS formation. Stress induces JNK activation, that can mediate the activation of ubiquitin ligase E3 Itch and p66Shc protein phosphorylation at serine 36. Activation of Itch and p66Shc induces the process of ferritin degradation and increases the LIP and iron-dependent ROS formation. Regular training may protect stress-induced JNK activation.

4. Iron metabolism and health

The prevalence of low levels of physical activity is systematically increasing in developed countries, and is becoming one of the main risk factors of chronic diseases and premature death [36, 37]. Recent calculations estimated that there are 5.3 million deaths per year due to little physical activity [38]. Moreover, chronic diseases such as cardiovascular disease and type 2 diabetes are classified as dominating health problems worldwide [39]. Consequently, physical inactivity has been identified as a risk factor stronger even than hypertension, hyperlipidemia, diabetes, and obesity for mortality [40]. Physical inactivity may also be responsible for body iron accumulation. However, up to day, there are no convincing evidences that this is a case. Studies on athletes consistently indicate that regular exercise is associated with lower levels of body iron. For example one study showed that both serum ferritin and bone marrow

hemosiderin (indicator of bone marrow iron stores) were lower in the athletes than in a group of non-athletic men of the same age [41]. Interestingly, iron deficiency anemia is rare among female athletes [42].

We hypothesize that changes in iron metabolism induced by regular exercise are essential for a healthy lifestyle and disease prevention. Moreover, we assume that one of the most important and pro-health effects of physical activity is associated with the reduction of total body iron. Although there is no direct evidence for such interdependency, numerous studies indirectly support this hypothesis. For instance, some study demonstrated that exercise improved insulin sensitivity [43]. A similar effect was also achieved by phlebotomy - a standard procedure in reducing body iron reserves [44]. On the other hand, Zacharski and co-workers demonstrated that in healthy men and women, whose iron reserves were reduced by phlebotomy and kept low (ferritin levels below 60 ng/ml), the incidence of neoplasia was reduced by 37% compared to the control group, during a 4.5 year time period [9]. In line with this data, there is a growing number of studies confirming that exercise is an effective way to reduce the risk of breast, colon, and lung cancers [45–47]. Manson and coworkers also observed that among a group of physically active women (3 hours of walking per week), the relative risk of a heart attack decreased by 50% compared to inactive subjects [48]. What is more interesting, Salonnen revealed that for each 1% rise in serum ferritin levels, there was more than a 4% increase in risk of a heart attack [8].

The relationship between iron accumulation in skeletal muscles and exercise capacity was investigated by Reardon and Allen [49]. In this study, authors applied a mouse model of iron overload (5 days of injections a week until day 31; 100 μl of iron dextran (10 mg iron) per injection). Compared with control mice, the iron-overloaded mice exhibited elevated levels of iron in the tibialis anterior muscle, and a four-fold increase in ferritin light chains. Elevated markers of oxidative stress were also observed in the iron group, compared with the control group. Moreover, the iron group exhibited more mass reduction of the fast-twitch extensor digitorum longus muscle than the slow-twitch soleus muscle, and a decrease in exercise capacity and muscle strength. As noted by authors, iron accumulation in skeletal muscle might play a significant role in reducing exercise capacity seen in iron overload disorders, as well as in aging. It may also be essential in skeletal muscle atrophy. Altogether, the data suggest that regular physical activity leads to diminished body iron stores, which can be one of the desirable adaptive responses to exercise.

5. Hepcidin - a key regulator of iron metabolism and the effects of exercise

Hepcidin is an iron-regulatory hormone that mediates the homeostasis of extracellular iron concentrations. It is a 25-amino acid peptide circulating in plasma and excreted in urine.

Hepcidin acts by regulating iron efflux into plasma from tissues engaged in iron storage or transport (duodenal enterocytes that absorb dietary iron, hepatocytes that store iron and macrophages that recycle iron from senescent erythrocytes). At the molecular level, hepcidin binds to ferroportin, and induces its internalization and lysosomal degradation. In addition to the tissues mention above, ferroportin is also present in skeletal muscle. Thus, muscle can be considered as a hepcidin target tissue. However, the exact function of ferroportin in skeletal muscle is not known as skeletal muscle is not considered to be an iron storing tissue. Hepcidin synthesis is physiologically increased by elevated plasma iron concentration, decreased by erythropoietic activity and during inflammation. On the other hand, hepcidin deficiency is the primary cause of iron overload in most cases of hereditary hemochromatosis and contributes to iron overload [6]. Recent studies have focused on the impact of exercise on blood hepcidin. Hepcidin levels have been shown to rise in response to running a marathon race. This type of exercise caused an increase in urine hepcidin levels in some athletes (responders), yet had no effect on other runners (nonresponders) [50]. In agreement with these data are observations on professional long-distance runners for whom decreased iron absorption and its increased elimination have been observed [51]. On the other hand, no changes in blood hepcidin levels were observed in ultra-marathon runners after running a distance of 100 km. It is worth noting that these athletes have low iron stores and blood ferritin was lower than 60 μg/L [11]. This study indicates that an exercise-induced rise in hepcidin levels may be dependent on the amount of stored body iron. According to our observations, some professional athletes have a higher concentration of hepcidin and lower body iron reserves compared to non-athletes [12]. In recent years much attention gained high intensity interval training which has been demonstrated to be a very effective method of training [52]. This method is able to induce adaptive changes similar to these observed in classical endurance training [52]. What is more important it also has pro-health effects, such as increase insulin sensitivity in type 2 diabetes

patients [53]. Interestingly, this kind of exercise also significantly increases blood hepcidin levels both in athletes and non-athletes [12]. However, in non-athletes the effects of this exercise lasted for 5 days, while in athletes hepcidin levels returned to baseline value after 24 hours [12]. In summary, this data indicate that both occasional exercise, as well as regular training, lead to increase in blood hepcidin concentration, which may cause a reduction in iron absorption from the duodenum. Currently, this seems to be the main mechanism responsible for the exercise-induced reduction of iron reserves in the human body.

6. Aging and age-related diseases

Human aging, associated with incorrect iron homeostasis, is a gradual process taking place over decades [54]. Age-related changes are associated with a progressive structural deterioration of biomolecules and cellular compartments, and a decline in function of human organs and tissues that leads to disease and mortality [55, 56]. There are plenty hypotheses regarding the pathophysiological mechanisms of aging, of which Harman's oxygen free radicals theory seems to be the most accepted. Harman proposed that oxygen free radicals (specifically hydroxyl, $^{\cdot}OH$, and hydroperoxyl, HO_2 radicals) are formed *in vivo* in oxygen-utilizing metabolic processes [57]. There is evidence confirming this theory, describing the accumulation of oxidatively-damaged macromolecules during aging [58, 59]. Oxidative stress is also associated with many age-related pathological conditions including cardiovascular disease, stroke, Alzheimers' disease, osteoporosis, sarcopenia, cancer, diabetes, and liver dysfunction [60, 61]. Each of these conditions is associated with a progressive functional decline, loss of independence, and ultimately, disability [55].

As mentioned above, iron is implicated in the pathogenesis of age-related diseases. Age-dependent, enhanced concentrations of iron have been observed in animals in the brain, liver, and heart [62]. Similarly, old rats showed significantly elevated cardiac (+72%) and liver iron level (+87%), and ferritin light chain (+59%) compared to adult animals [63]. In another study, greater levels of ferritin and iron, in spleens and livers of older rats were observed [64]. Butterfield's group has extensively elaborated on the role of oxidant radicals generated from Fenton reactions dependent on iron or copper, on protein and lipid peroxidation, brain aging, and neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis [65–69]. Excessive iron tissue levels are associated with an increase of oxidative or nitrative stress, which contributes to tissue damage that may also enhance the risk of diabetes [70]. It has been shown that iron overload obtained in control and diabetic rats by i.p. 300 mg/kg iron dextran, induced liver damage and oxidative/nitrative stress (increased liver lipid and protein peroxidation markers, and reduced liver glutathion peroxidase) [70]. Iron-dependent destructive effects were intensified in the diabetic group. Similar findings have been obtained in another model of iron overload [71]. Control female Golden Syrian hamsters (CI) received a standard diet supplemented with 0.83% carbonyl iron. The enhanced damage of the liver and elevated protein carbonyl content have been noted in CI as compared to control animals.

7. Caloric restriction and iron chelators

Dietary factors associated with the risk of high iron stores have been studied in the elderly (614 subjects aged 68–93 years) [72]. The results have demonstrated that intakes of highly bioavailable forms of iron (supplemental iron and red meat) promote high iron stores, whereas foods containing phytate (whole grains) decrease these stores. On the other hand, caloric restriction (CR) or chelators have an opposite effect on iron accumulation [63, 73]. Kastman et al. documented that CR decreases brain iron accumulation and preserves motor performance in older rhesus monkeys [73]. Monkeys from both groups (CR and control) showed age-related increases in iron concentrations in both the globus pallidus (GP) and the substantia nigra (SN), although the CR group had significantly less iron deposition in the GP, SN, red nucleus and temporal cortex. Neurodegenerative (age-related) diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), are associated with brain iron accumulation [74]. Authors suggested that sustained exercise and diet restriction may be the way to slow the rate of neurodegeneration, perhaps by promoting neurogenesis or through antioxidant-related pathways. Moreover, it has been suggested that CR and exercise cause the phosphorylation and activation of AMPK, and thus elevate the NAD+/NADH ratio which activates sirtuin 1 (SIRT 1)

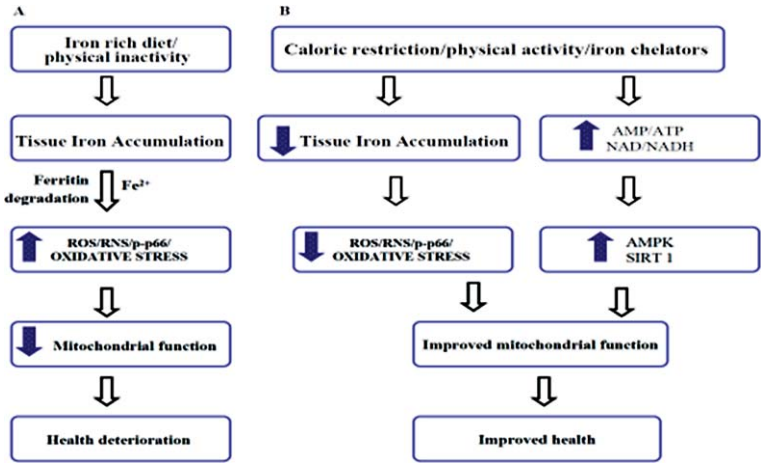

Fig. 2. Caloric restriction, exercise, and iron chelators induce iron reserve reduction, and improve health. An iron-rich diet is associated with iron tissue and cell accumulation. In the presence of fatty acids, iron can also accumulate in mitochondria, resulting in mitochondrial dysfunction, accelerated aging, and health deterioration (Fig. 2A). Caloric restriction, exercise, and iron chelators are able to reduce the accelerated iron accumulation within cell and decrease cytosolic and mitochondrial oxidative stress. Caloric restriction and exercise via the AMPK and SIRT 1 pathway also improve the mitochondrial function, age-related changes, and health (Fig. 2B).

(Fig. 2) [75, 76]. AMPK and SIRT 1 via activation of peroxisome proliferator-activated receptor α coactivator 1α (PGC-1α) increase mitochondrial biogenesis [75, 76].

Application of iron chelators is another possibility to reduce iron stores. Arvapalli et al. reported that deferasirox (iron chelator, 100 mg/kg body weight) treatment significantly reduced cardiac iron levels. It was also associated with a drop in the number of TUNEL-positive cells. Deferasirox treatment appeared to be an effective method in diminishing age-associated iron accumulation and cardiac apoptosis in the F344XBN rat model (Arvapalli et al. [63]).

Another interesting piece of data comes from Kitsati's et al. study [77]. Authors reported that several cinnamic acid derivatives (trans-cinnamic, p-coumaric, caffeic and ferulic acids, as well as caffeic acid-methyl and -propyl esters), present abundantly in plant-derived diet components, are able to protect cells from oxidative stress-induced DNA damage. According to the authors, the protection has been based on the ability of each compound to reach the intracellular space and chelate intracellular "labile" iron. The excess of iron in one's diet may contribute to iron tissue accumulation and may in consequence induce deleterious changes in tissues, cells and mitochondria, impairing one's health. On the other hand, CR, exercise, or iron chelators may reverse or slow these changes (Fig. 2A and B).

8. Conclusions

Our knowledge regarding iron metabolism has been growing exponentially for some time, however, we are far from its full understanding. In addition to being a metabolic co-factor for many proteins, iron stimulates ROS formation and is an important signaling molecule. This role of iron still lacks comprehensive data. On the one hand the level of labile iron is controlled by SAPK and transcriptional factors which influence ferritin gene expression such as c-myc, NF-κB or Nrf2 [78–81]. On the other hand the amount of iron released during ferritin degradation depends on the ferritin iron reserve. In this review, we present current evidence that regular exercise may protect the human body against excessive iron accumulation. We believe that pro-health effects of exercise are partially related to positive changes in iron metabolism. There is a growing body of evidence that reducing body tissue iron reserves via exercise, diet rich in iron chelators or phlebotomy ensures beneficial health outcomes, such as lower risk of cancer, diabetes and many others [9, 82].

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