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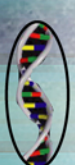
FERRITIN

Functions, Biosynthesis and Regulation

Protein Biochemistry, Synthesis, Structure and Cellular Functions



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PROTEIN BIOCHEMISTRY, SYNTHESIS, STRUCTURE AND CELLULAR FUNCTIONS

FERRITIN: FUNCTIONS, BIOSYNTHESIS AND REGULATION

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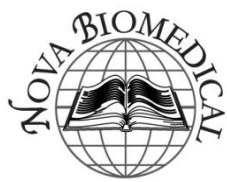

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PREFACE

Ferritins are a superfamily of iron-storage proteins that are found in all living organisms with the exception of yeast. They are ubiquitous, iron-binding proteins that play an important role in cellular iron homeostasis. This book discusses topical research in the study of the functions, biosynthesis and regulation of ferritin. Topics include ferritin as an iron source for pathogens and as part of the antioxidative machinery in plants under stress; the antioxidant role of ferritin and transferrin in elite athletes; ferritin and ferroxidase activity and increased serum ferritin in morbid obesity and bariatric surgery.

Chapter 1 - In this chapter the authors analyze the interaction between the host ferritin and pathogenic microorganisms, since this ferric protein can be used by invaders for their growth and, thus, colonization and invasion of tissues, causing disease. Iron is an essential nutrient for all living beings; however, this metal is toxic and must be captured by proteins, among them ferritin, the great intracellular storage of iron in the body. Pathogens living inside humans also need the vital iron; therefore, the iron availability in body tissues plays a crucial role in the host-pathogen relationship. In general, microorganisms living within a mammal have evolved several mechanisms to scavenge iron from the host iron-containing proteins; these mechanisms have been considered to be important virulence factors. Pathogens able to destroy cells and tissues can have easy access to ferritin and make use of its iron. Due to the high amount of iron atoms that ferritin is able to capture, this protein is really a remarkable iron source for every intracellular pathogen. In the literature, there are only a few reports about pathogens using ferritin as an iron source, but this is an exciting growing field of research. Each pathogen has developed its own manner to obtain iron from ferritin: for instance, the

bacterium *Neisseria meningitidis* triggers the host ferritin redistribution from cytosol to lysosomes within infected epithelial cells and accelerates the ferritin degradation by lysosomal proteases, thus providing the necessary iron for its own existence. On the other hand, in spite of being ferritin a stable supramolecular complex, *Burkholderia cenocepacia* secretes serine-proteases that degrade ferritin. Some strains of *Escherichia coli* and *Mycobacterium* secrete siderophores, ultra-high affinity iron-binding compounds able to confiscate iron to host ferritin. Another mechanism to get ferritin iron is the reported in *Listeria monocytogenes* and the mucosal pathogenic fungus *Candida albicans*, which use surface reductases to acquire iron from ferritin. In parasitic protozoa, there are practically no reports about the utilization of ferritin as a sole iron source. *Trichomonas vaginalis* uses ferritin iron but the mechanism by which it uptakes iron from this protein is still unknown. Recently, the authors reported that *Entamoeba histolytica* trophozoites endocytose ferritin by clathrin-coated pits and degrade this protein by means of specific cysteine proteases in the endosome/lysosome pathway. Concluding, pathogenic microorganisms capable of removing and acquiring iron from ferritin can obtain a plentiful source of this crucial metal to survive, colonize and invade the host.

Chapter 2 - Ferritin is a protein with multiple functions in the human being. Although traditionally related to iron metabolism status and iron deficiency, as the main protein to store iron, recently its role on oxidative stress has been an issue of concern. Iron deficiency (ID) is the most common nutritional deficiency disorder in the world, affecting more than two billion people, mostly infants, children and women of childbearing age. In general, iron deficiency symptoms are probably due not to lowered hemoglobin level, but to an insufficient supply of iron to the tissues, and, therefore, ferritin concentration is the most practical way of reporting iron deficiency in field and clinical studies. Increases in ferritin concentrations has been used to show improvements of fortification of food with iron with many different food vehicles, such as milk, cereals, etc. In such cases, subjects with infection or inflammation must be excluded from those studies, as ferritin acts as a positive acute-phase reactant protein, and thus its concentration is increased. Ferritin concentration measurement is also useful in studies when different iron compounds are compared in different conditions where iron deficiency or iron deficiency anemia is frequently seen, as in gastrectomized patients. Of note, ferritin concentration is usually different in patients with iron deficiency or iron deficiency anemia who will be submitted to gastrectomy or to bariatric surgery. In the former condition, patients will present low ferritin

concentration, whereas in the later ferritin can be increased, as abdominal fat in obese subjects may favor an inflammation milieu through pro inflammatory cytokines synthesis, and then ferritin concentration will act as a positive acute-phase reactant protein. On the other hand, it is well recognized that iron is the most powerful catalyst for the formation of highly toxic ROS. Iron in combination with ferritin is stable. However, in some overload iron conditions, such as chronic hepatitis C, ferritin is decomposed by lysossomes and hence an unstable iron ion is released. Therefore, reactive oxygen species are formed and they also facilitate iron release from ferritin. The aim of the present review is to show the importance that ferritin has acquired in the understanding of many processes, which will make easier the comprehension of the role of ferritin in both iron deficiency and oxidative stress mechanisms.

Chapter 3 - Ferritins are a superfamily of iron-storage proteins that are found in all living organisms with the exception of yeast. They are ubiquitous, iron-binding proteins that play an important role in cellular iron homoeostasis, storing up to 4,500 iron atoms in their central cavities. In plants, ferritins are preferentially localised in the plastids but are also found in mitochondria. Ferritin expression is developmentally regulated and plays a role in the synthesis of iron-containing proteins that are involved in photosynthesis in the early stages of plant development. It accumulates in seeds during embryo maturation to provide iron during germination, allowing for the proper formation of the photosynthetic apparatus. Ferritins are transcriptionally controlled by iron, which induces the production of reactive oxygen species (ROS) in the Fenton reaction in its free form and thus has a high potential for toxicity. Several reports have shown that the transcription of ferritin in plants may be induced by environmental factors, such as drought, salinity, cold, light intensity, pathogen attack, NO and ozone, which then stimulate ROS production. Studies that have analysed other stresses, such as those caused by herbicides and heavy metals, have also shown increased expression levels of the ferritin genes. These reports suggest that ROS induces ferritin transcription and that ferritin plays a protective role by capturing free iron and limiting oxidative damage to the cell. Recent evidence of ferritins effectively participating in the defensive machinery of plants under oxidative stress was obtained from transgenic plants that overexpressed ferritin genes. This review is focused on the protective role of ferritin in plants that are threatened by oxidative damage in response to biotic and abiotic stresses.

Chapter 4 - Vascular calcification plays a role in the pathogenesis of atherosclerosis, diabetes and chronic kidney disease. The mechanism of vascular calcification is not completely understood, but recent evidence

implicates factors involved in bone mineralization. In response to elevated inorganic phosphate transdifferentiation of vascular smooth muscle cells (VSMC) into osteoblast-like cells occurs. This process involves increased alkaline phosphatase activity, increased expression of the bone specific transcription factor, core binding factor-1, and the subsequent induction of osteocalcin. Mounting evidence suggests an essential role for ferritin to maintain homeostasis of vascular function. Recently the authors have found that both exogenous administration of ferritin and upregulation of endogenous ferritin production inhibit calcification and transdifferentiation of VSMC. Ferritin is a multifunctional protein, possessing ferroxidase activity and iron storing ability. In addition, as recently described, ferritin plays a role in transcriptional regulation of certain genes. Testing the inhibitory potential of different ferritins – L-ferritin, H-ferritin and a mutant form of H-ferritin lacking ferroxidase activity – revealed that ferroxidase activity of ferritin is essential to dampen Pi-induced calcification. Moreover, the authors have found that ceruloplasmin, a distinct protein with ferroxidase activity inhibited calcification and osteoblastic transdifferentiation of VSMC. In addition, they have shown that ferritin – via its ferroxidase activity – inhibits osteoblast activity, leading to decreased mineralization. These results suggest a novel role of ferritin/ferroxidase activity in inhibiting vascular calcification, VSMC-osteoblast transformation, osteoblast activity and mineralization.

Chapter 5 - Physical activity has been shown to increase the production of free radicals to a point that can exceed internal protective antioxidant system. The harmful effects of free radicals are neutralized by activity of antioxidant enzymes and numerous non-enzymatic antioxidants, including vitamins, glutathione, ubiquinone and flavonoids. Defence mechanisms against free radical-mediated oxidative damage also include the iron binding proteins such as transferrin and ferritin. Ferritin and trensferrin are able to restrict the availability of iron to participate in conversion of hydrogen peroxide to toxic hydroxyl radicals by Fenton reaction. Because the endogenous antioxidant system may not be sufficient to prevent exercise-induced free radical generation and consequent oxidative damage, supplementation of antioxidants may have important effect antioxidant status in athletes. The aim of the present work was to examine the association of physiological iron carriers with oxidative stress in elite athletes. One hundred and five elite athletes were divided into two groups: supplemented group which consisted of sixty-five athletes who regularly use antioxidant supplements and control group which consisted of forty athletes that were without antioxidants intake. Following parameters were measured: albumin, ferritin, transferrin, advanced oxidation

protein products (AOPP), lipid hydroperoxides (LOOH), biological antioxidative potential (BAP), superoxide dismutase (SOD) and total sulphydryl group concentration (SH groups). Multivariate analysis of covariance (MANCOVA, Wilks' lambda) was performed to test the hypotheses that supplementation (fixed factor) and antioxidative proteins (indicated via albumin, ferritin and transferrin) (covariates) have a significant effect on the oxidative stress parameters (dependent variables). The SOD activity (P=0.024) and sulphydryl group concentration (P=0.042) were significantly higher in supplemented athletes. Multivariate analysis of covariance indicated ferritin (P<0.001) and transferrin (P=0.001) as significant covariates, which have contributed 37.1 % and 22.8% to variability of oxidative stres parameters, respectively. The transferrin exhibited linear relationships with LOOH (R^2 = 0.127; P < 0.001) and AOPP (R^2 = 0.113; P < 0.001) while ferritin exhibited non-linear (logarithmic) relationships with this parameters (AOPP: R^2 = 0.201, y = 82.3 -15.9log(x), P<0.001; LOOH: R^2 = 0.256, y = 193 -33.3log(x), P<0.001). In conclusion, proteins that regulate iron transport and storage, transferrin and ferritin, were negatively related with oxidative damage in professional athletes regardless of antioxidant supplementation.

Chapter 6 - The proposal for this chapter is, first, to discuss iron metabolism disorder as a risk factor for obesity-related diseases and what increased serum ferritin means in obese individuals. Later, the effect of surgery on dietary iron absorption, the high prevalence of anemia among operated patients and the impact of the procedure on serum ferritin will be discussed. Ferritin is a nonspecific marker of a great number of disorders. One of the main causes for the increase of serum ferritin is non-alcoholic fatty liver disease (NAFLD). NAFLD is manifested mainly in obese, type-2 diabetic and dyslipidemic individuals. Increased serum ferritin concentration is associated with increased blood pressure, hyperglycemia, hyperinsulinemia and hypercholesterolemia, representing a marker of poor prognosis in acute stroke and coronary artery disease. High ferritin is associated with the metabolic syndrome and reflects the inflammation process highlighted in fat tissue metabolism, atherosclerosis and diabetes. In this context, ferritin can be involved as a pro-oxidant factor. Increased ferritin can contribute to NAFLD pathogenesis and associated diseases, but the hypothesis that its increase is only a marker of the ongoing inflammatory process cannot be discarded. Retrospective data on the prevalence of anemia and behavior of ferritin before and after bariatric surgery in operated morbidly obese patients will also be presented.

Chapter 7 - Serum ferritin level has been used as a standard measurement of body iron stores, and is the most reliable marker of iron status. The demand for iron is particularly critical in pregnant women, who need to expand their erythrocyte mass and generate the iron supply of the growing fetus. The amount of iron passing through the placenta increases as gestation progresses. This transfer of iron occurs against a concentration gradient from the placenta to the fetus, especially during the third trimester. During pregnancy, serum ferritin levels display a gradual decline, with a nadir at 35-38 weeks of gestation, followed by a slight increase in late pregnancy. In pregnant women, a favorable iron status is a prerequisite for a good perinatal outcome for both mother and infant. An elevated serum ferritin level can be a biomarker of body fat mass before and during pregnancy, especially in obese women. Some studies have shown an association between significantly increased ferritin levels during pregnancy and impaired glucose tolerance and gestational diabetes mellitus (GDM). However, these findings have been inconsistent. This article reviews the evidence for an association of elevated serum ferritin level and the risk of gestational diabetes mellitus. Its aim is to highlight the importance of maintaining good health because both GDM and iron deficiency anemia are enormous public health problems among reproductive age women.

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Chapter 1

FERRITIN AS AN IRON SOURCE FOR PATHOGENS

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ABSTRACT

In this chapter we analyze the interaction between the host ferritin and pathogenic microorganisms, since this ferric protein can be used by invaders for their growth and, thus, colonization and invasion of tissues, causing disease. Iron is an essential nutrient for all living beings; however, this metal is toxic and must be captured by proteins, among them ferritin, the great intracellular storage of iron in the body. Pathogens living inside humans also need the vital iron; therefore, the iron availability in body tissues plays a crucial role in the host-pathogen relationship. In general, microorganisms living within a mammal have

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evolved several mechanisms to scavenge iron from the host iron-containing proteins; these mechanisms have been considered to be important virulence factors. Pathogens able to destroy cells and tissues can have easy access to ferritin and make use of its iron. Due to the high amount of iron atoms that ferritin is able to capture, this protein is really a remarkable iron source for every intracellular pathogen. In the literature, there are only a few reports about pathogens using ferritin as an iron source, but this is an exciting growing field of research. Each pathogen has developed its own manner to obtain iron from ferritin: for instance, the bacterium *Neisseria meningitidis* triggers the host ferritin redistribution from cytosol to lysosomes within infected epithelial cells and accelerates the ferritin degradation by lysosomal proteases, thus providing the necessary iron for its own existence. On the other hand, in spite of being ferritin a stable supramolecular complex, *Burkholderia cenocepacia* secretes serine-proteases that degrade ferritin. Some strains of *Escherichia coli* and *Mycobacterium* secrete siderophores, ultra-high affinity iron-binding compounds able to confiscate iron to host ferritin. Another mechanism to get ferritin iron is the reported in *Listeria monocytogenes* and the mucosal pathogenic fungus *Candida albicans*, which use surface reductases to acquire iron from ferritin. In parasitic protozoa, there are practically no reports about the utilization of ferritin as a sole iron source. *Trichomonas vaginalis* uses ferritin iron but the mechanism by which it uptakes iron from this protein is still unknown. Recently, we reported that *Entamoeba histolytica* trophozoites endocytose ferritin by clathrin-coated pits and degrade this protein by means of specific cysteine proteases in the endosome/lysosome pathway. Concluding, pathogenic microorganisms capable of removing and acquiring iron from ferritin can obtain a plentiful source of this crucial metal to survive, colonize and invade the host.

Keywords: Bacteria, Ferritin, Fungi, Iron source, Parasites, Protozoa

1. Iron Is Vital for Mammals but also for Microorganisms Living in Mammalian Tissues

Iron (Fe) is a vital element for the cellular metabolism of virtually all living organisms, except some Lactobacillus species, which have copper or manganese in their enzymes [Archibald, 1983]. However, iron in the two oxidation forms (Fe2+, Fe+3) is toxic, thus it must not be free in cells and tissues. Free Fe2+ concentration, in the presence of oxygen, produces highly

reactive oxygen species (ROS) by the Fenton reaction, damaging all types of biological macromolecules [Chiancone et al., 2004]. Therefore, in all organisms, iron is part of or is bound to proteins. Specifically, mammals possess diverse mechanisms to maintain, perfectly regulated, the iron concentration in the body [Finch, 1994; Weinberg, 1999a; Sharp and Srai, 2007]. This delicate equilibrium is mainly accomplished by proteins that absorb, transport, bind, recycle, store, and use iron.

In humans, extracellular iron is bound to the iron-transporter transferrin, a protein found in serum that provides iron to all cells, mainly to erythroid precursors. Extracellular iron also binds to lactoferrin, protein that sequesters iron in mucosae and infection sites. Intracellular iron can be found in the heme group of electron-transporter proteins, such as cytochromes, the blood oxygen-transporter hemoglobin, and the muscle oxygen-store myoglobin. This element is also a cofactor in redox processes catalyzed by enzymes and in the Fe-S center of several proteins [Weinberg, 1978; Griffiths *et al.*, 1999; Conrad and Umbreit, 2000]. Excess iron is stored in ferritin, keeping this element available intracellularly when the cell needs it. When the intracellular iron concentration increases, ferritin sequesters iron and detoxifies the cell, avoiding its free form in cytosol [Andrews *et al.*, 1993; Arosio and Levi, 2002]. Ferritin controls the reversibility of the transition phase between Fe^{+2} (soluble) and Fe^{+3} (insoluble) mineralized inside the molecule [Theil, 1990; Koorts and Viljoen, 2007]. Ferritin is a versatile protein, mainly cytosolic, but localized also in other structures such as mitochondrion, nucleus, and in mammalian serum (in this case poor in iron). Ferritin is a heteropolymer composed of 24 subunits of two types, H and L, and the proportion of each subunit depends on the main function of the protein. For example, in the liver and spleen, where 50% of iron corporal reserves are kept (0.4g), ferritin is the most efficient protein for iron incorporation, being able to capture up to 4,500 iron atoms, and it has a proportion of 80% L and 20% H. However, in the brain, heart and lungs, ferritin is devoted to a detoxification function, capturing free iron and avoiding the ROS generation; the optimal proportion for this function is 80% H and 20% L. It is important to mention that ferritin is found in all tissues due to the iron requirements for metabolic processes and the necessity of avoiding the ROS production, toxic for the cell [Theil, 1990; Harrison and Arosio, 1996; Arosio *et al.*, 2009].

In addition to its role as an intracellular protein, ferritin can be an iron-carrier when it resides in extracellular sites. For example, serum ferritin and ferritin secreted by macrophages have a role in iron delivery to several kinds

of cells, and due to its high iron content, the iron supply can be more efficient than that of transferrin. Among the cells benefited by the ferritin-iron delivery pathway are erythroid precursor cells, hepatocytes, brain oligodendrocytes, lymphocytes, and enterocytes, all of which have a receptor to bind ferritin [Wang *et al.,* 2010]. Interestingly, in mouse oligodendrocytes, which do not have a detectable level of transferrin receptor, and then all iron is provided by ferritin, the ferritin-H endocytosis receptor has been identified as TIM-2: this is a transmembrane receptor, expressed in the liver, kidney, and in T and B cells, which regulates T helper type-2 responses and autoimmunity [Chakravarti *et al.*, 2005; Chen *et al.*, 2005].

Iron is also highly important for pathogens, and this element must be perfectly regulated in order to avoid toxicity to these microbes. They possess similar iron-dependent enzymes and proteins for metabolism to those found in humans. In most microbial species, complex systems for acquiring iron from the environment that function in response to iron-starvation have been described. In *Escherichia coli,* the bacterial species most studied in bacterial genetics, regulation of genes that participate in iron metabolism is mainly carried out at the transcriptional level by the repressor protein Fur and at the post-transcriptional level by the non-coding RNA RyhB, whose transcription is negatively regulated by Fe-Fur [Lee and Helmann, 2007].

Interestingly, parasitic protozoa must attain a higher quantity of iron than bacteria in order to grow, colonize, invade, and survive within a host. Some protozoa need even more iron, such as the amitochondrial protists (*Tritrichomonas, Trichomonas, Giardia, Entamoeba*), which have unusually high requirements for iron (50–200 μM), surpassing those of the majority of both eukaryotic and prokaryotic cells (0.4-4 μM) [Weinberg, 1974]. This is due to their energy metabolism, which relies heavily on Fe-S proteins [Tachezy, 1999; Vanacova *et al.*, 2001]. Iron storage is extremely important to all forms of life, thus ferritin is found in the three domains of living beings, *Archaea, Bacteria,* and *Eukarya*. However, some eukaryotic cells like yeasts and protozoa apparently do not have a ferritin molecule, but have evolved other ways to store and maintain their own iron homeostasis [Suchan *et al.*, 2003; Koorts and Viljoen, 2007; Arosio *et al.*, 2009]. Microorganisms therefore appear to be well-equipped for living in an iron-poor environment, and due to their imperious need for iron they have evolved different strategies for the capture of this element and supply themselves the iron requirement.

Table 1. Microorganisms capable of using ferritin as a sole iron source and their ferritin-iron acquisition system

Microorganism	Iron acquisition system from ferritin	Tissue or organ where microrganisms obtain ferritin	Reference
Bacteria			
Bacillus cereus	Iron acquisition system still unknown, it uses ferritin after direct binding to the surface receptor IlsA	Intestinal cells, oral epithelium, retinal tissue, CNS, blood, liver, striated muscle, skin, heart tricuspid-valve	[Daou *et al.*, 2009]
Burkholderia cenocepacia	Proteolytic mechanism: Secreted serine proteases that degrade ferritin	Lungs, macrophages, epithelial cells	[Whitby *et al.*, 2006]
Escherichia coli, and Yersinia pestis	Siderophores: Aerobactin is a siderophore used to obtain iron from ferritin	*E. coli* : intestinal cells, urinary tract, kidneys, CNS, blood *Yersinia pestis*: blood, lungs, lymph nodes, macrophages	[Brock *et al.*, 1991] [Perry and Brubaker, 1979; Sikkema and Brubaker, 1989]
Listeria monocytogenes	Surface-associated ferric reductase system: still unidentified	Intestinal cells, macrophages, hepatocytes, epithelial cells, fibroblasts, endothelial cells, neurons	[Deneer *et al.*, 1995; Barchini and Cowart, 1996; Jin *et al.*, 2006]
Mycobacterium spp	Siderophores: Carboximycobactin is a siderophore capable to remove iron from ferritin	Lungs, macrophages	[Gobin and Horwitz, 1996]

Table 1. (Continued)

Microorganism	Iron acquisition system from ferritin	Tissue or organ where microrganisms obtain ferritin	Reference
Neisseria meningitidis	Host ferritin redistribution by bacteria and Proteolytic process: Ferritin is hydrolyzed specifically in host lysosomes	Respiratory tract, blood, CNS	[Larson *et al.*, 2004]
Streptococcus pyogenes	Iron acquisition system still unknown, only it has been described the use of ferritin as an iron source	Lungs, throat epithelium, skin	[Eichenbaum *et al.*, 1996]
Parasite			
Entamoeba histolytica	Endocytosis through clathrin-coated vesicles and Proteolytic process: Cysteine-proteases through endosomal/lysosomal pathway	Blood, brain, intestinal cell, hepatocyte, lungs	[Lopez-Soto *et al.*, 2009]
Trichomonas vaginalis	Iron acquisition system still unknown, only it has been described the use of ferritin as an iron source	Vaginal mucosa	[Lehker and Alderete, 1992]
Fungi			
Candida albicans	Surface reductase: Als3 is the ferritin receptor, the identity of the associated reductase is unknown	Vaginal, oral-pharyngeal, esophageal, and gastrointestinal mucosae; blood, CNS, internal organs like lungs and heart	[Almeida *et al.*, 2008]

2. FERRITIN CAN BE AN ABUNDANT IRON SOURCE FOR PATHOGENIC MICROORGANISMS

Mammals have multiple sites rich in iron that can potentially support the iron requirement for the growth of pathogens. However, as a general strategy against invading microbes, mammals possess intricate iron-withholding systems for efficiently reducing the iron available to invaders. In this way, under normal conditions, the free-iron concentration in body fluids is negligible (~10^{-18} M) [Weinberg, 1978; Bullen, 1981]. As a consequence, pathogenic microbes surviving inside a host must successfully compete with the host's iron-containing proteins to supply their own iron needs in order to colonize, invade, and survive. Evidently, the host's iron availability plays a crucial role in the host-pathogen relationship.

In spite of this host strategy of keeping iron away from microbes, it has been well demonstrated that pathogens have evolved different mechanisms to achieve the host iron. Indeed, they can uptake extracellular and intracellular iron in the ferrous and ferric forms and, importantly, inorganic iron, as well as that bound to proteins. Microbes invading blood can lyse erythrocytes and have access to the four ferrous-iron ions supplied by hemoglobin; in this fluid, pathogens also can uptake the two ferric ions of transferrin. In mucosal tracts and infection sites, pathogens can bind lactoferrin and get its two ferric ions [Weinberg, 1999b]. In an enormous contrast, ferritin could provide more than 1000-fold the iron to pathogens than hemoglobin, transferrin, and lactoferrin, and definitively be a striking iron source for them. Since ferritin is mainly an intracellular protein, microorganisms may destroy the host cells, or invade them, or be phagocytosed by cells to have access to the ferritin iron. Serum ferritin is poor in iron. However, this protein can be iron-loaded in diseases involving iron overload and in infections, as a host response known as hypoferremia of infection, and then serum ferritin becomes a generous iron source for pathogens [Worwood *et al.*, 1976; Arosio *et al.*, 1977; Weinberg, 1978; Wang *et al.*, 2010]. In order to acquire the necessary iron, some bacteria and fungi produce siderophores, ultra-high affinity molecules devoted to scavenging iron from the environment and able to remove iron from host proteins such as transferrin, lactoferrin, and ferritin. In addition, bacteria express receptors that directly bind these three host iron-containing proteins for acquiring iron. Other mechanisms are the production of proteases that cleave these proteins, leading to release of iron, and of reductases, which reduce the ferric iron to the more soluble and assimilable ferrous form.

Next, we will describe some strategies employed by microbes to acquire iron from ferritin, which are summarized in Table 1.

2.1. Bacterial and Fungal Mechanisms for Iron Acquisition from Ferritin

2.1.1. Neisseria meningitidis Triggers Host Cell Ferritin Redistribution and Accelerates its Degradation by Lysosomal Proteases

Neisseria meningitidis is a human-specific Gram-negative diplococcal bacterium found frequently in the respiratory tract of healthy individuals but can cause bacterial meningitis. Meningococcal disease is a severe life-threatening infection often related with serious complications; it continues to be a major cause of childhood morbidity and mortality worldwide, the majority of cases occurring in developing countries. Infants and children who survive bacterial meningitis often suffer neurological and other disabling sequelae [Cartwright *et al.*, 1987; Orr *et al.*, 2003; Choudhuri *et al.*, 2011; Trivedi *et al.*, 2011]. In order to generate disease, the meningococci (MC) must disseminate to the bloodstream causing septicemia and then cross the brain-blood barrier to the cerebrospinal fluid and trigger meningitis. Meningococci express a wide range of virulence factors including capsular polysaccharide, lipopolysaccharide, and a number of surface-expressed adhesive proteins necessary to evade killing by host defense mechanisms [Nassif and So, 1995].

One key determinant in the MC pathogenesis is their ability to acquire iron from the human host proteins: MC can grow extracellularly and have access to iron of lactoferrin in mucosae and of transferrin in serum, in addition to the uptake of heme of the hemoglobin molecule by the destruction of erythrocytes in blood. MC can also invade and replicate within epithelial cells, and this fact may be a critical factor in both the establishment of a carrier state and the development of meningitis [Schryvers and Stojiljkovic, 1999; Stephens *et al.*, 2007; Jordan and Saunders, 2009; Virji, 2009]. Studies about intracellular replication of MC have been carried out in the human epithelial endocervical cell line A431. Interestingly, desferal inhibited the intracellular replication of MC; since this iron-chelator does not chelate heme iron, MC then could not use the iron incorporated with heme for intracellular growth. In addition, by the study of neisserial mutants defective either in porphyrin synthesis or in heme oxigenase, it was determined that heme is not required for the intracellular growth of MC. Also, a mutant affected in the use of

transferrin replicated normally inside the cell line A431. Therefore, intracellular MC use neither the iron from heme, nor from transferrin, for growth, and thus ferritin was the best candidate for the iron supply [Turner *et al.*, 1998; Larson *et al.*, 2002].

In 2004, Larson et al. demonstrated for the first time and through elegantly-designed experiments, that a pathogen is able to use the host intracellular ferritin as an iron source, in assays using the cell line A431 [Larson *et al.*, 2004]. First, this group of research demonstrated that the possibility of *N. meningitidis* used transferrin-derived iron indirectly after its removal from transferrin was null, and additionally, that iron-loaded transferrin inhibited the intracellular replication of MC in a dose-dependent manner. Moreover, the replication rate of MC was higher in iron-starved than in iron-replete cells, showing that intracellular MC replication is stimulated by the need for iron. Next, by using an ELISA, they monitored the levels of ferritin inside the cells, and found that ferritin levels declined rapidly in those cells infected with MC, and this fact was not due to a repression of ferritin transcription or transduction. Furthermore, by experiments of pulse-chase using [^{35}S]-cysteine and methionine, and immunoprecipitation with an anti-ferritin Ab, they determined that the declined ferritin levels were a result of a ferritin degradation triggered by MC infection. After infection of MC to epithelial cells, these bacteria induced aggregation of cytosolic ferritin and this led to the degradation of the ferric protein in a degradative compartment, perhaps lysosomes; this was observed by using indirect immunofluorescent microscopy. Interestingly, when holotransferrin (iron income prevents ferritin turnover), ascorbic acid (avoids the ferritin autophagy and degradation) or leupeptin (lysosomal proteases inhibitor) were added, the redistribution and degradation of ferritin, as well as the intracellular replication of MC, were diminished. With this set of experiments, the determination that ferritin degradation could be providing the iron source critical for growth and colonization of *Neisseria meningitidis* was overwhelmingly demonstrated. In many cell lines, degradation of cytosolic ferritin during iron starvation occurs to release iron in order to meet its own cellular metabolic needs [Ollinger and Roberg, 1997; Radisky and Kaplan, 1998; Tabuchi *et al.*, 2000]. MC disrupt transferrin uptake by epithelial cells, reducing transferrin receptor mRNA and slowing the receptor cycling [Bonnah *et al.*, 2000]. The authors propose that in this way MC induce an iron starvation response in host cells, resulting in the lysosomal degradation of cytosolic ferritin, a mechanism that has not been described in other pathogens [Larson *et al.*, 2004].

2.1.2. Burkholderia cenocepacia Secretes Serine-proteases that DegradeFerritin

Burkholderia cenocepacia is a motile, rod-shaped, metabolically diverse Gram-negative β-proteobacterium. This species is one of at least 17 phenotypically similar species known as the *Burkholderia cepacia* complex (Bcc), a group of genetically-related environmental microorganisms that cause chronic opportunistic infections in patients with cystic fibrosis (CF) and other underlying diseases [Vandamme *et al.*, 2003; Vanlaere *et al.*, 2008; Vanlaere *et al.*, 2009]. *B. cenocepacia* is the most common species isolated from CF patients and associated with the epidemic spread among these people [LiPuma *et al.*, 2001; LiPuma *et al.*, 2002]. *B. cepacia* complex organisms possess several factors that play a key role in pathogenesis, such as the ability to survive intracellularly within macrophages and respiratory epithelial cells, cable pili, flagella, a type-III secretion system, surface exopolysaccharide, production of melanin, catalase, up to four types of iron-chelating siderophores, proteases and other secreted enzymes, quorum-sensing systems, and the ability to form biofilms. Not all strains produce each of the proposed virulence factors and, to date, none of these individual factors have been clearly demonstrated to be a major contributor to human disease. Iron acquisition processes could be important in the lung colonization process during cystic fibrosis [Hunt *et al.*, 2004; Visser *et al.*, 2004; Loutet and Valvano, 2010].

The iron metabolism of the lungs differs from that of the rest of the body [Gutteridge *et al.*, 2001]. Each day, the human respiratory tract is exposed to a great quantity of airborne iron [Turi *et al.*, 2004], and, thus, it can suffer iron-mediated oxidative damage. The risk of damage to the lungs is additionally enhanced by the high partial pressure of oxygen and the presence of inhaled microorganisms. Thus, free iron is rapidly sequestered mainly by transferrin, lactoferrin and ferritin. Consequently, in the CF lung, ferritin is significantly up-regulated in comparison with normal healthy lungs [Stites *et al.*, 1998; Stites *et al.*, 1999; Turi *et al.*, 2004].

Whitby et al. (2006) demonstrated that the strain *B. cenocepacia* J2315T directly utilized iron from ferritin for its growth *in vitro*; this assessment was done in base to growth kinetics in media where ferritin was the only iron source [Whitby *et al.*, 2006]. Further studies examining the mechanisms of iron uptake from ferritin indicated that iron utilization resulted from a proteolytic degradation of this otherwise stable macromolecular structure. By using different types of protease-activity inhibitors, it was found that ferritin proteolysis was due to secreted serine proteases. Since ferritin concentration is

significantly higher in the CF than in healthy lungs, undoubtedly the ability of *B. cenocepacia* to use host ferritin may contribute to its colonization and persistence in the cystic fibrosis patient [Whitby *et al.*, 2006].

B. cenocepacia possesses another important iron acquisition system found under condition of iron depletion. This bacterium produces two main siderophores, ornibactin and pyochelin, acting to scavenge free or protein-bound iron from the surrounding environment. Ornibactin has been reported as the biologically more important siderophore, which is able to compensate for the function of pyochelin. Although it has not been reported that these siderophores are used by *B. cenocepacia* to get iron from ferritin, this mechanism could be possible, as occurs in *Mycobacterium* and other bacterial species. In addition to siderophore-mediated mechanisms of iron uptake, *B. cenocepacia* possesses mechanisms for acquiring iron from heme [Visser *et al.*, 2004].

2.1.3. Listeria monocytogenes and the Fungus Candida albicans Use Surface Reductases to Acquire Iron from Ferritin

Ferric-reductases, as a mechanism to obtain iron from ferritin, is interesting, since reducing Fe^{3+} to Fe^{2+} not only releases iron from the molecule, but also facilitates the assimilation of iron in the ferrous form by the pathogen.

Listeria monocytogenes

As a facultative intracellular pathogen and saprophyte, the Gram-positive bacterium *L. monocytogenes* can live in soil and decaying vegetation, but once it enters an animal or human host, it can cause severe disease. This species is not a usual constituent of the human flora, but its ability to grow at 4 °C allows it to contaminate foodstuffs. The great majority (99%) of the infections caused by *L. monocytogenes* are thought to be food borne [Mead *et al.*, 1999; Swaminathan and Gerner-Smidt, 2007]. Infection causes gastroenteritis in healthy human individuals [Orsi *et al.*, 2011]. However, groups at high risk for contracting invasive listeriosis are immune-compromised individuals such as HIV patients, the elderly, infants, and pregnant women [Schlech, 2000]. The most severe clinical manifestations of invasive human listeriosis include septicemia, encephalitis, meningitis, and spontaneous late-term abortion [Orsi *et al.*, 2011]. This pathogen is capable of invading cells within the host, multiplying within the cytoplasm, and spreading from cell-to-cell using a mechanism that exploits the host cell actin [Ramaswamy *et al.*, 2007].

Iron is required by *Listeria* in relatively large amounts to support growth *in vitro* and during experimental infections [Sword, 1966]. Consistently, a *L. monocytogenes* strain defective in hemin/hemoglobin uptake showed attenuated virulence in mice [Jin *et al.*, 2006]. The ability of this bacterium to acquire and utilize iron could be considered as an important virulence factor, since iron is essential to support its growth and survival in various environmental niches, especially during infection; this is why iron acquisition mechanisms in *L. monocytogenes* are thus diverse, complex and flexible. *L. monocytogenes* does not secrete siderophores; however, it mediates iron acquisition by at least five different systems: (i) ferric citrate uptake by a citrate inducible receptor [Adams *et al.*, 1990]; (ii) utilization of exogenous siderophores (xenosiderophores) or siderophore-like molecules [Simon *et al.*, 1995; Jin *et al.*, 2006] and iron-catecholamine complexes [Coulanges *et al.*, 1997; Coulanges *et al.*, 1998]; (iii) acquisition of iron from hemin and hemoglobin through the ATP-binding membrane permease HupC [Newton *et al.*, 2005; Jin *et al.*, 2006]; (iv) acquisition of iron by a cell surface of 126-kDa transferrin-binding protein [Hartford *et al.*, 1993]; and finally, **(v)** reduction of ferric to ferrous iron from several sources, one of them ferritin, by a bacterial surface-bound reductase or by an extracellular reductase [Cowart and Foster, 1985; Deneer *et al.*, 1995; Barchini and Cowart, 1996].

L. monocytogenes can utilize ferritin as an iron source to support its growth in iron deficient media [Jin *et al.*, 2006]. Reduction of Fe^{3+} from ferritin could be after direct contact of the bacterial surface with ferritin [Deneer *et al.*, 1995], or through an extracellular iron reducing activity [Barchini and Cowart, 1996]. This reduction does not involve ferritin degradation to release iron [Deneer *et al.*, 1995]. Slow reduction of iron was also observed from lactoferrin and transferrin, whereas the reduction to Fe^{2+} from ferritin proceeded relatively rapid [Deneer *et al.*, 1995]. However, the responsible reductase and the ferritin receptor remain to be identified.

The ferric reductase activity of *L. monocytogenes* using ferric ammonium citrate or Fe-NTA as an iron source was characterized, showing that the surface bound iron reductase and the secreted one could be the same enzyme, requiring FMN and NADH cofactors [Deneer *et al.*, 1995; Barchini and Cowart, 1996]. After iron reduction, ferrous iron was not released into the medium, suggesting that it is immediately internalized by bacteria. The reducing activity was not affected by the stage of growth, iron depletion or iron repletion [Deneer *et al.*, 1995], but it was increased when cells were grown in aerobic conditions or low temperature (4 ºC). This could be analogous to what happens with other virulence determinants of *L.*

monocytogenes [Czuprynski *et al.*, 1989; Stephens *et al.*, 1991]. Therefore, prolonged storage of *Listeria* at refrigeration temperatures would lead to increased ferric reductase activity and thereby contribute to increased virulence when consumed by a host. However, it is not possible to know if such characteristics are also applicable to the ferritin reducing activity, since it is not known if this is the same activity responsible for reducing iron from ferric ammonium citrate/Fe-NTA.

The listerial determinants favor the escape from the phagosome, which is considered to be iron-limiting, and permit proliferation in the host-cell cytosol, where iron-saturated ferritin is located [Gold *et al.*, 2001; Schaible and Kaufmann, 2004]. It has been suggested that *L. monocytogenes* can assess the intracellular iron concentration through the mechanism of iron-sensing: in the case of iron-limitation, this resulted in the increased expression of PrfA-regulated virulence factors listeriolysin (LLO) to lyse the membrane and ActA for phagosomal escape, movement in host cytosol and cell-to-cell spread, and in the case of iron-repletion, this resulted in the up-regulation of internalin proteins (InlA and InlB) required for invasion [Bockmann *et al.*, 1996; Conte *et al.*, 1996; Conte *et al.*, 2000; Gray *et al.*, 2006; Lungu *et al.*, 2009]. All the mentioned data allow us to conclude that *L. monocytogenes* is a well-adapted species capable of acquiring iron in both life styles, as saprophyte and as pathogen inside the host.

Candida albicans

This polymorphic yeast is the most important fungal pathogen of humans; however, it is normally a benign colonizer of human mucosal surfaces [Wilson *et al.*, 2009]. *C. albicans* is an opportunistic pathogen for immune-compromised people. It is responsible for painful mucosal infections, such as vaginitis in women and oral-pharyngeal thrush in AIDS patients [Kim and Sudbery, 2011]. The severity of candidiasis increases dramatically in people with predisposing factors, such as harshly impaired immunity, cancer, disruption of natural barriers, presence of indwelling catheters, dialysis, and solid organ transplantation [Ruhnke and Maschmeyer, 2002; Perlroth *et al.*, 2007]. In such vulnerable patients it causes severe, life-threatening bloodstream infections and subsequent infections in internal organs [Kim and Sudbery, 2011]. The extraordinary ability of *C. albicans* to successfully infect virtually every anatomical site reflects a remarkable potential to adapt to various microniches within the human host [Wilson *et al.*, 2009]. One of the key features of *C. albicans* is its ability to grow in different morphological forms, either as ovoid yeasts, filamentous hyphae, or as pseudohyphae

[Whiteway and Bachewich, 2007]. During the pathogenesis of oral infections, three different sub-stages have been identified: an early/colonization phase, characterized by adhesion of yeasts to upper layers of the oral tissue and fungal proliferation; an invasion phase, associated with hyphae formation and penetration of these layers; and a late phase, associated with extensive tissue destruction [Wilson *et al.*, 2009].

Several studies in mice and a number of clinical observations have shown the importance of iron to *C. albicans* virulence [Abe *et al.*, 1985; Iglesias-Osma *et al.*, 1995]. Iron overload may inhibit T-helper (Th) cell development in mice with candidiasis, thus negatively affecting the course and outcome of the infection [Mencacci *et al.*, 1997]. An undeniable role of iron in the virulence of this microorganism was demonstrated when the *FTR1* gene encoding for a surface iron permease, essential for iron uptake, showed to be crucial for *C. albicans* virulence in an experimental animal model of infection [Ramanan and Wang, 2000]. *In vitro C. albicans* was found to require approximately 0.2-0.5 μM iron for its complete unrestricted growth in a chemically defined medium [Sweet and Douglas, 1991; Holbein and Mira de Orduna, 2010]. Furthermore, transition to the hyphal growth, one of the important virulence factors, requires a greater amount of iron than that needed for yeast growth [Sweet and Douglas, 1991]. Therefore, one key factor for adaptation to the host environment is the ability of *C. albicans* to acquire iron within the host's iron-restricted sites. In consequence, *C. albicans* possesses more than one type of iron acquisition system: for hemoglobin [Santos *et al.*, 2003], for heterologous siderophores [Heymann *et al.*, 2002; Hu *et al.*, 2002] and the reductive uptake system for acquisition of transferrin, ferritin, and free iron [Hammacott *et al.*, 2000; Knight *et al.*, 2002; Knight *et al.*, 2005]. All three iron acquisition systems appear to be independent from each other [Almeida *et al.*, 2008], perhaps because each system is specifically adapted for an environmental niche or because redundancy is desired for this crucial function.

Ferritin promoted the *in vitro* growth of *C. albicans*. Utilization of iron from ferritin was independent of the siderophore and hemoglobin uptake systems and from secreted aspartic proteases. Ferritin use was also proposed occurring *in vivo* using oral epithelial cells enriched in intracellular ferritin, which are more susceptible to tissue damage by wild-type *C. albicans* than cells depleted of ferritin. The reduced damage of iron-depleted epithelial cells correlated with reduced invasion of *C. albicans* hyphal in these cells [Almeida *et al.*, 2008].

Ferritin iron uptake requires the reductive pathway since mutants lacking the high-affinity permease Ftr1 or the copper transporter Ccc2, both essentials for this pathway, were not able to grow in ferritin. This system is located in the plasma membrane and has three components. The first component is a ferric-reductase, which is able to reduce insoluble extracellular ferric (Fe^{3+}) ions into soluble Fe^{2+} ions; in the case of ferritin, this reduction step would be required to remove iron from the protein. The second and third components form a protein complex consisting of a ferroxidase and an iron permease, which together transport Fe^{3+} into the cell. Although the identity of the ferric-reductase is still unknown, 17 homologous genes encoding putative surface ferric-reductases have been identified in the *C. albicans* genome [Almeida *et al.*, 2009]. Ferric reductases already characterized in C. albicans, like Fre10, have been shown to act over structurally different substrates, increasing the possibility that an intermediate electron carrier performs reduction of ferric iron from ferritin [Knight and Dancis, 2006; Jeeves *et al.*, 2011]. Since Fre10 has FAD- and NADPH-binding motifs, this could mean that it uses an intermediate molecule, like O_2^- or Flavin, to mediate the electron transfer in analogy to what happens in other ferric reductases [Knight *et al.*, 2002; Schroder *et al.*, 2003; Knight and Dancis, 2006; Jeeves *et al.*, 2011]. Fre10 is increased in response to iron-restricted conditions, and regulated by the transcriptional repressor complex Tup1-Sfu1p (amongst other proteins), in iron replete conditions [Knight *et al.*, 2002; Lan *et al.*, 2004; Pelletier *et al.*, 2007].

The second component is a multi-copper oxidase. Reduced ferrous iron generated by surface reductase activity is toxic, and because of that Fe^{2+} needs to be re-oxidized to Fe^{3+} by multi-copper oxidase activity, thus preventing any damage [De Luca and Wood, 2000; Kosman, 2003]. This re-oxidation to Fe^{3+} may also serve to provide increased substrate specificity to iron import since iron permeases have shown to be highly specific for free Fe^{3+} [Schroder *et al.*, 2003]. The *C. albicans* genome contains five putative multi-copper oxidase genes, two of which, *FET3* and *FET99,* have been characterized [Eck *et al.*, 1999; Knight *et al.*, 2002]. Because of the copper requirement of the oxidase activity, the intracellular copper transporter Ccc2 is essential for this reductive pathway and for ferritin iron uptake [Weissman *et al.*, 2002].

The third component is the ferric permease. *C. albicans* has two iron permeases that are encoded by two highly homologous genes. The high-affinity iron permease gene, *FTR1,* is induced and essential for growth upon iron deprivation and for iron acquisition from ferritin and transferrin

[Ramanan and Wang, 2000; Knight *et al.*, 2005; Almeida *et al.*, 2008]. Fungal cells lacking *FTR1* lost their ability to damage oral epithelial cells and were completely avirulent in a mouse model of systemic infection [Ramanan and Wang, 2000; Almeida *et al.*, 2008]. These data demonstrate that the Ftr1 protein is an essential component of the reductive pathway both *in vitro* and *in vivo* and is thus involved in iron uptake in low-iron environments (such as within the host) and in iron acquisition from at least two different host proteins, including ferritin, making this permease crucial for *C. albicans* virulence [Almeida *et al.*, 2009]. Additionally, the fungus was only able to use ferritin as an iron source under conditions which allowed acid production (glucose, but not casaminoacids as a carbon source) and acidification of the surrounding environment (low concentrations of buffer at pH 7.4). The reduction of ferric iron from the ferritin core may be facilitated under acidic pH, since it is known that ferritin is unstable at acidic pH and natural recycling of iron from ferritin occurs in the acidic environment of lysosomes [Radisky and Kaplan, 1998; Dominguez-Vera, 2004; Kidane *et al.*, 2006]. A second potential link exists among the external pH, hyphal formation, and iron acquisition. It is well known that the external pH influences hyphal formation [Soll and Mitchell, 1983; Davis *et al.*, 2000]. Also, the balance between the soluble Fe^{2+} ion and the insoluble ferric form Fe^{3+} shifts towards the insoluble form in alkaline pH, therefore acidification facilitates iron acquisition. A close association between *C. albicans* cells and ferritin is required for the release of iron from ferritin and subsequent uptake into the fungal hyphae, but not yeast-phase cells. Hyphae can bind both purified ferritin, and ferritin contained within epithelial cells, which was demonstrated by electron microscopy [Almeida *et al.*, 2008].

Potential ferritin receptors were searched for among those genes known to encode hyphal-specific proteins that are cell surface localized. Only in the *Δals3* gene mutant was the ferritin binding dramatically reduced. Also the presence of both *ALS3* transcriptional factors, Tec1 and Bcr1, was necessary for *C. albicans* cells to bind ferritin. To confirm that *ALS3* gene is coded as the ferritin receptor, strains of *S. cerevisae*, which does not normally bind ferritin, engineered to express *C. albicans* Als3 (but not the closely related proteins Als1 or Als5), were able to bind ferritin. Most interestingly, hyphae of a *Δals3* mutant grew poorly on media containing ferritin as the sole source of iron, while uptake of free iron was normal. These observations together demonstrate that Als3 functions as a ferritin receptor and gives to *C. albicans* the capacity to obtain iron from the host ferritin [Almeida *et al.*, 2008].

ALS3 (Agglutinin-Like Sequence) gene encodes a hyphal-specific cell wall protein, which belongs to a family of adhesins (Als family). It is a multifunctional protein playing a key role in multiple processes that are necessary for the organism to colonize the host and cause disease [Liu and Filler, 2011]. In addition to its function as ferritin receptor, it plays an important role in biofilm formation on prosthetic surfaces. Als3 is one of two known *C. albicans* invasins. Consistent with Als3 being required for the uptake of ferritin iron, and all its other functions, it was found that the *Δals3* mutants lost their capacity to damage epithelial cells compared to wild type.

C. albicans hyphae, grown under iron-limiting conditions, or in the presence of excess iron, bound ferritin similarly. Also, they were able to bind ferric ferritin and apoferritin with similar efficiency, indicating that iron within the ferritin shell was dispensable for the binding of ferritin. Thus, these data indicate that the binding of ferritin by *C. albicans* is morphology associated, but not iron-regulated [Almeida *et al.*, 2008]. These agree with the fact that iron starvation did not increase the expression of Als3 [Liu and Filler, 2011].

Taken together, these data suggest that host ferritin can be used as an iron source by *C. albicans.* The mechanism proposed could be the direct binding by Als3 on the hyphae surface; iron release is then mediated by acidification and its uptake facilitated by the reductive pathway. Therefore, by using as a ferritin receptor, a protein expressed exclusively in hyphae, *C. albicans* employs an additional morphology specific and unique iron uptake strategy based on ferritin, while invading the host cells where ferritin is located [Almeida *et al.*, 2008].

2.1.4. Mycobacterium tuberculosis Siderophore Carboximycobactin Is Able to Remove Iron from Host Ferritin

Tuberculosis is a common and often lethal infectious disease caused by various strains of mycobacteria, usually *Mycobacterium tuberculosis*. Tuberculosis generally infects the lungs but can also affect other parts of the body. Bacteria spread among individuals primarily by aerosolized respiratory secretions. In the host, this microorganism is able to multiply both intracellularly in mononuclear phagocytes, especially in lung macrophages, and extracellularly in lung cavities [Gobin and Horwitz, 1996]. The initial foci of mixed inflammation that develop in the lung following aerosol exposure are called primary lesions [Smith *et al.*, 1970; Ho *et al.*, 1978]. A clinical and pathologic feature that characterizes tuberculosis lesions resulting from primary infection is calcification of granulomas of the lung and lymph node, which often appear on chest radiographs as discrete mineralized densities

[Stead *et al.*, 1968]. Dystrophic calcification replaces the foci of necrosis, which represents irreversible tissue damaged that can persist for the life of the patient.

Iron is essential for growth of *M. tuberculosis* and *M. leprae*. Pathogenic mycobacteria, in order to grow and cause disease within a host, must therefore compete against the host for its supply of iron. A general reaction exhibited by bacteria that face an iron-deficient environment is the synthesis of molecules devoted to iron acquisition. Like many bacteria, mycobacteria synthesize siderophores to capture iron. For mycobacteria, siderophores are of the hydroxamate and mixed-ligand type (hydroxamate mixed with phenolic siderophore structure). Mycobacteria, together with some species of *Noccardia* and *Rhodococcus* are unique amongst microorganisms in synthesizing both an intracellular siderophore, termed mycobactin, that is cell wall-associated [Snow, 1970], and carboxymycobactin, an extracellular siderophore [Ratledge, 2004]. *M. tuberculosis* siderophores are salycylate containing molecules. Two forms of mycobactin are produced, which differ in the length of an alkyl substitution and, hence in polarity and solubility. The more polar form is the carboxymycobactin that is released into the medium, whereas mycobactin, which is the less polar form, remains cell wall associated [Rodriguez, 2006]. Carboxymicobactin has the capacity to remove iron bound to the host iron-binding proteins transferrin, lactoferrin, and ferritin. Purified carboxymycobactin rapidly removed iron from transferrin; it was either 95 or 40% iron-saturated, and from human lactoferrin. Carboxymycobactin also removed iron, but at slower rate, from the iron storage protein ferritin [Gobin and Horwitz, 1996]. The uptake of carboxymycobactin is a process which is not energy-linked. Although nothing substantive is known about the energy-independent process, it could involve the participation of a porin protein such as that described in *M. smegmatis* and *M. chelonae* [Trias *et al.*, 1992; Trias and Benz, 1994] and which also may occur in *M. tuberculosis* [Liu *et al.*, 2009]. Calder and Horowits (1998) identified two iron-regulated proteins from *M. tuberculosis* that may be participating in the uptake of ferri-carboxymycobactin [Calder and Horwitz, 1998]. The two proteins, Irp10 and Mta72, could function as a two-component metal transport system. Recently, other researchers [Rodriguez and Smith, 2006] identified two genes (*irtA* and *irtB)* encoding an ABC transporter. IrtA and IrtB proteins are required for efficient utilization of iron from ferri-carboxymycobactin. These researchers postulate that IrtAB is a transporter of ferri-carboxymycobactin. Later, iron could be released from the carboxymycobactin by a reductase mechanism to other iron containing

proteins, such as bacterioferritin or could be transferred to mycobactin as the cell wall store of iron [Ratledge, 2004]. Gobin and Horowits (1996) have shown that exchange of iron occurs from ferri-carboxymycobactin into the mycobactin within the cell envelope of *M. tuberculosis*, even though both molecules probably have the same binding affinities for iron [Gobin and Horwitz, 1996]; this can be explained because the latter molecule could be at a greater concentration within the envelope. Mycobactin releases the iron by means of a ferric-mycobactin reductase in which the ferric iron is reduced in the presence of NAD (P) H to ferrous iron [Brown and Ratledge, 1975; McCready and Ratledge, 1979]. The exact mechanism of iron transfer is not understood, although it has been shown that salicylate can function as an acceptor of Fe (II) after reduction of mycobatin.

The importance of iron in tuberculosis is shown in an *in vivo* study by Basarba et al. (2008) in which they showed that ferric iron accumulates both intra- and extra-cellularly in the primary lung lesions of guinea pigs aerosol-infected with *M. tuberculosis* H37Rv strain [Basaraba *et al.*, 2008]. Iron was accumulated within macrophages at the periphery of the primary granulomatous lesions while extra-cellular ferric iron was concentrated in areas of lesion necrosis. Accumulation of iron within primary lesions was preceded by an increase in expression of H-ferritin, lactoferrin and receptors for transferrin, primarily by macrophages and granulocytes. The authors also found that the increased expression of intra-cellular H-ferritin and extra-cellular lactoferrin, more so than transferrin receptor, paralleled the development of necrosis within primary lesions. On the other hand, primary lung lesions from guinea pigs vaccinated with *Mycobacterium bovis* BCG prior to an experimental infection, reduced iron accumulation as well as H-ferritin, lactoferrin, and transferrin receptor expression. Then the amelioration of primary lesion necrosis was coincident with lack of extra-cellular ferric iron and lactoferrin accumulation.

2.1.5. Escherichia coli and Yersinia pestis Are Able to Obtain Iron from Ferritin

Escherichia coli and *Yersinia* spp are Gram-negative bacteria that produce intestinal diseases. *E. coli* secretes two types of siderophores, enterochelin (catechol) and aerobactin (hydroxamate). Brock et al. (1991) described that both siderophores may acquire iron from different sources, entereochelin scavenging predominantly transferrin-bound iron and aerobactin obtaining iron preferentially from cell or tissues (ferritin) [Brock *et al.*, 1991]. In the case of *Yersinia*, it comprises three pathogenic species. The most notorious

member is *Yersinia pestis*, the causative agent of bubonic and pneumonic plague [Perry, 1993]. *Y. pestis* possesses systems to obtain iron during transient intracellular or extracellular growth in mammals. Although *Y. pestis* uses a wide variety of heme containing compounds as iron sources, some molecules, such as ferritin, support the growth of this bacterium [Perry and Brubaker, 1979; Sikkema and Brubaker, 1989]. *Y. pestis* possesses a functional Fur protein that could participate in ferric iron transport as well as hemin, heme/hemopexin, heme/albumin, ferritin, hemoglobin and hemoglobin/haptoglobin utilization [Staggs and Perry, 1991]. Kirillina et al. (2006) proposed that the inorganic iron ABC transporter Yfe of *Y. pestis* is responsible for uptake of available iron in the spleen and liver, possibly for ferritin stores or other intracellular iron reservoirs [Kirillina *et al.*, 2006].

2.1.6. Streptococcus pyogenes Uses Ferritin as an Iron Source

Streptococcus pyogenes (β-hemolytic group-A *Streptococcus*) is a cause of significant morbidity and mortality worldwide. This Gram-positive respiratory and skin pathogen can be carried by humans asymptomatically or cause uncomplicated pharyngitis; however, *S. pyogenes* can also cause life-threatening diseases such as streptococcal toxic-shock syndrome and necrotizing fasciitis. *S. pyogenes* is also an important contributor to mortality associated with the influenza virus [Simonsen *et al.*, 2000; Morens and Fauci, 2007; Dmitriev and Chaussee, 2010].

S. pyogenes encodes a set of virulence factors necessary for the pathogenesis, such as adhesion to cells and evasion of the immune system. The *S. pyogenes* adhesion process is achieved by bacterial adhesins to several host molecules such as integrins, fibrinogen, collagen and extracellular matrix proteins [Cunningham, 2000; Nobbs *et al.*, 2009]. Another important virulence factor is the secreted lytic enzymes capable of hydrolyzing important host cell molecules involved in modulation of the human immune system [Collin and Olsen, 2003; Chiang-Ni and Wu, 2008]. There is only one report about the use of ferritin as an iron source by this pathogen [Eichenbaum *et al.*, 1996]. This research group observed that 5 mg of ferritin were able to support the growth of the *S. pyogenes* culture in an iron-deprived condition. However, the iron acquisition mechanism by which this bacterium uses ferritin is still unknown. *S. pyogenes* secretes molecules known to produce cellular lysis such as proteases and streptolysin; in this way, several iron containing proteins from infected cells can be released, such as ferritin, hemoglobin, and myoglobin, which could be used as iron source by this bacterium. Surely *S. pyogenes* will be studied in this aspect in the close future since it may be of interest to know

whether ferritin use is a virulence mechanism utilized by this important pathogen to invade the human host.

2.1.7. Bacillus cereus Uses Als3 as a Ferritin Binding Protein

Bacillus cereus is a Gram-positive, motile, aerobic-to-facultative, spore-forming rod widely distributed environmentally. The bacterium exists as a spore former and vegetative cell in nature and only as a vegetative cell when colonizing the human body [Bottone, 2010]. *B. cereus* is generally regarded as a pathogen causing food-borne gastroenteritis due to the production of diarrheal or emetic toxins [Granum and Lund, 1997; Kotiranta *et al.*, 2000]. In addition to food poisoning, *B. cereus* causes a number of systemic and local infections in both immunologically compromised and immunocompetent individuals. Among those most commonly infected are neonates, intravenous drug abusers, patients suffering traumatic or surgical wounds, and those with indwelling catheters. The spectrum of infections include fulminant bacteremia, central nervous system (CNS) involvement (meningitis and brain abscesses), endophthalmitis, pneumonia, and gas gangrene-like cutaneous infections, to mention a few [Bottone, 2010].

Larva infection of the lepidopterans, like *Galleria mellonella,* is used as a model to study *B. cereus*, since this bacterium can cause infection in insects, mice and other mammals [Salamitou *et al.*, 2000; Vilas-Boas *et al.*, 2007]. A mutant strain of *B. cereus*, lacking a ferric dicitrate transporter, showed attenuated virulence in a lepidopteran infection model, highlighting the importance of iron and iron uptake systems to the virulence of *B. cereus* [Harvie and Ellar, 2005]. Accordingly, *B. cereus* has developed strategies to use iron-containing proteins from the host. It is able to use hemoglobin (2 μM), hemin (16.5 μM) and ferritin (0.3 μM) as iron sources for growth in iron-depleted media. The use of transferrin and lactoferrin is controversial, and might be strain dependent [Sato *et al.*, 1999; Park *et al.*, 2005; Daou *et al.*, 2009].

B. cereus lacking IlsA protein showed significant growth defects in iron-depleted media that had been supplemented with hemoglobin, hemin, or ferritin. *ilsA* mutants had no problem with the uptake of inorganic iron, suggesting that IlsA is involved in iron acquisition from these three host proteins during infection [Daou *et al.*, 2009]. *In vivo,* disruption of *ilsA* decreased the growth and virulence of *B. cereus* after oral inoculation or injection into the insect hemocoel [Fedhila *et al.*, 2006; Daou *et al.*, 2009]; these observations indicate that IlsA is an important factor required for adaptation within an insect host. Using a plasmid with a construction fusioning

the *ilsA* promoter (*pIlsA*) with the reporter gene of the green fluorescent protein (GFP*)*, it was observed that *ilsA* is highly expressed in the hemocoel during infection of the insect larvae. Actually, in insects, notably in *G. mellonella*, ferritin is present in high amount in hemolymph and in hemocytes and may play a role in iron transport in addition to iron storage [Kim *et al.*, 2001; Ji-Eun *et al.*, 2005]. In others hosts, where ferritin is mostly intracellular, an extracellular pathogen such as *B. cereus* would need to release ferritin in order to have access to it. In fact, it has been shown that *B. cereus* produces a large variety of cytotoxic proteins (Hbl, Nhe, CytK, Clo, and HlyII), all of them able to lyse various eukaryotic cells [Lindback *et al.*, 1999; Andreeva *et al.*, 2006; Fagerlund *et al.*, 2008].

ilsA (regulated leucine-rich surface protein) gene possesses a Fur-binding box in the promoter region. The protein has an N-terminal peptide signal, and three conserved domains: NEAT domain (N-terminal iron transport-associated domain), LRR (Leucine-Rich Repeat), and SLH (S-Layer Homology) [Fedhila *et al.*, 2006]. *ilsA* is expressed in iron-depleted conditions, which is consistent with the presence of a Fur binding box for iron regulation in its promoter [Fedhila *et al.*, 2006]. It is present in the surface of the bacteria, accumulating at the division site [Daou *et al.*, 2009]. The surface localization may be due to a SLH-domain that presumably binds the protein to peptidoglycan [Fouet and Mesnage, 2002; Fedhila *et al.*, 2006].

Direct interaction between ferritin and IlsA was demonstrated using binding studies with ferritin immobilized on ELISA plates and the purified recombinant protein GST-IlsA. By using Surface Plasmon Resonance (SPR), it was shown that these interactions occur with soluble proteins in real time and under flow conditions. Therefore, *B. cereus* should use hemoglobin, hemin and ferritin, after direct binding to the surface receptor IlsA [Daou *et al.*, 2009]. The NEAT domain is suggested to mediate heme binding to IlsA. However, the interaction with ferritin is much less evident. LRR domains are known to bind structurally unrelated protein ligands [Kobe and Kajava, 2001; Bierne *et al.*, 2007], so it could be speculated that LRR domains are involved in IlsA binding to ferritin. Although both heme and ferritin use the same receptor IlsA, the mechanism of iron uptake should be different for each protein. The authors hypothesize that IlsA might be able to destabilize the ferritin structure, via a possible interaction with the LRR domains [Daou *et al.*, 2009]. The structural modification may permit other factors, such as reductases-like, as occurs in *Listeria* spp. [Deneer *et al.*, 1995], or proteases-like, as occurs in *Burkholderia* [Whitby *et al.*, 2006], to liberate iron (Fe^{3+}) that could be captured by siderophores produced by *B. cereus*, such as

petrobactin or bacillibactin [Wilson *et al.*, 2006] and transferred by an iron uptake-system into the cytosol [Daou *et al.*, 2009]. Actually, bacillibactin, like related tris-catecholate siderophore, binds iron with high affinity ($K_a=10^{-47.6}$) [Dertz *et al.*, 2006] that is markedly greater than that of ferritin [Ratledge and Dover, 2000]. However, this is only a hypothesis and needs to be confirmed.

2.2. Parasitic Protozoa Must Get Iron from the Host Resources to Survive

Parasitic protozoa are broadly spread pathogens responsible for important and often fatal diseases worldwide [Lambert and Barragan, 2010]. Parasites and their corresponding hosts have co-evolved during their life in the Earth, in such a way that parasites have been adapted to only live inside a host, and in several cases, in an exclusive host. Some parasites spend one part of their life cycle in an invertebrate vector and the other part in a vertebrate host in which express the pathogenicity determinants [Bannister *et al.*, 2000; Sacks, 2001; Macedo *et al.*, 2004; Lodge and Descoteaux, 2005; Garcia *et al.*, 2007; Carvalho *et al.*, 2009]. Parasites can live in intracellular niches [Simpson *et al.*, 2006; Ronnebaumer *et al.*, 2008; Ueno *et al.*, 2009; Landfear, 2011], but some species can live extracellularly in fluids and secretions [Felleisen, 1999]. In both sites, parasites express nutrient uptake systems such as those to get iron, among other diverse virulence mechanisms [Burchmore and Barrett, 2001; Azema *et al.*, 2004; Ronnebaumer *et al.*, 2008; Blume *et al.*, 2009; Landfear, 2011]. As such, parasitic life requires a repertoire of adaptations to assure entry/exit from the cell and evade the immune responses to prevent clearance [Sibley, 2011]. The mechanisms of intracellular iron transport and its delivery to organelles are poorly understood in parasitic protists. Several authors propose that iron is transported within the cell through a complex with low-molecular mass ligands; this mobile iron is referred as labile-iron pool (LIP), which consists of weakly-bound iron associated with compounds of low molecular mass (5-30 kDa)). Parasitic protozoa must also store the iron excess; however, in the parasites studied, a ferritin has not been found; instead, perhaps iron is stored in cytosol as LIP [Suchan *et al.*, 2003].

2.2.1. Ferritin as an Intracellular Iron Source for Parasitic Protozoa

Host ferritin is an abundant intracellular-iron store and could be an important source of this element for parasites growing inside a cell. In addition, ferritin could be easily accessible to parasites that produce

lytic enzymes that destroy cells. As protist unicellular protozoa, the use of cationic ferritin has facilitated the study of binding and endocytosis of this protein in experiments with electronic microscopy; due to ferritin being electron-dense it has been used as a microscopy tracer. Reports about pathogenic parasites using ferritin as an iron source are few, including *Trichomonas vaginalis* and *Entamoeba histolytica*. In the case of *Tritrichomonas foetus* and *Trypanosoma brucei,* there are reports about endocytosis of ferritin, but its use as an iron source has not been demonstrated to date.

2.2.1.1. *Tritrichomonas foetus* Can Endocytose Ferritin

Tritrichomonas foetus is a sexually transmitted protozoan that infects the female genital tract of cattle resulting in abortion, endometritis, and infertility [Manning, 2010; Pereira-Neves *et al.*, 2011]. This parasite causes significant economic losses to cattle producers worldwide. Also, some strains of this parasite have been recognized as causing diarrhea in cats [Gookin *et al.*, 1999] and mild-rhinitis in swine [Lun *et al.*, 2005]. As an amitochondrial anaerobic parasite, *T. foetus* depends on high iron requirements (50–100 μM) [Tachezy *et al.*, 1996], surpassing those of eukaryotic cells, although comparable to other anaerobic amitochondrial protists. To face its iron requirement, *T. foetus* is able to utilize lactoferrin, transferrin, or LIP complexes [Tachezy *et al.*, 1996; Tachezy *et al.*, 1998]. Involvement of iron in *T. foetus* virulence has been examined in an experimental mouse infection in which the administration of ferric ammonium citrate to infected mice increased the mortality rate [Kulda *et al.*, 1999].

Previous studies of endocytic processes in *T. foetus* have described that this parasite has a high endocytic activity through vesicles of different size and shape [Affonso *et al.*, 1994]. At 4º C, the temperature at which the endocytic processes are stopped, ferritin was only observed in patches; however, at 37 ºC, no labeling of the plasma membrane was observed. Cytoplasmic positive compartments were observed as pleiomorphic or round structures generally filled with ferritin; the internalization of ferritin was followed for 60 min. Thus, this parasite is capable of internalize ferritin [Affonso *et al.*, 1997]. The mechanism by which ferritin is endocytosed is still unknown.

2.2.1.2. *Trypanosoma brucei* Endocytoses and Digests Ferritin

Trypanosoma brucei causes African trypanosomiasis or sleeping-sickness in humans and nagana in cattle [Roberts *et al.*, 2005]. An estimated 60 million people are infected [WHO, 2001] and 48,000 deaths were reported in 2002

[WHO, 2004]. In addition, 46 million cattle are at risk of contracting nagana with a high cost to people working in livestock production [Kristjanson *et al.*, 1999]. The life cycle of this parasite requires a tsetse fly (*Glossina* spp.) that feeds on blood from an infected host. The parasite metacyclic stage is the infective form to the mammalian host. After transmission, protozoa multiply extracellularly in the blood, lymphatic system and interstitial spaces and then, during latter stages of infection, in the central nervous system [Breidbach *et al.*, 2002; Roberts *et al.*, 2005; Peacock *et al.*, 2011]. Bloodstream trypanosomes are highly pleomorphic forms [short stumpy (SS), intermediate short-stumpy (ISS) and dividing long-slender (LS) forms]; during the life cycle, the LS forms transform in SS forms in the blood and SS transform into the midgut forms in the fly [Langreth and Balber, 1975]. The endocytosis of transferrin by *T. brucei* has been well-demonstrated [Schell *et al.*, 1991]: holotransferrin, after being bound to its receptor at the flagellar pocket (the main site of uptake of exogenous proteins), is endocytosed and delivered to an endosomal system and, subsequently, to dissociation of the receptor-ligand complex [Maier and Steverding, 1996]. The unoccupied receptor is recycled [Steverding *et al.*, 1995], whereas transferrin is transported to lysosomes and proteolytically degraded [Grab *et al.*, 1992; Steverding *et al.*, 1995]; Steverding determined that accumulation of only 40,000 iron atoms/cell during the generation-doubling time of 10 hours appears to be sufficient to support their multiplication [Steverding, 1998]. A single trypanosome contains 1.4 x 10^6 iron-atoms as determined by atomic absorption spectrometry [Schell *et al.*, 1991]; thus bloodstream forms of *T. brucei* are capable of storing iron.

Concerning cationic ferritin, it was demonstrated the uptake and digestion of this protein by transmission electron microscopy [Langreth and Balber, 1975]. Ferritin was observed to be up taken rapidly in the flagellar pocket of the SS bloodstream trypanosomes at 25 ºC and then appeared in the cell in large vesicles, which appeared invaginate from the pocket. Since ferritin was not observed in these vesicles at 0 ºC, the process requires energy. After that, ferritin was observed in large vesicles fusioned with smooth straight tubules of the collecting system and at a later time in acidic vesicles with digestive activity, probably lysosomes. In this way, *T. brucei* is most probably capable of using ferritin iron, which is released at acidic pH, since it is known that ferritin is unstable at acidic pH and natural recycling of iron from ferritin occurs in the acidic environment of lysosomes [Goswami *et al.*, 2002; Nadadur *et al.*, 2008; Zhang and Enns, 2009].

2.2.1.3. *Trichomonas vaginalis* Utilizes Ferritin in Cultures in Vitro

Trichomonas vaginalis is a sexually transmitted extracellular flagellated single-cell parasitic protozoan that lives in the female lower reproductive tract and the male urethra. Unique genetic and structural features place the parasite at the base of the eukaryotic phylogenetic tree and suggest an intriguing evolution toward mucosal parasitism [Wolner-Hanssen *et al.*, 1989; Shafir *et al.*, 2009]. *T. vaginalis* selectively adheres to the human vaginal epithelial cells, surviving for years in the hostile vaginal environment that is typically acidic, contains a plethora of microbicidal innate-immune factors, and is reinforced by the presence of a complex commensal bacterial consortium [Lehker and Sweeney, 1999; Van der Pol, 2007]. Iron concentration is a key factor in the virulence of this parasite, since its metabolic activity, cytoadherence, and resistance to complement lysis are increased by iron [Tsai *et al.*, 2002; Garcia *et al.*, 2003].

In 1992, it was reported that *T. vaginalis* is capable of using ferritin as an iron source in cultures *in vitro*. The group of research demonstrated that ferritin and lactoferrin (each 250 µM), but not transferrin, were used by this parasite *in vitro* as iron sources and suggested that these iron-containing proteins could be used as an iron source necessary for growth and colonization of the vaginal mucosa. The iron acquisition system from lactoferrin and ferritin is very important for the infection process, due to the iron-limiting environment in the vagina. Currently, the iron acquisition process from host cellular ferritin by *T. vaginalis* is unknown [Lehker and Alderete, 1992].

2.2.1.4. *Entamoeba histolytica* Possesses a Ferritin Binding-Protein and Endocytoses Ferritin by Clathrin-coated Pits

Entamoeba histolytica is an enteric parasitic protozoan that causes amoebiasis, a cosmopolitan infection that affects only human beings. Cyst is the infective stage transmitted by the fecal-oral route through the intake of contaminated water and foods. When cysts are ingested they can pass throughout the acidic pH of stomach, and in terminal ileum excystation occurs producing the invasive stage or trophozoites. These primitive eukaryotic cells are able to adhere and invade the colon-intestinal mucosa causing dysentery, fever and abdominal pain; severe cases can lead to ulcerative colitis. By unknown reasons, trophozoites can travel to the liver via portal vein, producing liver abscesses, which can be fatal if not treated. Amoebae can also invade other organs, especially the brain and lungs [Espinosa-Cantellano and Martinez-Palomo, 2000]. Amoebiasis is the third cause of death by parasites

worldwide. An estimate of 50 million people are symptomatically infected and 100,000 people die annually, mainly in developing countries because of the poor hygienic conditions [Stanley, 2003; Ali *et al.*, 2008]. In response to unknown stimuli, amoebae undergo morphological and biochemical changes leading to the formation of cysts, which are eliminated in the feces, closing the cycle [Martinez-Palomo, 1987].

E. histolytica requires a large amount of iron for growth *in vitro* (80-100 µM Fe), and this fact probably reflects its iron necessity *in vivo*. This parasite is able to use several host iron-containing proteins for growing *in vitro*, such as hemoglobin (Hb), ferric transferrin (holoTf) and ferric lactoferrin (holoLf) [Serrano-Luna *et al.*, 1998; Reyes-Lopez *et al.*, 2001; Leon-Sicairos *et al.*, 2005]. Recently, our group reported the interaction between the amoebic trophozoites and ferritin. By using growth kinetics in iron-chelated medium to which different concentration of ferritin was added as a sole iron source, we found that *E. histolytica* cultures developed at an optimal concentration of 100 µM Fe provided by ferritin. Uptake of this molecule could be mediated by a ferritin binding-protein (EhFbp), since the binding of FITC-ferritin to amoebae was dependent on the ligand concentration, time, and temperature of incubation. Furthermore, the binding was highly specific for ferritin, since other iron-proteins such as Hb, holoTf and holoLf did not compete with ferritin for the binding. Trophozoites surface was saturable at 46 nM of ferritin [Lopez-Soto *et al.*, 2009].

To investigate whether ferritin is endocytosed by trophozoites, we used some inhibitors of diverse endocytosis pathways and demonstrated the ferritin endocytosis by confocal laser-scanning microscopy (CLSM) using FITC-ferritin. Amoebae quickly internalized ferritin (in the first 2 min of interaction). Ferritin entrance was found constrained only by inhibitors of clathrin-coated pits such as chloroquine, NH_4Cl, sucrose, and chlorpromazine. Filipin, which disrupts caveolae structures, or wortmannin, which affects fluid-phase endocytosis, did not inhibit ferritin endocytosis. This result was confirmed by electronic microscopy: cationic ferritin was observed inside clathrin-covered vesicles by using an anti-bovine brain clathrin Ab. Interestingly, after 30 min of incubation, ferritin co-localized with an anti-rat LAMP-2 Ab in amoebic lysosomes by CLSM, suggesting its degradation in these organelles. In amoebic extracts analyzed by electrophoresis of gels copolymerized with ferritin (substrate gels), three internal neutral cysteine-proteases (100, 75, and 50 kDa) of *E. histolytica* were observed cleaving ferritin [Lopez-Soto *et al.*, 2009]. The results together suggest that *E.*

histolytica possesses vesicular proteins related to clathrin and lysosome-like structures that are used by trophozoites for ferritin endocytosis and degradation, a process that seems to be similar to that used by mammalian cells. However, the amoeba receptor and thus its similarity to the mammalian receptor, remain yet to be identified. This mechanism of endocytosis for use of ferritin as an iron source has not been described in other parasites.

As we mentioned before, trophozoites from this parasite are able to invade the human liver causing fatal hepatic abscesses. Furthermore, cysteine-proteases, amoebopores, cytolysins and other factors contribute to liver tissue degradation in hepatic amoebiasis [Perez-Tamayo *et al.*, 2006]. Therefore, although the use of this iron-rich protein was not investigated *in vivo*, ferritin could be exposed to the environment by the alteration of liver tissue and being used as an iron source for amoebae, recrudescing the extra-intestinal phase of the disease.

CONCLUSION

As we can see, microorganisms have developed numerous ways to acquire iron from ferritin. The mechanism could be as simple and general as the iron acquisition from a xenosiderophore, which the same microorganism does not even need to synthesize, or it could be much more elaborate, like *Neisseria*, which manipulates the host cell so magnificently in order to get ferritin iron. When the microorganism has several mechanisms in order to get iron from ferritin, it could seem a redundant function; however, it is only implying the importance of this rich iron source for the infection success.

Only a few ferritin receptors or surface binding proteins have been studied in microorganisms; moreover, there is no obvious structural homology between them, for example, Als3 from *B. cereus* and IlsA from *C. albicans*, or even between the microorganism receptors and the mammalian receptor Tim2. Thus, depth structural studies related to the interaction between those receptors and ferritin are needed to elucidate this aspect.

Also, we can perceive that microorganisms do not need to produce specific proteins dedicated to bind ferritin to acquire its iron; in the case of Als3 it also binds hemin and hemoglobin to resolve this trouble. Interestingly, Als3 is a multifunctional protein with many roles in addition to capturing iron. This fact is in agreement with what has been found in some pathogens' transferrin receptors, like staphylococcal GAPDH or *E. histolytica* EhADH2.

Both are enzymes working like receptors of a host iron-protein; this is telling us how microorganisms are "economic beings," taking advantage of proteins with other functions to acquire the host iron.

Finally, given the importance of the iron acquisition mechanisms and the fact that they have no parallel in the host, inhibition of iron binding and transport might be an attractive therapeutic target.

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Chapter 2

FERRITIN: FUNCTIONS, BIOSYNTHESIS AND REGULATION

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ABSTRACT

Ferritin is a protein with multiple functions in the human being. Although traditionally related to iron metabolism status and iron deficiency, as the main protein to store iron, recently its role on oxidative stress has been an issue of concern. Iron deficiency (ID) is the most common nutritional deficiency disorder in the world, affecting more than two billion people, mostly infants, children and women of childbearing age. In general, iron deficiency symptoms are probably due not to lowered hemoglobin level, but to an insufficient supply of iron to the tissues, and, therefore, ferritin concentration is the most practical way of reporting iron deficiency in field and clinical studies. Increases in ferritin concentrations has been used to show improvements of fortification of food with iron with many different food vehicles, such as milk, cereals, etc. In such cases, subjects with infection or inflammation must be excluded from those studies, as ferritin acts as a positive acute-phase reactant protein, and thus its concentration is increased. Ferritin concentration measurement is also useful in studies when different iron compounds are compared in different conditions where iron deficiency or iron deficiency anemia is frequently seen, as in gastrectomized patients. Of note, ferritin concentration is usually different in patients with iron deficiency or iron deficiency anemia who will be submitted to gastrectomy or to bariatric surgery. In the former condition, patients will present low ferritin concentration, whereas in the later ferritin can be increased, as abdominal fat in obese subjects may favor an inflammation milieu through pro inflammatory cytokines synthesis, and then ferritin concentration will act as a positive acute-phase reactant protein. On the other hand, it is well recognized that iron is the most powerful catalyst for the formation of highly toxic ROS. Iron in combination with ferritin is stable. However, in some overload iron conditions, such as chronic hepatitis C, ferritin is decomposed by lysossomes and hence an unstable iron ion is released. Therefore, reactive oxygen species are formed and they also facilitate iron release from ferritin. The aim of the present review is to show the importance that ferritin has acquired in the understanding of many processes, which will make easier the comprehension of the role of ferritin in both iron deficiency and oxidative stress mechanisms.

INTRODUCTION

Iron deficiency (ID) is the most common nutritional deficiency disorder in the world, affecting more than two billion people, mostly infants, children, and

women in childbearing age (Stolzfus, 2001). The most practical way to detect ID is through serum ferritin values, as bone marrow aspiration is an invasive procedure and there are several conditions besides ID in which serum iron levels may have decreased values.

In general, the symptoms of ID are considered to be due probably not to lowered hemoglobin level, but to an insufficient supply of iron to the tissues (Hallberg, 2001). The human brain develops up until about 20 years of age, and brain iron content also increases concomitantly up to this age. It may be difficult to repair negative effects of iron deficiency (ID) developed during this period (Hallberg, 2001).

Currently, the role of iron in neurocognitive and neurobehavioral development has deserved much attention and several reviews (Grantham-McGregor & Ani, 2001; Beard & Connor, 2003) and reports (Alkman et al., 2004; Konofal et al., 2004) have shown negative effects of ID related to mental symptoms in infants, children and even in adolescents (Bruner et al., 1996). Therefore, the outcome of current evidence strongly suggests that to assure optimal health and development and to avoid permanent deficit in cognitive development which could result in prejudicial social and general life achievements, it is important to prevent and treat even mild ID of growing individuals, at least up to around 20 years of age, as well as during pregnancy (Beard & Connor, 2003; Armano et al., 2010).

In the meantime, anemia is one of the most common nutritional problems in the world. The World Health Organization (WHO) estimates that some two billion people are anemic defined as hemoglobin concentrations that are below recommended thresholds; iron deficiency anemia (IDA) is the most prevalent, followed by chronic disease anemia (CDA) in which changes in iron metabolism also occur. Hence, to choose the best indicators to assess iron status is fundamental (WHO, 2008).

Considering that transport and iron storage are mediated by three proteins, transferrin, transferring receptor 1 (TfR1), and ferritin, the most used parameters to assess iron status are serum iron, t*otal iron-binding capacity* (TIBC), which is a laboratory test that measures the *capacity* to bind iron with transferrin, transferrin saturation, soluble transferrin receptor (sTfR1), and serum ferritin. In addition, these indicators are also important in diagnosing iron overload mainly due to iron increased absorption conditions and repeated blood transfusions (WHO, 2004). The bone marrow aspiration (Pearl's Prussian blue reaction) is still considered the "gold standard" for evaluation of iron storage; however, as an invasive procedure, it was widely substituted for serum ferritin, and currently is only used in certain conditions, such as

diagnosing sideroblastic anemia. Serum ferritin is usually determined by immunological methods, such as by enzyme-linked immunosorbent assay (ELISA) or immunoturbidometry (WHO, 2004).

Although traditionally related to iron metabolism status and iron deficiency as the main protein to storage iron, ferritin has been has been considered an issue of concern due to its role on oxidative stress (Arosio, Ingrassia, Cavadini, 2009).

Therefore, the aim of the present review is to show the importance that ferritin has acquired in the understanding of many processes, which will make it easier the comprehension of both iron deficiency and oxidative stress mechanisms.

UTILIZATION OF FERRITIN IN CLINICAL AND EPIDEMIOLOGICAL STUDIES

Increases in ferritin concentrations has been used to show improvements of fortification of food with iron with many different food vehicles, such as milk, cereals, etc. (Miglioranza et al., 2008; Miglioranza, Breganó, Dichi, 2011). In such cases, subjects with infection or inflammation must be excluded from those studies, as ferritin behavior as a positive acute-phase reactant protein, and thus its concentration is increased. Ferritin concentration measurement is also useful in studies when different iron compounds are compared in different conditions where iron deficiency or iron deficiency anemia is frequently seen, as in gastrectomized patients (Mimura et al., 2008). Of note, ferritin concentration is usually different in patients with iron deficiency or iron deficiency anemia who will be submitted to gastrectomy or to bariatric surgery (Dichi, Breganó, Miglioranza, 2011). In the former condition, patients will present low ferritin concentration, whereas in the later ferritin can be increased, as abdominal fat in obese subjects may favor an inflammation milieu through pro inflammatory cytokines synthesis, and then ferritin concentration will act as a positive acute-phase reactant protein. On the other hand, it is well recognized that iron is the most powerful catalyst for the formation of highly toxic ROS. Iron in combination with ferritin is stable. However, in some overload iron conditions, such as chronic hepatitis C, ferritin is decomposed by lysossomes and hence an unstable iron ion is released (Venturini et al., 2010). Therefore, reactive oxygen species are formed and they also facilitate iron release from ferritin.

STRUCTURE, FUNCTION AND REGULATION OF FERRITIN

Ferritin is formed by a protein complex with 24 peptide subunits (L and H forms) assembled into a hollow spherical shell; it is hydrosoluble with 465000d molecular weight. Apoferritin, a ferritin not bound to iron, can sequester up to approximately 4500 iron atoms (Harrison & Arosio, 1996).

The main function of ferritin is to store intracellular iron in a compact, safe way, and make it available when necessary. This function is due to a very high iron binding capacity and its ferroxidase activity, which converts Fe^{++} to Fe^{+++} (Arosio, Ingrassia, Cavadini, 2009).

Iron regulatory protein (IRP) mediates ferritin, transferrin receptor (TfR1), and divalent metal transporter (DMT-1) synthesis. IRP has affinity for iron responsive elements (IREs), located in ferritin, TfR1, and DMT-1 mRNA (Torti & Torti, 2002; Arosio, Ingrassia, Cavadini, 2009).

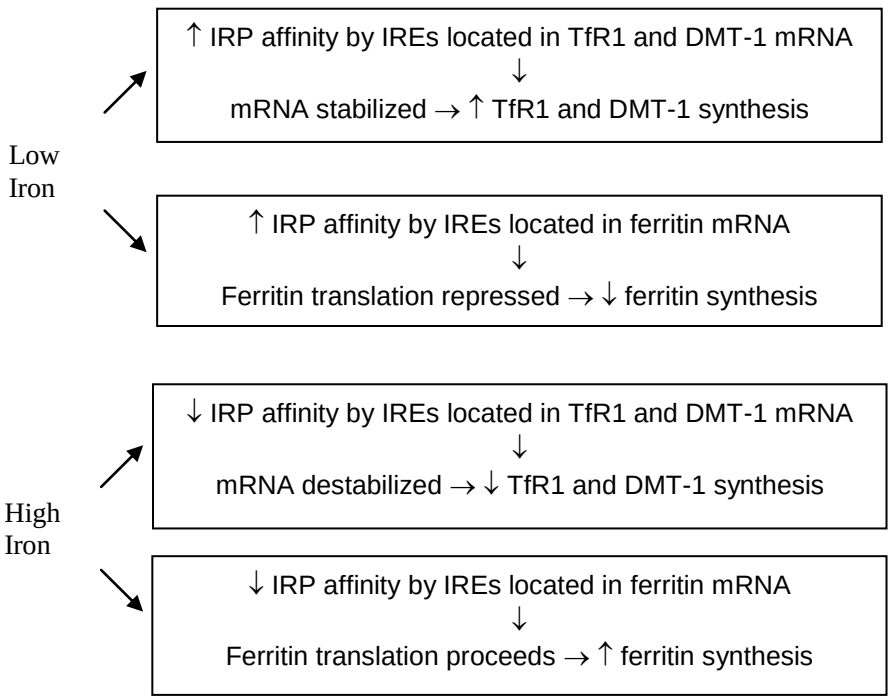

Figure 1. Influence of iron status on ferritin, transferrin receptor (TfR1), divalent metal transporter (DMT-1) synthesis, iron regulatory protein (IRP) affinity, and iron responsive elements (IREs).

DMT-1 is a glycoprotein located in enterocytes and macrophages cell membranes and transport divalent metals, including Fe^{++}, to the inner cell, being involved in iron absorption regulation by enterocytes in duodenum. When iron deficiency occurs, there is an increase in DMT-1 expression and an enhancement in iron displacement from intestinal lumen to these cells (Mimis e Prchal, 2005).

TfR1 promotes cell entrance of iron bound to transferrin by endocitosis, which is considered the most important mechanism by which cell entrance occurs. TfR1 is also considered an excellent erythropoiesis marker and can be evaluated by measuring plasma soluble TfR1 (sTfR1) (Punnonen, Irjala & Rajamaki, 1997; Beguin, 2003; Skikne, 2008). sTfR1 level varies with age, gender, and pregnancy (Choi et al,1999; Choi et al 2000) .

ROLE OF FERRITIN ON IRON DIFICIENCY AND IRON DEFICIENCY ANEMIA

Serum ferritin is a marker of iron storage both in iron deficiency (ID) and IDA. Despite being measured in plasma, its value reflects variations in iron body storage. References values range between 15 and 300 μg/L and vary with age and gender. Iron storage is considered depleted when ferritin values are below 12.5μg/L (WHO, 2005). Nevertheless, it has been demonstrated that patients with normocytic anemia who have ferritin levels above 50 μg/L should not be automatically considered to have adequate iron stores (Koulaouzidis et al,2009).

Iron storage depletion (stage I) is a precocious ID marker and precedes the development of microcytic and hypochromic (stage II) with reduction in mean corpuscular haemoglobin concentration (MCHC), mean cell hemoglobin (MCH), and mean cell volume (MCV) (stage III). Red Cell Distribution Width (RDW) is increased due anisocytosis. Of note, whereas serum tranferrin levels increase in IDA, in inflammatory processes transferrin concentration decrease and ferritin increase, even with low iron storage, making difficult the interpretation of these markers (Feelder, et al, 1998; Wish, 2006). Tumor necrosis factor alpha (TNF-α), interleukin 1α (IL-1α), IL-6 and IL-10 are pro inflammatory cytokines which induce ferritin expression and iron retention inside macrophages (Weiss e Goodnough, 2005).

TfR1, which is increased in IDA, is another marker used to evaluate iron metabolism and it seems that, differently from transferrin and ferritin, it is not

influenced by infectious or inflammatory processes (Joosten et al, 2002; Skikne, 2008). TfR1 may be used isolated or associated with serum ferritin, propitiating the calculation of sTfR1 and serum ferritin log index (sTfR/log ferritin-Index). In stage I, sTfR1 remain stable, despite serum ferritin concentration decrease, whereas in stage II, when ID begins to decrease hemoglobin synthesis (iron-deficient erythropoiesis) and other iron physiologic functions, sTfR1 begins to increase, being an important marker of this stage. In the last stage (stage III), there is a more accentuated decrease in hemoglobin levels, ferritin concentrations, and a marked increase in sTfR1. As sTfR/log ferritin-Index is influenced by both parameters, its augmentation can be even detected in stage I. Therefore, interpretation of both ferritin and sTfR concomitantly helps to recognize the distinct stages of IDA (Suominen, Punnonen, Rajamäki e Irjala, 1998) and in areas with high infection prevalence, sTfR/log ferritin-Index best predicted iron deficiency (Phiri et al,2009).

ROLE OF FERRITIN IN CHRONIC DISEASE ANEMIA

CDA is much prevalent in patients with chronic infectious disease, such as tuberculosis and osteomielitis, in patients with chronic inflammatory disease, such as rheumatoid arthritis, systemic lupus erythematosus, and Chron`s disease and also in patients with carcinoma and lymphoma. There is a decrease in serum iron and transferrin and an increase in iron in macrophages and erytrhroid cells. Serum ferritin concentration is at normal range or increased, thus reflecting increased iron retention and storage in reticulo-endothelial system. There is also an enhancement in hepcidin and in IL-6, IL-1, IL-10, and TNF-α which stimulate ferritin synthesis (Torti et al, 1988; Rogers, 1996; Weiss and Goodnought, 2005).

Hepcidin is considered the most important iron metabolism hormonal regulator; hepcidin, which is stimulated by IL-6, inhibits iron disposal by macrophages and intestinal epithelial cells due to ferroportin reduction, an iron exporting protein (Nemeth et al, 2004). Iron increased retention in macrophages results in lower iron absorption, higher iron storage in endothelial system, and higher ferritin storage. On the other hand, ID, hypoxia, and inefficient erythropoiesis result in hepatocytes decreased hepcidin synthesis and secretion with a higher iron absorption and bioavailability (Nicolas et al,2002).

Other mechanisms, hepcidin apart, also contribute to CDA: IL-1 inhibits erythropoietin production; besides inhibition in erythropoietin production, interferon-γ also increases DMT-1, and thus enhances iron retention by macrophages; TNF-α and interferon-γ decrease ferroportin synthesis with a reduction in iron disposal. IL-10, an anti-inflammatory cytokine, increases ferritin and TfR1 with an augmentation of iron retention by macrophages (Ludwiczek et al, 2003).

Table 1. Differential diagnosis between iron deficiency, iron deficiency anemia, anemia of chronic disease, and both anemia of chronic disease and iron deficiency anemia based on ferritin and other parameters.

Parameters	Iron deficiency	Iron-deficiency anemia	Anemia of chronic disease (ACD)	Iron-deficiency anemia and anemia of chronic disease
Hemoglobin	Normal	Reduced	Reduced	Reduced
Ferritin	Reduced	Reduced	Normal to increased	Reduced to normal
Serum Iron	Normal	Reduced	Reduced	Reduced
Transferrin saturation	Normal	Reduced	Reduced	Reduced
Transferrin	Normal	Increased	Reduced to normal	Reduced
sTfR1	Normal	Increased	Normal	Normal to increased
STfR/log ferritin-Index	High(>2)	High(>2)	Low (<1)	High(>2)
Hepecidin	Reduced	Reduced	Increased	lower hepcidin levels than ACD
Cytokine levels	Normal	Normal	Increased	Increased
Marrow iron	Absent	Absent	Present	Reduced to normal

Differential diagnosis between CDA versus IDA and CDA association is important; iron supplementation only has beneficial effects on IDA, whereas in CDA patients, in contrast, may have deleterious effects (Weiss e Goodnought, 2005). This association has been considered when besides anemia, transferrin saturation values are below 16%, serum ferritin below 100ng/mL, and sTfR/log ferritin-Index is above 2 (Punnonem, Irjala, Rajamaki,1997). Hepcidin determination has been also shown to contribute in CDA versus IDA and CDA association differential diagnosis. Patients with ACD/IDA would respond to treatment with oral iron supplementation, as

hepcidin levels are low and duodenal ferroportin expression and duodenal iron absorption are increased. Moreover, hepcidin determination may be useful to monitor the therapeutic success of iron supplementation therapies, even before an increase of hemoglobin levels can be observed. (Theurl et al, 2009).

Differential diagnosis between ID, IDA and other anemia related diseases based on ferritin and other parameters are shown in Table 1.

FERRITIN AND STRESS OXIDATIVE: PRO OR ANTIOXIDANT?

Ferritin is an protein in which thousands of iron atoms are maintained in solution, avoiding the toxicity that could occur if iron atoms remained free. Ferritin concentration is mainly intracellular and has a role on iron metabolism reactions which occur in the intracellular milieu. Iron flux in ferritin occurs in agreement with cellular needs (HARRISON and AROSIO, 1996). The main regulatory system of ferritin expression is the post-transcriptional, iron-dependent machinery based on the interaction by the iron regulatory proteins (IRPs) and iron responsive elements (IRE) located on the target mRNAs (HENTZE an KUHN, 1996).

The H-ferritin is primarily responsible for the ferroxidase activity of the ferritin complex and this activity is essential for uptake of free iron; whereas the L-ferritin facilitates the storage of iron into the ferritin core. The efficient storage of iron requires the cooperation of both ferritin subunits (ZHAO et al., 2001). These subunits are directly related to ferritin functions and an imbalanced reaction would result in unneeded stress. This reaction was shown by the production of mouse erythroleukemic cell clones transfected with the mouse H-chain, which showed an iron-deficient phenotype with reduced labile pool (LIP), upregulation of transferrin receptor and IRP activity, and a decrease in hemoglobin synthesis (PICARDI, et al. 1996). These cells also showed a reduced production of reactive oxygen species (ROS) after exposure to hydrogen peroxide when compared to control cells (EPSZTEJN, et al. 1999). The important finding was that the presence of an excess of H-chain induced an iron deficient phenotype similar to that observed in the transfected mouse erythroleukemic (MEL) cells, reduced cell proliferation rate, and also increased cell resistance to oxidative damage induced by hydrogen peroxide. All these manifestation were abolished by iron supplementation, indicating that they are all associated with the relative iron deficiency.

Several studies reported a significant modulation of ferritin levels in conditions often associated with cellular stress. In addition to iron levels, ferritin synthesis is regulated by inflammation (cytokines) and oxidative stress. The expression of the ferritin subunits is under transcriptional and translational regulation (White & Munro, 1988). Their mRNA contains iron response elements that can increase the translation of ferritin when iron is abundant and vice-versa (Munro et al., 1993). Transcriptional regulation allows for a different distribution of the two subunits of ferritin within cells enabling them to adapt to changes in iron bioavailability. The ratio between H- and L-subunits in a ferritin shell varies widely in different tissues. L-subunit-rich ferritin predominates in iron storage organs such as the liver and spleen, and H-subunit-rich ferritin predominates in organs of low iron content such as the heart and pancreas (Alkhateeb & Connor, 2010). The ability of cells to induce rapid ferritin synthesis prevents the effects of free radical damage to cellular components.

Ferritin expression, on a transcriptional level, is modulated by a variety of conditions associated with oxidative stress that act either directly on gene expression or indirectly via the modification of IRPs activity. Inflammatory cytokines, particularly TNF-α and interleukin-2, upregulate ferritin synthesis in various mammalian cells, including mesenchimal cells, hepatocytes, and monocyte-macrophages (MILLER et al. 1991). The regulatory element for this response, which is specific for the H-chain and is named FER2, has been located 4.8 kbp upstream the start of transcription in the mouse H-ferritin gene and encompasses a binding site for the transcription nuclear factor kappa B (NFκB) (KWAK et al. 1995). H-ferritin was found to be regulated by NFκB and to act as an essential mediator of the antioxidant and protective activities of NFκB. H-ferritin is induced downstream NFκB and protects against apoptosis triggered by TNF-α by sequestering iron and inhibiting ROS formation (Pham et al., 2004). Therefore, it has been suggested that a restriction of cellular iron availability is a possible additional pathway that contributes to the p-53-mediated growth arrest. (Arosio & Levi, 2010). Ikegami et al. (2009) showed that adipocyte-derived protein adiponectin upregulates H-ferritin in skeletal muscle cells. The protective role of adiponectin was attributed to the induction of H-ferritin, which reduced ROS levels and oxidative damage.

Ferritin expression is regulated by oxidative stress that acts either directly on gene expression or indirectly via modifications of IRPs activity. Oxidants, such as H_2O_2, transcriptionally activate the mouse ferritin H gene by directly targeting a 75-bp cis-acting element that is located upstream of the

transcription initiation site. In addition, the responses of both IRP1 and IRP2 to oxidants have been shown by many studies. Cairo et al. (1996) observed that oxidative stress induced ferritin translation by inactivating IRP1. However, Pantopoulos and Hentze (1998) showed that H_2O_2 activates IRP1, possibly through direct disassembly of 4Fe-4S cubane cluster. The activated IRP1 enhanced IRE-binding activity and represses biosynthesis of ferritin, potentially leaving the cells more susceptible to oxidative injury.

Most of the stimuli related to inflammation and directed to ferritin synthesis seem to upregulate H-ferritin preferentially over the L, thus determining an increase of catalytic sites and a reduction of cell iron availability. Cytokines from inflammation may also affect ferritin translation indirectly through their ability to induce nitric oxide synthase (iNOS) and hence increase nitric oxide (NO) concentration. NO in turn causes the activation of both IRP1 and IRP2. Mechanisms hypothesized to underlie NO-mediated induction of IRP binding activity include cluster disassembly (IRP1), intracellular iron chelation (IRP1 and IRP2), or increased de novo synthesis (IRP2). Thus, NO stimuli upregulate H- and L-ferritin synthesis at a transcriptional level (MARZIALI, et al., 1997; Torti & Torti, 2002)

NO chelates labile iron in a form which decreases its potential to yield reactive intermediates, and NO reacts with and scavenges free radicals. The prevention of ferritin stimulation by NO suggests that the antioxidant capacity of NO could also involve its ability to decrease the activity of iron-heme compounds, preventing the release of catalytic active iron from storage forms such as ferritin. Thus, the cellular ability to generate free radicals involved in cytoxicity would be decreased (Puntarulo, 2005).

Ferritin plays an important role in the protection against oxidative damage. The biological evidence of an antioxidant role of ferritin is compelling. This antioxidant role likely explains the presence of mitochondrial and nuclear ferritin. It is possible that ferritin may play specialized roles within organelles. Unbound intracellular ferrous iron is capable of generation free radicals and ROS through Fenton chemistry (?) causing lipid peroxidation, DNA breaks, and others forms of cellular damage (Halliwell & Gutteridge, 1984). It has been shown that the H-ferritin can sequester Fe (II) in the solution and reduce lipid peroxidation induced by Fenton-type reaction, and that iron-loaded ferritin can bind large amounts of nitric oxide molecules in different sites of the structure (Arosio & Levi, 2002).

The existence of an iron storage protein inside mithocondria was speculated on several years ago, but its presence was confirmed only at the beginning of this century. This new form of ferritin, mithocondrial ferritin, is

encoded by an introless gene on Chromosome 5q23.1. It is expressed as a 30 KDa precursor with a 58 N-terminal amino acid leader sequence that ensures efficient mithocondrial targeting. The mitochondrial ferritin is important because this organelle is tightly involved in iron trafficking, and has a key role in essential cellular activities, including respiration, the production of ROS, and the regulation of apoptotic pathways. Thus, this ferritin is an important regulator of local iron trafficking and defense against the possible interaction between free iron and ROS, both abundantly in the organelle (Arosio & Levi, 2002). The mithocondrial ferritin protects mithocondria from oxidative damage, probably by regulating local iron availability, making the cells more resistant to iron-mediated oxidative damage (Arosio & Levi, 2010). Although the important role of mitochondrial ferritin has been shown in cellular defense, contradictory results have been reported in the literature. When mithocondral ferritin overexpressing cells were treated with tert-butyl-hydroperoxide, it was associated with decreased mithocondrial metabolic activity, reduced glutathione, and a concomitant increase in reactive oxygen species levels and apoptosis, indicating that mithocondrial ferritin may increase cell sensitivity to oxidative stress. Thus, like cytossolic ferritin, mithocondrial ferritin might have different roles under various circumstances.

It is generally thought that ferritin iron is in a safe form not available for oxidative damage. However, ferritin can have pro-oxidant activity due to iron release under non-physiological conditions, and, with the production of strong reductants, ferritin iron can be released to catalyze the production of free radicals. It has been demonstrated that reducing natural agents, such as sulfide, superoxide ion and hydrogen peroxide, reduces ferritin iron. Therefore, Li and Hu (2009) showed that incubating of ferritin with hydrogen peroxide produces DNA damage similar to that obtained from Fenton reaction and this can be inhibited by strong iron chelators. Under conditions in which ferritin iron capacity to store iron is overwhelmed or impaired by either chemical or genetic reasons, ferritin iron can contribute to tissue damage. Thus, the relationship is complex, since ferritin iron may recycle and become pro-oxidant in pathological conditions or even during aging. In addition, the switch from the most common protective roles to a more rare pro-oxidant may be relevant in some disorders.

Over the last two decades, several studies have reported the presence of ferritin in cell nuclei. Nuclear ferritin is comprised of the same ferritin found normally in the cytoplasm. RNA interference studies in SW1088 astrocytoma cells revealed that both nuclear and cytoplasmatic ferritins are the product of

the same gene (Surguladze et al., 2005). It is possible that nuclear ferritin has a specialized role in DNA protection. In addition, ferritin allows for controlled DNA nicking, which is necessary for transcriptional elongation and DNA replication (Windon, 1998)

Hyperferritinemia is associated with inflammation, infections, and malignances. In these conditions, increases in cytokines and oxidative stress may induce ferritin expression (Zandman-Godard & Shoenfeld, 2007).

Also, ferritin and iron homeostasis have been implicated in the phatogenesis of many diseases, including hemochromatosis, atherosclerosis, autoimmune disease, diabetes melitus, chronic hepatitis C, etc (You & Wang, 2005; Zandman-Goddard & Shoenfeld, 2007; Rajpathak et al., 2009; Venturini et al., 2010). Nevertheless, controversies exist on the functional role of ferritin in these conditions, whether pro or antioxidant.

CONCLUSION

Whereas the role of ferritin, as an iron storage protein in ID and IDA is well known, the same does not occur with the role of ferritin in the oxidative stress process. Ferritin has an ambiguous role and depends on various circumstances to be considered pro or antioxidant. The understanding of these mechanisms will be essential for the development of different strategies to prevent and treat many diseases, mainly those related to iron overload. Further basic and clinical studies are much recommended to explore this complex picture.

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Chapter 3

FERRITIN AS PART OF THE ANTIOXIDATIVE MACHINERY IN PLANTS UNDER STRESS

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ABSTRACT

Ferritins are a superfamily of iron-storage proteins that are found in all living organisms with the exception of yeast. They are ubiquitous, iron-binding proteins that play an important role in cellular iron homoeostasis, storing up to 4,500 iron atoms in their central cavities. In plants, ferritins are preferentially localised in the plastids but are also found in mitochondria. Ferritin expression is developmentally regulated and plays a role in the synthesis of iron-containing proteins that are involved in photosynthesis in the early stages of plant development. It accumulates in seeds during embryo maturation to provide iron during germination, allowing for the proper formation of the photosynthetic apparatus. Ferritins are transcriptionally controlled by iron, which induces the production of reactive oxygen species (ROS) in the Fenton reaction in its free form and thus has a high potential for toxicity. Several reports have shown that the transcription of ferritin in plants may be induced by environmental factors, such as drought, salinity, cold, light intensity,

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pathogen attack, NO and ozone, which then stimulate ROS production. Studies that have analysed other stresses, such as those caused by herbicides and heavy metals, have also shown increased expression levels of the ferritin genes. These reports suggest that ROS induces ferritin transcription and that ferritin plays a protective role by capturing free iron and limiting oxidative damage to the cell. Recent evidence of ferritins effectively participating in the defensive machinery of plants under oxidative stress was obtained from transgenic plants that overexpressed ferritin genes. This review is focused on the protective role of ferritin in plants that are threatened by oxidative damage in response to biotic and abiotic stresses.

Keywords: Ferritin; oxidative stress; iron

1. INTRODUCTION

Laufberger was the first to purify and describe ferritin in 1937 by crystallisation with cadmium salt (Laufberger, 1937). Since then, it has been widely characterised and has become the most widely-studied iron protein after haemoglobin (Arosio et al., 2009). Plant and animal ferritins have very similar structures and are composed of 24 subunits that come together to form spherical protein shells. They are ubiquitous iron-binding proteins that play important roles in cellular iron homoeostasis because these multimeric complexes are able to store up to 4,500 iron atoms in their central cavities (Arosio et al., 2009; Briat et al., 2010a).

Ferritin is present in all living organisms with the exception of yeast. In animals, it is found in the cytoplasm and mitochondria, and the expression of the heavy (H) and light (L) subunits are regulated at the transcriptional and translational levels via interactions between iron responsive elements (IREs) and iron regulatory proteins (IRPs); in addition, both subunits respond to environmental signals (Bottcher et al., 2011; Theil, 2007; Xi et al., 2010). In bacteria, ferritin is located in the cytoplasm and the DNA complex, and its synthesis is controlled at the transcriptional level (Hintze and Theil, 2006). In plants, the expression of ferritin is only controlled at the transcriptional level by cis-acting elements (Wei and Theil, 2000). In both plants and bacteria, ferritin genes respond to different environmental and/or developmental signals (Theil, 2007).

Plant ferritin is encoded by a small gene family and is preferentially located in the plastids but can be also found in mitochondria; it has never been

detected in the cytoplasm (Arnaud et al., 2006; Briat and Lebrun, 1999; Zancani et al., 2004). Ferritin is found in small amounts in the leaves in the early stages of development because its presence is related to the synthesis of iron-containing proteins that are involved in photosynthesis, and larger amounts are found in the seeds during embryo maturation (Bottcher et al., 2011; Ravet et al., 2009). The role of ferritin in seeds is to provide iron during germination because it is essential for the proper formation of the photosynthetic apparatus and thus for autotrophic plant life (Lobreaux and Briat, 1991; Ravet et al., 2009). In *Arabidopsis*, four ferritin genes (*AtFer1*, *AtFer2*, *AtFer3*, and *AtFer4*) have been identified, and it was suggested that three of them (*AtFer1*, *AtFer3*, and *AtFer4*) are preferentially expressed in vegetative tissues, whereas *AtFer2* is seed-specific (Petit et al., 2001). It is also well-known that ferritin expression is developmentally regulated and responds to environmental factors in addition to being transcriptionally controlled by iron (Briat and Lebrun, 1999).

It has long been suggested that the capacity of ferritins to store free iron enables them to protect plant cells against iron toxicity, and these proteins have been included in the plastids for protection against oxidative stress (Briat and Lebrun, 1999). The aim of this review is to describe recent reports that support the role of ferritins in the protection of plant cells against biotic and abiotic-generated oxidative stress.

2. IRON

After aluminium, iron (Fe) is the second most abundant metal in the earth's crust, but its availability depends on soil oxygenation. In aerobic soils, Fe forms insoluble ferric hydroxide complexes, but under anaerobic conditions, such as flooded soils, ferrous iron (Fe^{2+}) concentrations increase and they are freely taken up by plant roots (Majerus et al., 2009).

Fe is an essential element for all plants. It is required for important biological processes, particularly those related to chloroplast development, chlorophyll biosynthesis, and photosynthesis, because of the presence of abundant iron-containing proteins in the photosynthetic apparatus (Long and Merchant, 2008). As a transition element, Fe participates in electron transfer through reversible redox reactions and cycling between Fe^{2+} and Fe^{3+} (Bottcher et al., 2011; Nagajyoti et al., 2010).

In plants, the absence of Fe may result in typical interveinal chlorosis that leads to markedly decreased productivity, but at high levels and in the free

form, Fe is toxic because it enables the production of free oxygen radicals that irreversibly impair cellular structures and damage membranes, DNA and proteins (Donnini et al., 2009). Although rice has adapted to flood conditions, elevated Fe^{2+} may result in Fe toxicity, which is characterised by small brown spots, stunted growth, and limited tillering (Majerus et al., 2009).

Excess Fe generates oxidative stress in plants (Briat et al., 2010b) because free Fe is able to act catalytically via the Haber-Weiss reaction to produce hydroxyl radicals ($^{\bullet}OH$ - Figure 1) that can oxidise organic molecules, leading to serious cellular damage and, ultimately, cell death (Briat and Lebrun, 1999).

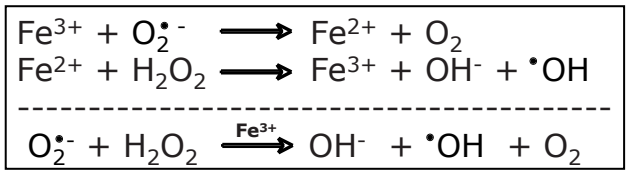

Figure 1. Relevant reactions of the Haber-Weiss reaction.

In addition, the photosynthetic apparatus actively produces the anion superoxide ($O_2^{\bullet-}$), which is a substrate of the Haber-Weiss reaction. Therefore, Fe homeostasis must be strictly controlled to avoid Fe deficiency and toxicity, which are both known to drastically harm the physiology of plants, affecting their growth and development (Briat et al., 2010a).

The study of multiple knock-out *Arabidopsis* mutants that were devoid of ferritin (*fer1-3-4*) confirmed that this protein is involved in protecting plants against oxidative stress (Briat et al., 2010a). The absence of ferritins led to the increased production of reactive oxygen species (ROS) and enhanced activity of several enzymes involved in ROS depletion. In some ways, oxidative stress may be considered to be an imbalance between the production of free radicals and their defence mechanisms.

Previous reports have shown that ferritin gene transcription in plants is able to be induced not only by Fe overload but also by oxidative stress caused by drought, cold, light intensity, pathogen attack, NO and ozone. These reports suggest that ROS play active roles in the induction of ferritin transcription (Briat et al., 2010a; Briat et al., 2010b; Murgia et al., 2001).

Bottcher et al. (2010) studied the effects of the exposure of *Coffea arabica* cell suspension cultures to excess iron. A western blot analysis showed increased levels of ferritin in addition to increased expression levels of *CaFER1* but not *CaFER2*. The increase in ferritin gene expression was

followed by increases in the activities of superoxide dismutase, guaiacol peroxidase, catalase, and glutathione reductase, while the activity of glutathione S-transferase decreased. Altogether, these data suggest that ferritin participates in the coffee antioxidant system to protect cells against the oxidative damage caused by Fe.

3. PHOTOINHIBITION

Photoinhibition is an ordinary phenomenon that occurs during the life cycle of a plant during exposure to excess light and results in reduced activities of the enzymes involved in the Calvin cycle and, consequently, in the deficiencies of reducing agents (Hegedus et al., 2008; Murgia et al., 2001). Large amounts of ROS are produced during photoinhibition and are responsible for damaging some components of the photosynthetic apparatus, such as proteins and pigments (Murgia et al., 2001).

To verify the role of light-induced photoinhibition in the induction of ferritin genes, Murgia et al. (2001) exposed an *Arabidopsis* wt strain and a mutant strain, *vtc1*, which is more sensitive to oxidative stress, to excess light. The *Arabidopsis* wt strain accumulated abundant ferritin transcripts in response to excess light; even at very low irradiation levels (100 μmol photons$\cdot m^{-2}\cdot s^{-1}$), moderate ferritin transcript accumulation was observed. However, the accumulation of ferritin proteins was not observed (Murgia et al., 2001). In the mutant *Arabidopsis*, significant ferritin mRNA accumulation occurred following exposure to excess light, but there was only a small amount of ferritin protein observed. In both cases, ferritin mRNA transcription abruptly decreased after the interruption of the photoinhibitory treatment, indicating that ferritin transcription is dynamically regulated because it expands rapidly when the photosynthetic apparatus is damaged and subsequently decreases (Murgia et al., 2001). These observations are physiologically relevant and the results support the idea that ROS are involved in the accumulation of ferritin.

Murgia et al. (2001) also used two transgenic tobacco lines constitutively overexpressing soybean ferritin to investigate the behaviour of ferritin during light-induced photoinhibition (Murgia et al., 2001). The constitutive overexpression of soybean ferritin in tobacco did not cause the photochemical apparatus to be more tolerant to photoinhibition. In the same report, these authors used cold temperatures to study photoinhibition and observed that

under moderate light, plants that overexpressed ferritin were more affected than control plants, and recovery was also impaired (Murgia et al., 2001). Although consistent decreases in chlorophyll concentrations (approximately 20%) were observed in the mutant tobacco plants compared to the controls, the reason for the increased susceptibilities to photoinhibition of the ferritin-overexpressing plants compared to those of the control remains to be elucidated (Murgia et al., 2001). It is likely that the sequestration of Fe by ferritin affects processes that require free Fe, such as chlorophyll synthesis, chloroplast development and photosystem repair after photoinhibitory stress (Murgia et al., 2002).

The co-occurrence of cold temperatures and high irradiance levels are some of the major oxidative stresses experienced by plants (Hegedus et al., 2008). In contrast to the results obtained by Murgia et al. (2001), two transgenic tobacco lines that overexpressed alfalfa ferritin showed better tolerances to photoinhibition that was induced by low temperatures compared to the control and did not show phenotypic alterations or decreases in chlorophyll concentrations (Hegedus et al., 2008).

The contradictory results obtained by Murgia et al. (2001) and Hegedus et al. (2008) using transgenic tobacco may be due to differences in the way that the treatments were applied. Murgia et al. (2001) used small leaf discs, and Hegedus *et al.* (2008) used intact transgenic plants grown in a growth chamber. Furthermore, the plants were transformed with ferritin from different sources; while Murgia et al. (2001) used soybean ferritin, Hegedus et al. (2008) used alfalfa ferritin. The stress tolerance observed in the transgenic plants overexpressing ferritin may be related to an increased capacity of alfalfa ferritin proteins to sequester Fe, resulting in lower levels of available, intracellular, free Fe and the subsequent inhibition of the Haber-Weiss reaction (Hegedus et al., 2008).

Chlamydomonas reinhardtii, an unicellular green algae, showed decreased levels of ferritin mRNA under conditions of photo-oxidative stress (Long and Merchant, 2008). However, when wild and *ferritin1* knock-down strains were exposed to Fe-deficient and photo-oxidative conditions, ferritin gene expression was higher in the wild type, and the knock-down strains were more susceptible to the photo-oxidative stress (Busch et al., 2008). Low ferritin levels seem to cause cells to be more photosensitive in Fe-deficient conditions, either because the remodelling of the photosystems is not as efficient and/or the free Fe that is released during the remodelling process is not adequately buffered, which allows for ROS to be produced (Busch et al., 2008).

4. WATER DEFICIENCY AND SALINITY

Water deficiency is the most serious environmental stress affecting crop productivity. Under natural conditions, plants are frequently exposed to drought, which often disturbs plant development and metabolism (Clement et al., 2008). However, water shortages may also occur in many different conditions, including altered salinities and low temperatures (García-Mata and Lamattina, 2001).

In the leguminous family, soybean (*Glycine max*) is one of the species that is the most sensitive to drought stress, and wide variations in soil water concentrations have been shown to lead to significant reductions in biological N_2 fixation (Clement et al., 2008). The nodule response to drought was studied by Clement et al. (2008) using soybean and *Bradyrhizobium japonicum* as models. These authors demonstrated that 56 cDNA fragments were induced by drought, including ferritin, which was greatly stimulated in infected cells and vascular bundles, suggesting the relevant trafficking of Fe to stressed nodules. Ferritin accumulation in the nodules in response to drought may be involved in oxidative cellular protection, and increased gene expression may play a role in preventing cellular damage due to the Fe release that was induced by the degradation of leghaemoglobin (Clement et al., 2008).

Fobis-Loisy et al. (1995) carried out drought experiments with maize (*Zea mays*) plantlets and analysed two members of the ferritin gene family (*ZmFer1* and *ZmFer2*). They observed the accumulation of *ZmFer2* mRNA, while *ZmFer1* transcripts were only weakly detected at basal levels. Ferritin also accumulated in response to water stress (Fobis-Loisy et al., 1995).

Approximately 20% of the cultivated land in the world and approximately 50% of all arable lands are affected by salinity (Li et al., 2008). In plants, high salinity causes decreases in productivity and death because excess Na^+ is toxic and can result in both ionic and osmotic stresses, which in turn negatively affect a number of plant processes, including photosynthesis, protein synthesis, and energy and lipid metabolism (Gao et al., 2011; Li et al., 2008). It has been shown that plants exposed to high levels of salt have enhanced antioxidant enzyme activities (Li et al., 2008).

Rice is particularly sensitive to salinity, and adaptation to salt stress involves alterations in gene expression. An analysis of salt-stressed rice showed significant changes in the expression of many genes, including those involved in antioxidant metabolism (Parker et al., 2006). Among them, ferritin transcripts were shown to be approximately 3.5- and 5.2-fold higher after short- and long-term salt stress.

Wheat is an important cereal crop that, like rice, is sensitive to salinity, which can significantly affect growth and grain yield (Gao et al., 2011). A two-dimensional gel electrophoresis of wheat leaf proteins from plants that had been subjected to salt stress showed the increased expression of glutathione S-transferase, an important enzyme in antioxidative stress metabolism. A ferritin protein was also identified as being present in elevated concentrations following salt treatment (Gao et al., 2011). The storage of Fe^{2+} by ferritin during salt stress prevents the formation of $OH^{\bullet}$ as a result of the reaction between Fe^{2+} and H_2O_2 (Parker et al., 2006). Thus, the increased expression of ferritin could help to neutralise damage resulting from the ROS (Gao et al., 2011).

Pyrus pyrifolia plants exhibited high expression levels of all four ferritin genes (*PpFer1*, *PpFer2*, *PpFer3*, *PpFer4*) following salt treatment (200 mM NaCl), showing two peaks at 6 h and 24 h from the beginning of the treatment with the exception of *PpFer2*, which showed no band for the second peak (Xi et al., 2010). *PpFer3* showed the highest expression levels after salt stress.

Li et al. (2008) also investigated the changes in antioxidant enzymes and ferritin accumulation in the leaves of barley (*Hordeum vulgare)* plantlets subjected to salt stress. Additionally, they applied exogenous nitric oxide (NO), which is a signalling molecule that mediates several metabolic processes in plants, including responses to both biotic and abiotic stresses (Li et al., 2008). Some reports have suggested that NO plays an important role in protecting plants from oxidative stress. NO seems to be involved in germination induction, the growth and development of plant tissues, Fe homeostasis, acceleration senescence, and responses to water deficit, salinity, heat stress, disease resistance, and apoptosis (Shi et al., 2005). Depending on the concentration and situation, NO can either be protective or toxic (Shi et al., 2005). The aim of the study that was conducted by Li et al. (2008) was to determine the ability of NO to promote adaptive responses to cope with water stress through the increased activities of antioxidant enzymes and ferritin accumulation. They observed that salt stress induced oxidative stress and that the NO treatment prevented ion leakage and decreased lipid peroxidation. NO seems to be a regulator of ferritin levels in barley, as was also observed by Murgia (2002) in *Arabidopsis*, by controlling mRNA and protein levels. Although the salt treatment enhanced ferritin synthesis, the combined presence of salt stress and NO resulted in a more significant ferritin increase of over

98% compared to the control (Li et al., 2008). Thus, ferritin is involved in antioxidant metabolism and NO may mediate its accumulation in chloroplasts under salt stress (Li et al., 2008).

However, in *Chorispora bungeana*, a typical alpine plant that can survive under frequently fluctuating, freezing temperatures, the presence of exogenous NO resulted in the slightly increased expression of ferritin (*CbFer*) with no obvious effects on NO (Zhang et al., 2009).

5. TEMPERATURE

Temperature is an environmental factor that can affect the distribution of nutrients, growth, and development in plants. Several reports have shown that variations in temperature (high and low) may lead to oxidative stress in plants (Apel and Hirt, 2004).

Following exposure to cold temperatures, the tissues of *C. bungeana* plants exhibit symptoms of oxidative stress (Zhang et al., 2009). The expression patterns of *C. bungeana* ferritin (*CbFer*) were determined at 4°C and –4°C to correlate gene expression with cold tolerance. After 24 h at 4°C, *CbFer* expression was 152% higher compared to the control plants, while at –4°C, the highest expression was observed only 6 h after the onset of treatment, and the expression was 192% greater than that observed in the control (Zhang et al., 2009). Thus, ferritin may play a relevant role in protecting plants against the oxidative damage caused by low temperatures.

As previously mentioned, transgenic tobacco lines overexpressing alfalfa ferritin that are exposed to low temperatures combined with excess light show increased tolerances to oxidative stress (Hegedus et al., 2008).

Low and high temperature variations, however, seem to provoke different responses in ferritin expression levels. *P. pyrifolia* plants that were maintained at 40^{o}C showed elevated expression levels of ferritin genes (Xi et al., 2010). *PpFer1* and *PpFer3* reached the highest levels after 12 h and *PpFer4* after 6 h from the onset of the heat treatment. However, compared to the control, the cold stress treatment did not significantly increase ferritin gene expression. It was suggested that the cold temperatures may have inhibited gene expression directly by impeding metabolic reactions and indirectly by cold-induced osmosis, inhibiting water uptake and cellular hydration (Xi et al., 2010).

6. BIOTIC STRESSES (BACTERIA, FUNGI AND VIRUSES)

Biotic stresses are major limiting factors to plant productivity. ROS are produced by plants when they are invaded by pathogens, such as bacteria, fungi and viruses, and these reactive molecules have been related to signal transduction, the restriction of pathogen invasion, and the induction of plant tissue necrosis (Déak et al., 1999). Plants also display other defences against pathogens; e.g., the decomposition of localised cell wall components and production of pathogenesis-related proteins (Liu et al., 2007).

Seven-week-old transgenic tobacco plants that were inoculated with the tobacco necrosis virus (*Alternaria alternate* or *Botrytis cinerea*) and overproduced an alfalfa ferritin showed reduced numbers of necrotic lesions and smaller diameters of necrotic leaf areas compared to the control (Déak et al., 1999). These results indicate that the transformed plants exhibited an increased tolerance to the necrosis that was caused by both pathogens, and thus, it may be suggested that the ferritin can act as an antioxidant to protect plants against pathogen-induced necrosis (Déak et al., 1999).

García Mata et al. (2001) investigated the impact of deferoxamine (DFO), an Fe-specific chelator that inhibits the Fe-catalysed generation of $OH^{\bullet}$, on the severity of disease caused by *Phytophthora infestans* in potato (*Solanum tuberosum*). It is known that ROS production occurs when the potato is infected by *P. infestans* (García Mata et al., 2001). Ferritin gene expression differed in the leaves compared to the tubers. It is likely that the *P. infestans* infection resulted in different rates of Fe release in the leaves and tubers, explaining the differences in ferritin gene expression (García Mata et al., 2001). Additionally, ROS production was low in the leaves treated with DFO that were inoculated with *P. infestans*. These results suggest that the molecules that regulated the availability of Fe in the cells, such as DFO and ferritin, may play protective roles against the deleterious effects of *P. infestans* in potato (García Mata et al., 2001).

In bacterial infections, plant responses must be tightly regulated in accordance with the disease process and elicitors produced by the invading microorganism. An analysis of *A. thaliana* that was infected with *Erwinia chrysanthemi* or *Pseudomonas syringae* revealed an upregulation of the ferritin *AtFer1* gene within 24 h after the onset of infection, which suggested the possible role of ferritin in plant basal resistance. Moreover, a knock-out mutant for *AtFer1* was more susceptible to infection (Dellagi et al., 2005).

Thus, the increase in gene transcripts and production of ferritin proteins may be considered to be a defensive reaction against the proliferation of the two pathogens (Dellagi et al., 2005).

7. PARAQUAT

Paraquat (Pq) induces the generation of the anion superoxide in the chloroplast, leading to proteolysis, the peroxidation of lipids, and ultimately, cell death (Déak et al., 1999; Van Wuytswinkel et al., 1999).

Damage caused by this herbicide includes the bleaching of leaf tissues. Therefore, it is expected that cells containing high intracellular levels of superoxide dismutase are more resistant to Pq. Free Fe is required for the methylviologen (the active molecule of Pq) to exhibit toxic effects (Stein et al., 2009).

Resistance against cellular damage caused by Pq was tested in the leaf discs of progenies of transgenic tobacco plants that overexpressed an alfalfa ferritin (*MsFer*). It was observed that control leaves completely lost their photosynthetic functions, and the transgenic progenies showed variable degrees of activity (Déak et al., 1999). The application of Pq resulted in decreased chlorophyll concentrations; however, this effect was less prominent in the transgenic plants (Déak et al., 1999).

Tobacco plants overexpressing soybean ferritin were also subjected to Pq treatments. The total protein concentrations of the control plants decreased significantly (63%), while proteolysis was less pronounced in the transgenic plants. Lipid peroxidation increased two-fold in the control plants, but no changes were observed in the transgenic plants. It was presumed that the sequestration of free Fe by ferritin allowed for the greater tolerance because its overexpression resulted in an increased resistance to the methylviologen treatment (Van Wuytswinkel et al., 1999).

The potential protection to oxidative stress that is conferred by *MsFer* was also tested in grapevines. Plants of the rootstock variety *Vitis berlandieri* X *Vitis rupestris* cv. 'Richter 110' that overexpressed *MsFer* showed enhanced tolerances following Pq treatments (Zok et al., 2010). Additionally, *Oryza sativa* seedlings that were simultaneously exposed to Cu and Pq, which are both known to be inducers of oxidative stress, also showed increased expression levels of *OsFER2*, thus limiting cell damage (Stein et al., 2009).

CONCLUDING REMARKS

Plant productivity is strongly influenced by environmental conditions, and different abiotic and biotic stresses limit crop production. Salinity, drought and extreme temperatures are among the most prominent environmental stresses, but other conditions, such as soil contamination with heavy metals, high irradiation levels, and infection by viruses, bacteria or fungi, may also strongly limit plant growth and crop productivity (Atienza et al., 2004; Déak et al., 1999).

Plants that are subjected to different stresses may show similar responses; e.g., frost and dehydration result in osmotic stress and destabilise cellular membranes, low temperatures in the presence of moderate to high light levels result in the excessive excitation of the photosynthetic apparatus, and drought and salinity modify the water availability of soil and may also cause osmotic stress (Atienza et al., 2004). The development of genetic and molecular tools has enabled the identification of a considerable number of plant genes that are related to different stress responses (Atienza et al., 2004). ROS production seems to be a typical response that is elicited by different stresses and provokes a concomitant increase in the production of antioxidative machinery that is composed of antioxidant molecules and ROS scavenging enzymes to keep the ROS at normal levels (Jaspers and Kangasjärvi, 2010).

Ferritin gene expression increases in plants as a result of oxidative stress (Lobreaux and Briat, 1991). Accordingly, several recent reports have described the potential of ferritin to act as a cytoprotective antioxidant, which suggests that in addition to being an important Fe source for plant nutrition and maintaining Fe homeostasis, this protein also plays relevant roles in plant defence against oxidative stress.

The close interaction between Fe homeostasis and ROS has been well-characterised in bacteria and animals, in which the regulation of Fe homeostasis has been shown to be modulated by oxidative stress. This relationship has only recently been proposed as a result of the cumulative evidence that has been gathered from physiological and molecular studies in plants (Ravet et al., 2009). Toxic Fe concentrations increase oxidative stress responses (Donnini et al., 2009). Moreover, pro-oxidants, such as NO, H_2O_2, ozone, water deficits, high light intensities, extreme temperatures, and biotic stresses, also induce ferritin synthesis (Briat et al., 2010b; Déak et al., 1999;

Murgia et al., 2001; Murgia et al., 2002). In addition, these results support the interaction between Fe and ROS production in plants, but a direct relationship has not yet been established (Ravet et al., 2009).

During their life cycles, plants experience numerous stresses that can differ in intensity, location and duration, and induce Fe in its free form, which in turn increases the potential for ROS generation. Thus, the presence of ferritins that buffer excess Fe can prevent such adverse and deleterious effects if no protection system has been activated (Ravet et al., 2009).

The identification of ferritin as part of the plant antioxidant machinery indicates that the development of transgenic plants that overexpress ferritin genes may be an effective way to increase tolerance to biotic and abiotic stresses and influence Fe concentrations in plants (Xi et al., 2010). The further manipulation of ferritin-centred Fe metabolism may provide new tools for interfering with oxidative damage and improving plant stress tolerances to adverse situations (Déak et al., 1999).

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Chapter 4

FERRITIN/FERROXIDASE ACTIVITY: A POTENT INHIBITOR OF VASCULAR CALCIFICATION, OSTEOBLASTIC TRANSDIFFERENTIATION OF VASCULAR SMOOTH MUSCLE CELLS AND OSTEOBLAST ACTIVITY

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ABSTRACT

Vascular calcification plays a role in the pathogenesis of atherosclerosis, diabetes and chronic kidney disease. The mechanism of vascular calcification is not completely understood, but recent evidence implicates factors involved in bone mineralization. In response to elevated inorganic phosphate transdifferentiation of vascular smooth muscle cells (VSMC) into osteoblast-like cells occurs. This process involves increased alkaline phosphatase activity, increased expression of the bone specific transcription factor, core binding factor-1, and the subsequent induction of osteocalcin. Mounting evidence suggests an essential role for ferritin to maintain homeostasis of vascular function. Recently we have found that both exogenous administration of ferritin and upregulation of endogenous ferritin production inhibit calcification and transdifferentiation of VSMC. Ferritin is a multifunctional protein, possessing ferroxidase activity and iron storing ability. In addition, as recently described, ferritin plays a role in transcriptional regulation of certain genes. Testing the inhibitory potential of different ferritins – L-ferritin, H-ferritin and a mutant form of H-ferritin lacking ferroxidase activity – revealed that ferroxidase activity of ferritin is essential to dampen Pi-induced calcification. Moreover, we have found that ceruloplasmin, a distinct protein with ferroxidase activity inhibited calcification and osteoblastic transdifferentiation of VSMC. In addition, we have shown that ferritin – via its ferroxidase activity – inhibits osteoblast activity, leading to decreased mineralization.

These results suggest a novel role of ferritin/ferroxidase activity in inhibiting vascular calcification, VSMC-osteoblast transformation, osteoblast activity and mineralization.

INTRODUCTION

Ferritin

Ferritin is the most ancient molecule of iron homeostasis that carries fundamental functions and new roles arose during evolution. The properties of these molecules have been described in various recent reviews [1-2]. The major role of ferritin is to bind Fe(II), oxidize it, and sequester it in a safe less reactive form inside its cavity and thereby reducing its pro-oxidant activity [3]. This role is linked to the ferroxidase activity, a common feature to all ferritins. Ferritins are made of 24 subunits, which form an almost spherical shell

delimiting a large cavity that can accommodate up to 4,000 Fe atoms as ferric oxide. Mammals have three functional ferritin genes: FTH on human chromosome 11 encodes the cytosolic heavy chain (H-chain) of 183 amino acids, FTL on chromosome 19 encodes the cytosolic light chain (L-chain) of 175 amino acids and the intronless FTMT gene on chromosome 5 that encodes the precursor of the mitochondrial ferritin (FtMt) of 242 residues. The H and L chains co-assemble in different proportions originating a large number of isoferritins ($H_{24}L_0$, $H_{22}L_2$,..H_0L_{24}) with a tissue specific distribution [4]. In contrast, FtMt is specifically taken up by mitochondria, which cleave the N-terminal targeting sequence and produce a mature subunit of a size similar to that of the cytosolic ferritin, which assembles in homopolymers [5]. The 3 subunit types have conserved 3D structures, composed of a 4-helical bundle and a fifth short helix. The ferroxidase site is embedded inside the 4-helical bundle of the H and FtMt chains [6]. The L chain lacks of such site [7]. Ferroxidase activity of ferritin was revealed to prevent cellular damage provoked by reactive oxygen species [8]. This site catalyzes the oxidation of Fe(II) with the production of H_2O_2, which is used as an oxidant for further oxidation of Fe(II). Thus, the ferroxidase activity can consume both reagents of the Fenton reaction: H_2O_2 and Fe(II) [9]. The L chain has no enzymatic activity but its presence even in small proportions (2-4 chains per shell) accelerates the transfer of iron to the iron core and improves the overall iron sequestering process [10]. Consequently the presence of the two subunit-types enhances the functionality of the molecule. In mammals the H and L chains assemble in the proportion determined by the tissue and the cellular development. The H-rich ferritins are found in heart and brain, have higher ferroxidase activity and have a more pronounced anti-oxidant activity, while the L-rich ferritins of spleen and liver are physically more stable, may contain larger amount of iron and a more pronounced iron storage function [4].

Ferritin expression in mammals is regulated by iron mainly at a post-transcriptional level [11]. The mRNAs of H and L chains contain in the 5'UTR an Iron Regulatory Element (IRE), which binds the repressors Iron Responsive Proteins IRP1 and IRP2. The IRPs undergo conformational changes in response to the iron and the redox status of the cell: when iron levels are low the binding affinity is strong and translation is repressed while under high iron availability the IRPs lose RNA binding activity [12-13]. Moreover ferritins respond transcriptionally to oxidant agents with a mechanism that involves an upstream antioxidant responsive element (ARE) present in both ferritin promoters [13-14]. Heme is a strong inducer of the cytosolic ferritin since it

acts both at a transcriptional (via Bach1 binding) and translational level (via IRP2 binding) [15-16], and its effect on ferritin expression is not reduced by the inhibition of heme oxygenase, which releases the heme iron [8].

There are indications that ferritins play functions other than the maintenance of iron homeostasis and are involved in signal transduction. For example it was shown that FTH enhances the granulocyte colony-stimulating factor (G-CSF) induction in hematopoietic cells [17], and that FTH is involved in the signalling pathway induced by the chemokine CXCL12 and its receptor CXCR4 [18]. This seems to be important also in rat primary neurons, where opioids inhibit the CXCR4-mediated survival signaling by inducing FTH and its interaction with the chemokine receptor [19].

Clinical Context and Pathophysiology of Vascular Calcification

Vascular calcification occurs in many pathologic conditions and can lead to devastating clinical consequences. For example, it has been related to increased risk for cardiovascular morbidities and complications such as atherosclerotic plaque burden [20-22], myocardial infarction [23-24], coronary artery disease [25-26], postangioplasty dissection [27], and increased ischemic episodes in peripheral vascular disease [28]. Studies also have indicated that coronary calcification may be predictive of or associated with sudden cardiac death [29-30]. Indeed, coronary calcification score measured by electron beam computed tomography has been shown to have a prognostic value for cardiovascular events comparable to that of the Framingham risk index [30]. Vascular calcification follows three distinct patterns: (1) Intimal calcification is associated with atherosclerosis and leads to focal calcification of atherosclerotic plaques, (2) medial calcification, which is characterized by diffuse calcification of the media, particularly at the level of the internal elastic lamina that does not necessarily accompany atherosclerosis and (3) calciphylaxis which is a cutaneous ischemic small vessel vasculopathy also known as calcific uremic arteriolopathy. These vascular calcification patterns are frequently seen in patients with chronic kidney disease (CKD), who commonly exhibit hyperphosphatemia.

The mechanism of vascular calcification is not completely understood, although recent evidence suggests that during vascular calcification trans-differentiation of VSMC into osteoblast (OB)-like cells occur [31-32]. This notion is strongly supported by demonstration of structures similar to mineralized bone like cartilage, as well as cell types responsible for normal

bone metabolism i.e. OB, chondrocytes and osteoclasts being present in calcified vessels [33-34].

OB is a versatile secretory cell that retains the ability to divide and proliferate. It secretes collagen and ground substance that constitute the initial unmineralized bone. OB expresses specific genes involved in proper differentiation, maturation and bone integrity. Among these, core binding factor alpha-1 (cbfa-1) is a transcription factor that acts as an essential regulator of OB differentiation, bone matrix gene expression, and consequently, bone mineralization [35]. Deficiency of cbfa-1 in *cbfa-1*$^{-/-}$ mice leads to maturational arrest of OB resulting in complete blockade of the intramembranous and endochondral ossification [36]. The mineralization process is initiated by the secretion of small matrix vesicles rich in alkaline phophatase (ALP) into the matrix [37]. ALP is an important enzyme in early osteogenesis since it releases phosphate (Pi) ions from molecules of the matrix and contributes to local elevation of Pi, which results in precipitation and deposition of hydroxyapatite crystals [38].

Many studies have demonstrated the role of high extracellular Pi to induce calcification of vascular cells *in vitro* [39-44] in a process mediated by a sodium-dependent Pi co-transporter that facilitates entry of Pi into vascular cells [44]. Elevated intracellular Pi concentration is accompanied by increased expression of Cbfa-1, the OB specific transcription factor [35]. During vascular calcification there is increased expression of Cbfa-1-regulated gene products such as ALP and osteocalcin that highlights the active nature of VSMC transdifferentiation into OB [45].

Iron and Osteoblast Activity

Several studies have suggested a link between excessive and misplaced iron and secondary decreased bone mass leading to osteoporosis and osteopenia [46-48]. In a study of iliac crest biopsies from 21 individuals with severe osteoporosis (at least one vertebral fracture), iron concentrations of bone (cortical and trabecular) were evaluated. A significant increase in iron content in cortical bone was found in osteoporotic patients' vs. 12 controls [46]. There is a growing body of evidence that suggests excessive iron can play a deleterious role in bone. In genetic hemochromatosis, previous human studies have found Perl's prussian blue staining (which unambiguously identifies iron) in bone trabeculae of patients [47-48]. Accordingly, bone mineral density is decreased in patients with genetic hemochromatosis and

severe iron overload [49] and negatively correlates with hepatic iron concentration (a good index of total body iron overload) and bone mineral density at the femoral neck [50]. There are also a number of animal models that support the damaging effect of iron overload in bone. The first study of iron overload with intramuscular iron dextran was conducted in pigs over 36 days [51]. The main effect of iron overload was a decrease in bone formation, without significant changes in bone resorption. Similarly, osteopenia was induced in Sprague-Dawley rats fed a diet containing iron lactate (5%) for 2 or 4 weeks [52]. Very recently, it has been shown that iron overload causes bone loss in mice via iron-mediated oxidative stress and inflammation [53]. Studies were performed to confirm the close relationship between iron levels and osteogenesis by modulating iron metabolism to the opposite direction by iron chelation. It has been reported that lactoferrin an iron-binding glycoprotein present in epithelial secretions, such as milk, and in the secondary granules of neutrophils is a potent regulator of OB activity and increases bone formation *in vivo* [54-55]. Additionally there is evidence that a green tea iron chelator, epigallocatechin-3-gallate stimulates mineralization of murine bone marrow mesenchymal stem cells [56]. Clinical reports also suggest that several intracorpuscular hemolytic anemias including thalassemia and sickle cell eventually develop hemochromatosis. There is strong relationship between such elevated iron levels and occurrence of osteopenia and osteoporosis in these patients [57-60]. Deferoxamine (DFO) is a powerful chelator of iron, zinc, cobalt and copper and is commonly used to prevent iron overload. Given the overwhelming evidence suggesting an inverse relationship between iron overload and OB activity, it is reasonable to assume that iron chelation would lead to enhanced OB activity and a subsequent reduction in the severity of osteoporosis in these patients. However, some studies suggest that in addition to the growth retardation due to untreated thalassaemia, DFO produces a further negative effect on growth velocity by causing bone dysplasia. The decrease in growth velocity mainly affects the long bones, in particular the distal femoral physis, which normally accounts for 70% of femoral growth. It should be noted that these trace metals are more likely to be chelated in the presence of reduced iron levels. Actually serum zinc levels were below normal limits in 37% of chelated patients in one study [32]. Zinc deficiency is associated with delayed skeletal maturation and a reduction in growth, as well as bone matrix and collagen synthesis. In fact iron chelation with DFO and simultaneous zinc supplementation has been shown to increase growth in some thalassaemic patients. This further verifies that decreased iron levels can have beneficial effect on OB activity.

Iron and Vascular Calcification

There are very few investigations addressing the possible relationship between vascular calcification and iron metabolism. In a study of mineral composition of throatic aortas iron content of calcified and control aortas were determined. A significant decrease in iron content was found in calcified throatic aortas, but the causality was not addressed [61]. Vascular calcification and impaired iron metabolism are common complications in CKD, but the question of whether there is an association between these two pathological events is yet to be elucidated. Iron deficiency in CKD is a consequence of impaired gastrointestinal absorption and blood loss during hemodialysis. Moreover, CKD is frequently associated with functional iron deficiency, a defect of mobilization of iron from iron stores that is a consequence of inflammation and increased hepcidin levels. Iron deficiency leads to anemia in CKD that is frequently corrected by stimulation of erythropoiesis with erythropoietin and iron supplementation (reviewed in[62]).

Accumulation of Pi starts relatively early in kidney disease, but overt hyperphosphatemia does not develop until the later stages of CKD [63]. Clinical, animal, and *in vitro* studies proved that such elevated Pi level is an important inducer of vascular calcification (reviewed in [64]). Cardiovascular events are the major cause of mortality in CKD patients and account for about 50% of all deaths. Analyzing data from hemodialysis patients reveals that the extent of elevated serum Pi is positively correlated with mortality [65-66]. In particular, development of calciphylaxis, which is a syndrome of vascular calcification and skin necrosis, is almost exclusively seen in patients with stage 5 CKD and correlates with extremely high fatality rates (reviewed in [67]).

Ferritin as an Inhibitor of Osteoblast Function

To better understand and explore the molecular mechanism behind iron-mediated inhibition of osteoblast function we examined the process of osteogenesis *in vitro* by culturing human OB in a Pi-rich medium, and in particular studied the effect of iron (Figure 1). Extracellular matrix formation is potently induced by elevated Pi as assessed by measuring Ca content of the matrix. We found that administration of iron inhibits Pi-mediated Ca deposition by OB in a dose responsive manner [68].

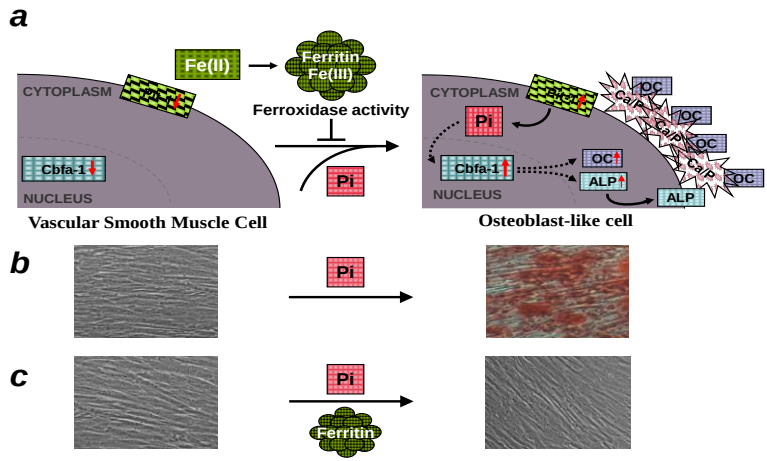

Figure 1.*The inhibitory effect of ferritin on VSMC osteoblastic transdifferentiation. a)* Scheme of Pi-mediated osteoblastic transformation of VSMC. Red arrows represent response to elevated Pi. Elevated Pi increase the expression of Pit-1 resulting in increased uptake and elevated cytosolic Pi concentration followed by upregulation of the bone specific transcription factor Cbfa-1. Subsequently, expressions of Cbfa-1-regulated target genes, e.g. osteocalcin (OC) and alkaline phosphatase (ALP) are increased. ALP and is then secreted in matrix vesicles and initiate mineralization. Extracellular matrix build up of Ca/P and OC. Ferritin via its ferroxidase activity inhibits the main steps of VSMC transdifferentiation into osteoblast type cells. Pi-induced phosphate uptake, upregulation of Pit-1, Cbfa1, ALP and OC expression as well as Ca deposition are all inhibited by ferritin. b) Pi-mediated mineralization of Human Aortic Smooth Muscle Cells (HAoSMC). Ca deposition assessed by alizarin red staining of HAoSMC (100x) cultured under normal or elevated Pi (4 mmol/L) conditions. c) Effect of ferritin on Pi-mediated mineralization of HAoSMC. Representative images of alizarin red staining of HAoSMC (100x) exhibiting elevated ferritin expression cultured under normal or elevated Pi conditions.

Iron is a strong inducer of ferritins (H and L), therefore we asked whether ferritin could be responsible for the inhibitory effect of iron. We have shown that apo-ferritin suppresses Pi-mediated Ca deposition similarly to that of iron [68]. On the other hand, we have found that iron chelation with DFO leads to down-regulation of both ferritin subunits that is accompanied by increased deposition of Ca in the extracellular matrix of OB. These results suggested that there is a linear inverse relationship between intracellular ferritin content of the OB and Ca deposition of the extracellular matrix. Next, we have examined whether such suppression is restricted to inhibition of calcification or OB specific genes are also regulated by iron and/or ferritin.

To initiate mineralization OB are rich in ALP protein and high ALP activity is one of the characteristic features of OB. Elevated Pi levels induce a further increase in both expression and activity of ALP. We found that iron is a strong regulator of ALP leading to an almost complete down-regulation of ALP expression and activity [68]. Intriguingly, the inhibitory effect of iron on ALP expression and activity can be mimicked by apo-ferritin. Osteocalcin is another OB specific protein that is highly expressed in OB cultured in Pi-rich conditions. We showed that iron as well as apo-ferritin suppresses the expression of osteocalcin [68]. Finally we have targeted Cbfa-1, the OB-specific transcription factor. Similarly to that of ALP and osteocalcin, expression of Cbfa-1 is also down-regulated by apo-ferritin [68].

Our study has focused on shedding new light on better understanding the mechanism by which iron causes repression of OB activity. In agreement with previous studies, we confirmed that iron inhibits OB activity *in vitro*. However, our investigations clearly indicated that such inhibition is mainly due to iron induced up-regulation of ferritin (Fig. 1). This notion is supported by the fact that apo-ferritin which contains very little iron, if any, causes a dose responsive decrease of OB gene expression and subsequent calcification.

Ferritin as an Inhibitor of Vascular Calcification

For decades there was a general consensus that vascular calcification is a passive process and simply a consequnce of Ca-Pi precipitation. Based on numerous well designed studies it is now evident that vascular calcification shares similarities with bone mineralization, which led us to investigate whether ferritin solely inhibits OB function or it suppresses the phenotype transition of VSMC into OB-like cells. One of the triggers of such transdifferentiation is elevated level of Pi, that has been shown to induce calcification *in vitro* [39-43] and *in vivo* (reviewed in [69]). We used this Pi-mediated *in vitro* vascular calcification model to test whether iron or ferritin can inhibit this process. We found that iron – via the induction of ferritin – inhibits Pi-induced calcium deposition of VSMC [70]. Transdifferentiation of VSMC into OB-like cells is accompanied by up-regulation of certain genes characteristics for OB such as ALP, osteocalcin and Cbfa-1. We have shown that ferritin potently inhibits the Pi-induced up-regulation of these OB-specific genes [70] (Figure 1).

The Central Role of Ferroxidase Activity in Inhibiting Vascular Calcification and Osteoblast Function

Ferritin shell made of 24 polypeptide chains of 2 types H and L subunits. The H subunit carries a ferroxidase activity that promotes iron incorporation, and oxidizes Fe(II) into Fe(III). The L subunit is associated with iron nucleation and long-term iron storage, but does not possess ferroxidase activity. We sought to investigate whether one or both of the subunits are responsible for the inhibitory effect of apoferritin on OB activity and VSMC osteoblastic transdifferentiation. Therefore, we examined the effect of the single subunits, i.e. H and L on OB function and on Pi- mediated VSMC transdifferentiation. We found that inhibitory effect was possessed almost exclusively by the H subunit whereas L subunit had minimal effect on OB function (68) or VSMC transdifferentiation [70].

To further examine if ferroxidase activity of the H subunit could be responsible for the calcification inhibition we tested a recombinant mutant form of H ferritin lacking ferroxidase activity. We found that neither OB function nor Pi-mediated osteoblastic transformation of VSMC was affected by the mutant H ferritin [68, 70]. As an alternative approach to demonstrate the central role of ferroxidase activity behind the observed inhibition we have tested whether an unrelated protein possessing ferroxidase activity, namely ceruloplasmin, can suppress OB activity and/or VSMC osteoblastic transformation. We found that ceruloplasmin mimicked the effect of H ferritin, suggesting that ferroxidase activity plays a major role in inhibiting OB activity [68] and in the transdifferentiation of VSMC into OB-like cells [70].

CONCLUSION

In this chapter we described a novel function of ferritin, the ancient molecule of iron homeostasis. We found that ferritin inhibits OB function as well as transdifferentiation of VSMC into OB-like cells. The mechanism via which ferritin exerts its effect is not completely understood at this point, but our results suggest that ferroxidase activity plays a crucial role in the inhibition.

The contribution of reactive oxygen species to vascular calcification has recently emerged. It has been shown that ROS production is increased around calcifying foci in human sclerotic aortic valve [71]. Moreover it was recently

described that mitochondrial ROS-mediated p65 nuclear translocation is involved in Pi-induced vascular calcification [72]. In contrast, antioxidant properties of ferritin were first described by Balla et al [8]. Ferroxidase center of H-ferritin catalyzes the oxidation of Fe(II) with the concomitant production of H_2O_2. The formed hydrogen peroxide then is used as an oxidant for further oxidation of Fe(II).Thus, the ferroxidase activity can consume both reagents of the Fenton reaction: H_2O_2 and Fe(II). This could serve as a plausible explanation with regards to the inhibitory effect of ferritin on vascular calcification that remains to be confirmed.

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Chapter 5

THE ANTIOXIDANT ROLE OF FERRITIN AND TRANSFERRIN IN ELITE ATHLETES

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ABSTRACT

Physical activity has been shown to increase the production of free radicals to a point that can exceed internal protective antioxidant system. The harmful effects of free radicals are neutralized by activity of antioxidant enzymes and numerous non-enzymatic antioxidants, including vitamins, glutathione, ubiquinone and flavonoids. Defence mechanisms against free radical-mediated oxidative damage also include the iron binding proteins such as transferrin and ferritin. Ferritin and trensferrin are able to restrict the availability of iron to participate in conversion of hydrogen peroxide to toxic hydroxyl radicals by Fenton

reaction. Because the endogenous antioxidant system may not be sufficient to prevent exercise-induced free radical generation and consequent oxidative damage, supplementation of antioxidants may have important effect antioxidant status in athletes. The aim of the present work was to examine the association of physiological iron carriers with oxidative stress in elite athletes. One hundred and five elite athletes were divided into two groups: supplemented group which consisted of sixty-five athletes who regularly use antioxidant supplements and control group which consisted of forty athletes that were without antioxidants intake. Following parameters were measured: albumin, ferritin, transferrin, advanced oxidation protein products (AOPP), lipid hydroperoxides (LOOH), biological antioxidative potential (BAP), superoxide dismutase (SOD) and total sulphydryl group concentration (SH groups). Multivariate analysis of covariance (MANCOVA, Wilks' lambda) was performed to test the hypotheses that supplementation (fixed factor) and antioxidative proteins (indicated via albumin, ferritin and transferrin) (covariates) have a significant effect on the oxidative stress parameters (dependent variables). The SOD activity (P=0.024) and sulphydryl group concentration (P=0.042) were significantly higher in supplemented athletes. Multivariate analysis of covariance indicated ferritin (P<0.001) and transferrin (P=0.001) as significant covariates, which have contributed 37.1 % and 22.8% to variability of oxidative stres parameters, respectively. The transferrin exhibited linear relationships with LOOH (R^2 = 0.127; P < 0.001) and AOPP (R^2 = 0.113; P < 0.001) while ferritin exhibited non-linear (logarithmic) relationships with this parameters (AOPP: R^2 = 0.201, y = 82.3 -15.9log(x), P<0.001; LOOH: R^2 = 0.256, y = 193 -33.3log(x), P<0.001). In conclusion, proteins that regulate iron transport and storage, transferrin and ferritin, were negatively related with oxidative damage in professional athletes regardless of antioxidant supplementation.

Keywords: free radicals, ferritin, transferrin, advanced oxidation protein products, lipid hydroperoxides

INTRODUCTION

Because of elevated metabolic rate associated with exercise, elite athletes often come to a condition leading to increased oxidative stress. Free radicals generated during exercise originate from the mitochondrial respiratory chain, xanthine oxidase-catalysed reactions and neutrophil activation (1). Although iron is essential for heme synthesis, metabolic processes and the function of many enzymes, an excess of free ferrous ions can be toxic for cellular systems.

The toxicity of iron is a explained by its participation in Fenton and Haber-Weiss reactions. In these reactions H_2O_2 is reduced by ferrous ion to hydroxyl radical and iron is oxidized to ferric ion. Hydroxyl radical produced in iron-mediated process is one of the most dangerous oxidizing species in biological systems and it can directly damage various molecules (2,3). The detectable consequences of increased free radical generation are the oxidative modifications of lipids, proteins and DNA. Lipid hydroperoxides are markers of the initial reaction of free radicals and they measure the rate of peroxidation of the membrane. Oxidatively damaged proteins can be assessed as advanced oxidation protein products or carbonyl contents (4). The prolonged exposure to reactive oxygen species, wich is comon in elite athletes, can lead to oxidative stress-related injury and health threatening state (5). Antioxidative defense enzymes (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic antioxidants (vitamin C, vitamin E, retinol and reduced glutathione) are in charged to keep an equilibrium between life-supporting oxygen utilization and the deleterious effects of reactive oxygen species (ROS) (6). Antioxidative defense system also include the iron binding proteins, such as ferritin and transferrin, which can reduce lipid peroxidation, oxidative damage of DNA and proteins.

Many proteins possess antioxidant features and contribute to plasma antioxidant capacity. Albumin with its thiol groups, bilirubin and uric acid are nonspecific chain-breaking antioxidants and they are responsible for the major part of the total ability of plasma to trap free radicals (7). Albumin and caeruloplasmin reduce reactive oxygen species generation by Fenton's reaction because they participate in copper transport (8,9). Transferrin is iron-binding glycoprotein, which is mainly synthesized in the liver and its primary function is iron transport and sequestration. It consists of two structurally similar domains and each domain contains a single iron-binding site (10,11). The most of the iron in the plasma is bound to transferrin. Considering that free radical-induced damage involve catalytically active iron ions, antioxidative role of transferrin is based on its capability to make iron unavailable for this kind of reaction in plasma (12). Iron transport to cells is achieved by transferrin receptor-mediated endocytic pathway (13) (Figure 1). Once inside the cell, iron is available for utilization which depends of type of the cell. The largest amounts of iron are required in bone marrow for hemoglobin synthesis. Also, iron plays significan role in many cellular metabolic pathways and enzymatic reactions in cytoplasm and mitochondria. The excess intracellular iron is sequestered in ferritin, its major intracellular pool.

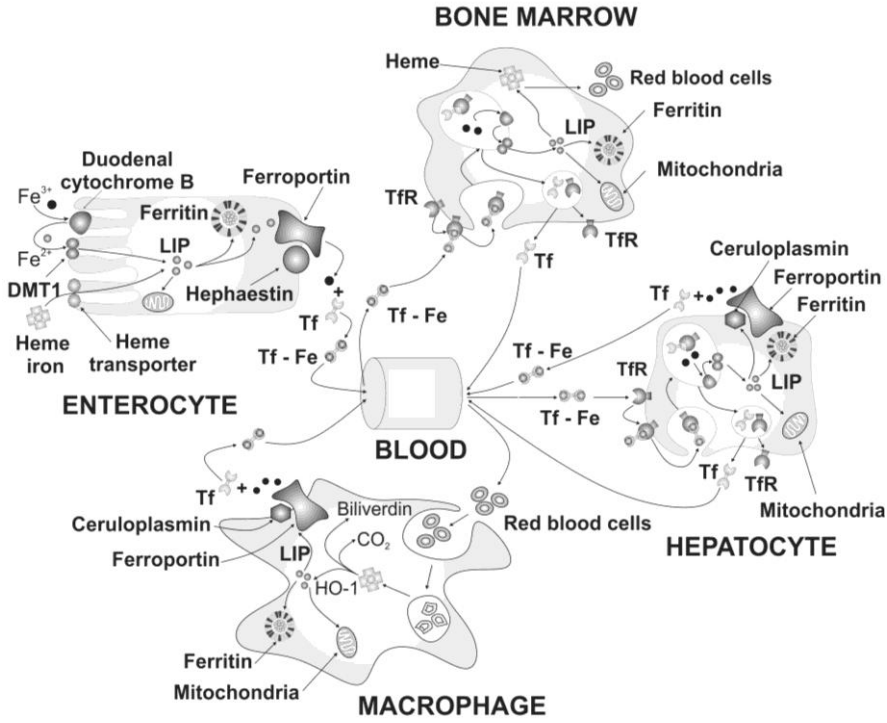

Figure 1. Iron absorption, transport and storage. Iron absorption occurs via enterocytes in the small intestine. An enzymatic ferric reductase on apical surfaces of enterocytes (Duodenal cytochrome B) reduces Fe^{3+} to Fe^{2+}. Ferrous ions are further transported across membrane into the cytoplasm by protein divalent metal transporter 1 (DMT1). The majority of absorbed iron from enterocytes is transported via ferroportin to plasma. A membrane ferroxidase hephaestin convert Fe^{2+} to Fe^{3+} prior to the transport of iron outside the cell. When bound to transferrin (Tf), Fe^{3+} is available for utilization in other cells. Iron-saturated transferrin (Tf–Fe) enters cells via the transferrin receptor (TfR). Transferrin-transferrin receptor complex undergoes endocytosis, allowing iron to be released from transferrin. In the cell, iron is incorporated into ferritin, enzymes, mitochondria and some amount of iron is in cytoplasm as labile iron pool (LIP). The empty transferrin and transferrin receptors are returned to the cell's surface. By analogy to enterocytes, iron release from macrophages and hepatocytes accros their plasma membrane occurs via ferroportin and it is accompanied by Fe^{2+} oxidation to Fe^{3+} by ceruloplasmin and followed by transferrin loading.

Ferritin is protein consisted of 24 subunits of the heavy (H) and light (L) type (14). In order to store iron into the ferritin core, catalytically active H subunit induces oxidation of ferrous (Fe^{2+}) to ferric iron (Fe^{3+}) whereas the L

subunit plays a role in iron nucleation, mineralization and long term storage (15,16,17). (Figure 2). Ferritin capacity to prevent intracellular iron-mediated oxidative damage to biomoleculs is based on H subunit ferroxidase activity and iron removal from cytoplasm (18,19,20). However, some amount of iron is present in cytoplasm as cellular labile iron pool (LIP) whose transient rise can lead to cellular protein oxidation (21).

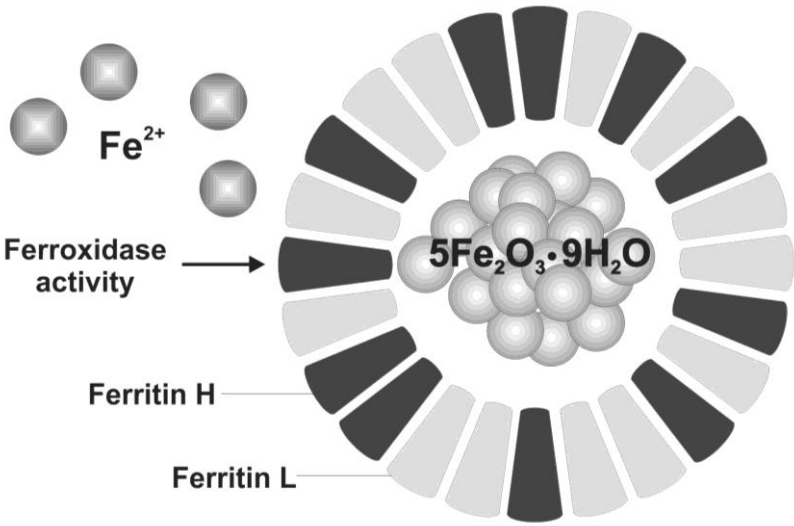

Figure 2. Ferritin structure. Ferritin is heteropolymer composed of H and L subunits and mineral core. H subunit is catalytically active and responsible for oxidation of ferrous (Fe^{2+}) to ferric iron (Fe^{3+}).

In many occasions, the increase in reactive oxygen species during exercise might overwhelm endogenous antioxidant defense and result in oxidative damage. Several human studies showed that supplementation with antioxidants can be helpful, especially in athletes at elite performance level (22,23). Iron depletion given through ferritin decrease is also common in elite athletes (24). The mechanism underlying iron depletion in athletes involve a number of factors including hemolysis, hematuria, sweating and gastrointestinal bleeding (25). As exercise is accompanied with aforementioned conditions and considering the antioxidative role of proteins that regulate iron transport and storage, we attempted to determine the association of physiological iron carriers with oxidative stress in supplemented and nonsupplemented elite athletes.

METHODS

A total of 105 athletes (male: 11 karate professionals, 10 wrestlers, 8 kick boxers, 7 rowers, 6 triathletes; female: 30 volleyball players, 13 water-polo players, 9 karate professionals, 11 swimmers) were admitted into the study. All athletes attended routine health checks and gave written informed consent to participate in the study. Any individual having suspect pathological findings during physical examination, recent history of disease or injuries, altitude exposure or intake of iron supplements or other medications were excluded. Two days prior to study participation all participants refrained from strenuous physical training. The athletes were divided into two groups: supplemented group which consisted of sixty-five athletes who regularly use antioxidant supplements and control group which consisted of forty athletes that were without antioxidants intake. A supplemented group received a two antioxidants: vitamin E (300 mg/day) and vitamin C (200 mg/day) for six weeks prior blood sampling. In both groups the approximate number of female and male athletes was included (Male: supplemented n=24, without supplementation n=18, Female: supplemented n=41, without supplementation n=22). All study procedures were in accordance with the Helsinki declaration and were approved by the Faculty of Pharmacy Ethics Committee for Clinical Trails (University of Belgrade, Belgrade, Serbia).

Blood sampling took place under standard conditions between 7 and 8 am after a 12-hour overnight fast. Blood samples were transported and stored in the laboratory where analyses were performed strictly following international guidelines (26). Transferrin (g/L) was analysed using a Behring Nephelometer (Dade-Behring, Marburg, Germany) (the intra-assay CV was 1.3% and the inter-assay CV was 1.9% for transferrin). Serum ferritin (μg/L) (intra-assay CV was 2.7% and the inter-assay CV was 5.0%) was determined using an Access 2 Immunoassay System (Beckman-Coulter Inc, Chaska, MN, USA). Albumin concentration was measured with an automatic biochemistry analyzer (BT 2000 Analyzer, Biotehnica, Milan, Italy) equipped with reagents purchased from bioMerieux (Marcy l'Etoile, France). The following oxidative stress parameters were measured: advanced oxidation protein products (AOPP), lipid hydroperoxides (LOOH), biological antioxidative potential (BAP), superoxide dismutase (SOD) and total sulphydryl group concentration (SH groups).

The AOPPs were spectrophotometrically detected at 340 nm and expressed as chloramine-T equivalents (μmol/L) (the intra-assay CV was 4.6% and the inter-assay CV was 5.7%) (27). LOOH were quantitated by the xylenol

orange method (the intra-assay CV was 3.5 % and the inter-assay CV was 5.8%) (28). The BAP test provides an estimation of the global antioxidant capacity of blood plasma, measured as its reducing potential against ferric ion. The BAP test is based on the ability of a coloured solution containing ferric ions bound to a chromogenic substrate (a thiocyanate derivate) to decolour when its ferric ions are reduced to ferrous after adding herarinised plasma. Solution discolouration was detected spectrophotometrically at 505 nm and is directly proportional to the concentration of all substances able to reduce ferric ion (expressed in μmol/L). For the BAP intra-assay coefficient of variation was 5.8% and the inter-assay CVs was 6.1%. Plasma SOD activity (U/L) was measured according to a previously published method (29). One unit of SOD activity is defined as the activity that inhibits the auto-oxidation of adrenalin by 50% (the intra-assay CV was 4.1% and the inter-assay CV was 6.2%). The concentration of sulphydryl groups (SH groups, mmol/L) in plasma was determined using 0.2 mmol/L 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) reported by Ellmann (the intra-assay CV was 3.5% and the interassay CV was 5.5%) (30). Measurements were performed in duplicate and the results were averaged. For oxidative stress parameters quality control was provided by using quality control samples (pooled plasma).

Statistics

Kolmogorov–Smirnov tests were employed to determine if the distribution of the variables was normal and equality of variances was controlled by Levene's test. The AOPPs and ferritin were not normally distributed ($P < 0.05$), so logarithmic transformations were performed for both of these variables. The transformed values were used in all subsequent analyses. Student t-test was empoloyed to determine whether there was a statistically significant difference between supplemented athletes and controls. The X^2-test was used to compare distributions of males and females in the two experimental groups, supplemented athletes and controls. To determine whether there was a statistically significant difference between supplemented athletes and controls we used the general linear model analysis of variance.

Multivariate analysis of covariance (MANCOVA, Wilks' lambda) was performed to test the hypotheses that supplementation (fixed factor) and antioxidative proteins (indicated via albumin, ferritin and transferrin) (covariates) have a significant effect on the normally distributed oxidative stress parameters (dependent variables). Univariate ANCOVA was then

performed for each of the individual parameters. Partial eta-squared (η^2) values, which describe the proportion of variability attributable to a factor, were included to provide an intuitive measure of effect size. Regression was performed to evaluate the linear relationships among covariates and oxidative stress parameters.

RESULTS

Oxidative stress parameters, antioxidative proteins and anthropomorphological characteristics of the athletes are shown in Table 1. Student t-test indicated that SOD activity (P=0.024) and sulphydryl group concentration (P=0.042) were significantly higher in supplemented athletes.

Table 1. Oxidative stress parameters, antioxidative proteins and anthropomorphological characteristics of the athletes (male and female).

Parameter	Supplemented (n=65)	Control group (n=40)	All athletes (n=105)
Age, years	22.4 ± 2.8	21.50 ± 2.3	21.9 ± 2.5
Weight, kg	66.2 ± 4.5	69.5 ± 3.7	67.8 ± 3.1
Height, cm	177.1 ± 5.6	176.5 ± 4.2	176.8 ± 4.7
BAP, μmol/L	2416 ± 318	2358 ± 287	2394 ± 307
SOD, IU/L	112.8 ± 34.7[a]	87.1 ± 37.1	102.7 ± 33.6
SH groups, μmol/L	0.544 ± 0.085[a]	0.501 ± 0.101	0.527 ± 0.097
AOPP, μmol/L	1.26 ± 0.33	1.33 ± 0.31	1.29 ± 0.36
LOOH, μmol/L	120 ± 49	125 ± 46	122 ± 48
Albumin, g/L	45.4 ± 3.2	44.8 ± 3.3	45.2 ± 3.2
Ferritin, μg/L	1.39 ± 0.34	1.44 ± 0.27	1.41 ± 0.36
Transferrin, g/L	2.4 ± 0.5	2.2 ± 0.5	2.3 ± 0.53

Values are given as mean ± SD.

When we tested the distribution of supplemented athletes and controls according to gender, no significant differences between male and female athletes were found. (Figure 3).

MANCOVA revealed that supplementation (p=0.009), transferrin (p=0.001) and ferritin (p<0.001) were significant covariates (Table 2). Based upon η^2 values, gender accounted for the largest proportion of variability for

all oxidative stress parameters (31.7%). ANCOVA, however, found that, in addition to the covariates mentioned above, albumin was significant for BAP (p =0.047). Transferrin and ferritin provided negative model coefficients (*B*) and this equated to a negative relationship between covariates and oxidative stress parameters. The supplemetation-negative model coefficient was equated to higher SOD values in supplemented athletes relative to controls. The positive model coefficient for the albumin implied a positive relationship between this covariate and dependent variable (BAP).

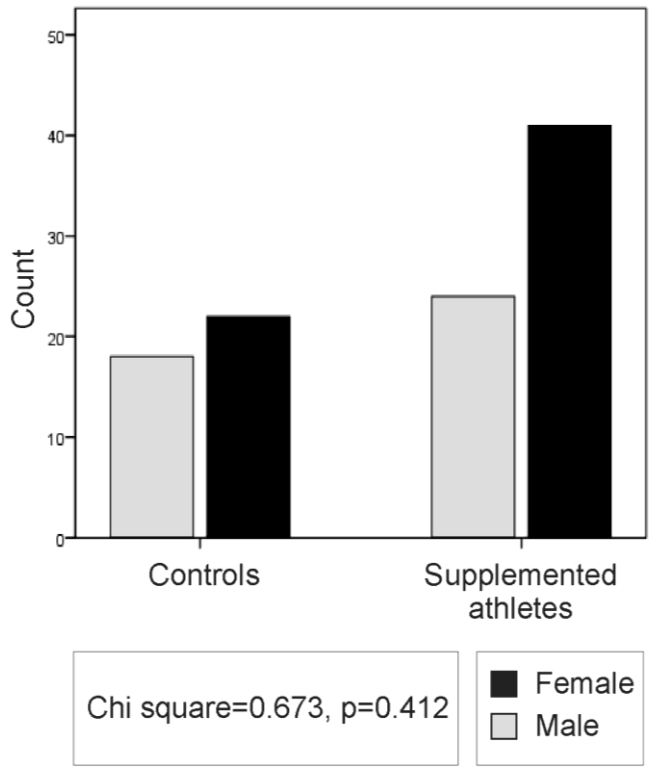

Figure 3. Males and females distribution in controls and supplemented athletes.

Linear regression analysis indicate that SOD activity showed a linear relationship with transferrin, but only in supplemented athletes (R^2=0.161, p=0.001).

Table 2. Multivariate, univariate and parameter estimates results with the effect size.

Multivariate					
Effect		Wilks' Lambda	F	Partial η2	p
Albumin, g/L		0.917	1.515	0.083	0.194
Ferritin, µg/L		0.683	7.795	0.317	<0.001
Transferrin, g/L		0.772	4.95	0.228	0.001
Supplementation		0.837	3.281	0.163	0.009
Univariate - parameter estimates					
Dependent variable	Parameter	B	Observed Power	Partial η2	p
BAP, µmol/L	Albumin, g/L	23.917	0.514	0.044	0.047
SOD, U/L	Transferrin, g/L	-19.124	0.564	0.050	0.035
	Supplementation	-34.595	0.972	0.149	<0.001
AOPP, µmol/L	Ferritin, µg/L	-0.475	1.000	0.256	<0.001
	Transferrin, g/L	-0.290	0.996	0.197	<0.001
LOOH, µmol/L	Ferritin, µg/L	-68.267	1.000	0.276	<0.001
	Transferrin, g/L	-28.648	0.914	0.114	0.001

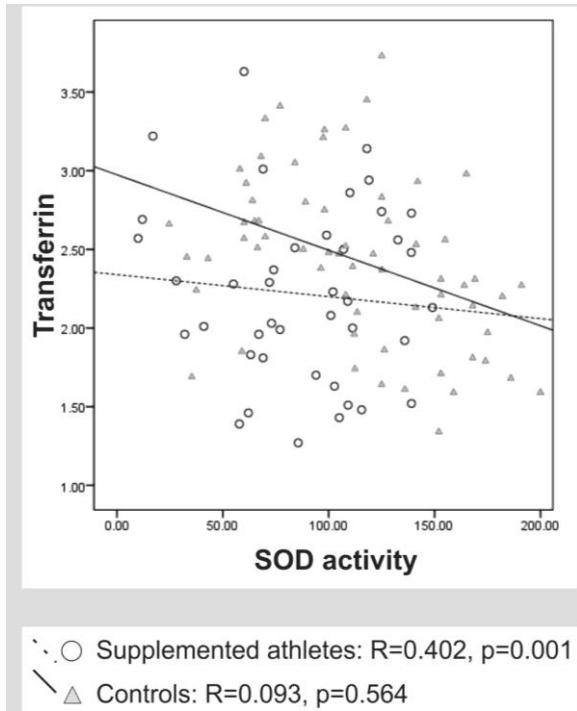

Figure 4. The transferrin exhibited linear relationships with SOD activity but only in supplemented athletes.

Figure 5 indicates bivariate scatter plots of LOOH and AOPPs *versus* significant covariates, ferritin and transferin. The transferrin exhibited linear relationships with LOOH (R^2 = 0.127; p < 0.001) and AOPP (R^2 = 0.113; p < 0.001) while ferritin exhibited non-linear (logarithmic) relationships with this parameters (AOPP: R^2=0.201, y=82.3 -15.9 log(x), p<0.001; LOOH: R^2 = 0.256, y = 193 -33.3log(x), P<0.001).

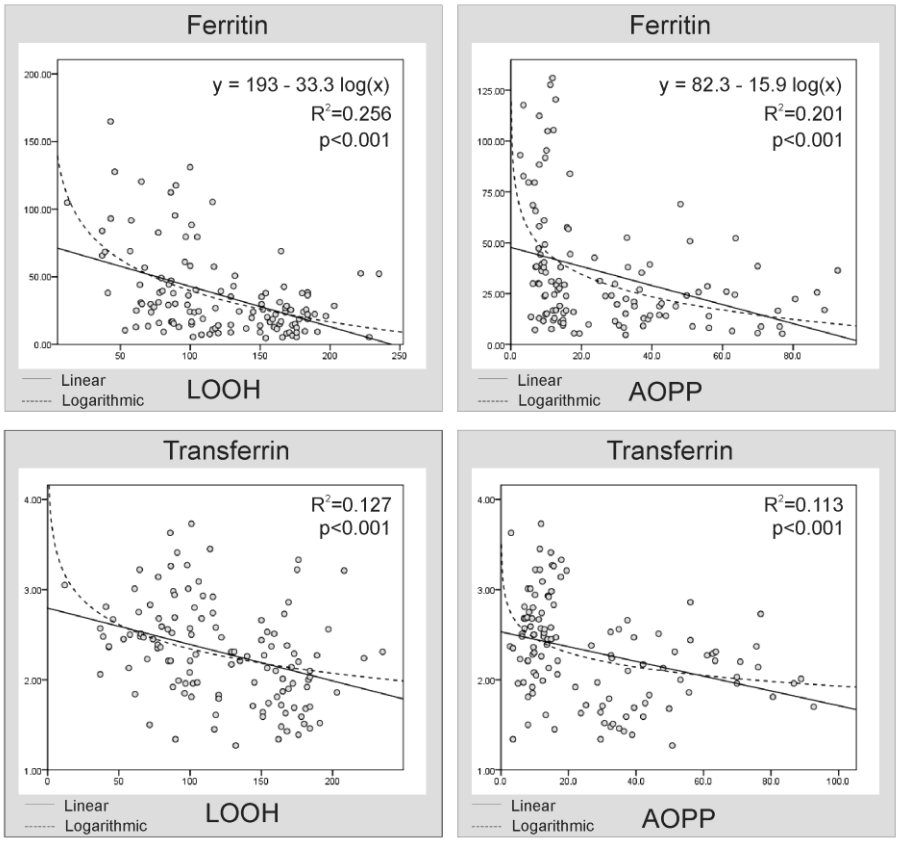

Figure 5. Bivariate scatter plots of LOOH and AOPPs *versus* ferritin and transferrin.

DISCUSSION

Vitamin C is plays important antioxidant role in human plasma and protects against lipid peroxidation (31). However, the vitamin C has other

biological functions some of them are related to its ability to reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron (32). Antioxidant substances can exhibit a synergistic effect, especially vitamin E and C. Vitamin E is the major lipid-soluble antioxidant in cell membranes, and it protects against lipid peroxidation by reacting with a variety of oxygen radicals. The consequence of this reaction is the formation of a tocopherol radical, which can be reduced by vitamin C (33,34).

In present work, we aimed to investigate whether oxidative stress in athletes, in addition to antioxidant supplements use, depended on proteins that regulate iron transport and storage. In previously published articles a relatively high incidence of low antioxidant dietary intakes and exercise-induced modifications on the endogenous antioxidant system has been reported in elite athletes (35,36). Also, it was demonstrate that antioxidant supplements provide protection against the negative health consequences of free radicals produced during exercise. Several reports have suggested the enhancement in antioxidant enzyme activity of superoxide dismutase after antioxidant supplementation and our finding is consistent with this studies (37,38). The athletes who received antioxidant had enhanced enzymatic antioxidant defense expressed via higher superoxide-dismutase activity comared to control group. Also, the higher superoxide-dismutase activity in supplemented athletes was accompanied by higher sulphydryl group content. This leads us to suppose that interaction of enzymatic and nonenzymatic antioxidant defense with dietary antioxidants could contribute to a better oxidative status of athletes and superoxide-dismutase activity. Supplementation contributed 16.3% to variability of oxidative stress parameters with the largest single influence in superoxide-dismutase activity (14.9%)

Although significant, the impact of transferrin on 5.0 % of superoxide-dismutase variability suggests that iron binding protein that scavenges free iron from the circulation have a relatively modest influence on enzymatic antioxidant defense. That means that even when significant changes in the plasma transferrin level occur, superoxide-dismutase activity will not change more than five percent. It is noteworthy that rerelationship between transferrin and superoxide-dismutase activity was linear and significant only in supplemented athletes (Figure 4). The same applies for albumin BAP, which accounted for only 4.4% of variability for biological antioxidative potential. It is also noteworthy, that BAP test provides an estimation of nonenzymatic category of antioxidant defenses that includes ascorbate, glutathione, α-tocopherol, bilirubin and uric acid. Lack of significant differences in BAP

values bettwen supplemented athletes and controls indicated that applied supplements were already utilized in free radical scavenge.

Almost all iron is sequestered by proteins and in plasma is it bound to transferrin. Transferrin has proved to be a significant negatively related factor to oxidative stress in general (22.8 %). In both supplemented athletes and controls lower serum transferrin was associated with increased LOOH and AOPPs and this relationship was linear. The 19.7 % of AOPP variability was explained by changes in the transferrin concentration. The accumulation of AOPP may accelerate inflammatory mediators release through enhanced oxidative stress and trigger activation of oxidative "bursts" in neutrophils, monocytes and T-lymphocytes (39). This correlation between transferrin and advanced oxidation protein products confirms the importance of maintaining the normal level of bound iron in plasma in order to protect proteins from oxidative damage. The 11.4 % of changes in LOOH were caused by different transferrin level. Our earlier research showed that a reduction in transferrin saturation levels is linked to lipid peroxidation (40). Lipid hydroperoxides are primary oxidation products and their measurement allow us to detect molecular reorganization of polyunsaturated fatty acids during the initial phase of lipid peroxidation. At physiological pH iron is bound with high affinity to transferrin and we assume that exercise-induced acidosis can cause a mild iron dissociation from its plasma carrier. We suppose that amount of dissociated iron could be enough to start a chain reaction and subsequent lipid oxidation (41).

In previously published work it was show that gender-dependent differences in ferritin could cause different levels of oxidative stress in male and female athletes (42). Due to the aforementioned, supplemented athletes and controls were equally distributed according to gender in both experimental groups. Ferritin accounted for the largest proportion of variability for all measured oxidative stress parameters (31.7 %). Ferritin was significant covariate at the general level but also it was found to be a significant negatively related factor for AOPP and LOOH. A negative correlation was expected, considering that ferritin does not only account for cellular iron storage, but also contribute to antioxidative defense. In both, controls and supplemented athletes, this relationship was strong but non-linear (logarithmic). That practicaly means that decrease in ferritin level for one unit lead to approximately tenfold increase in AOPP or LOOH. Ferritin is in equilibrium with a labile iron pool (LIP) which represents a cytosolic fraction of redox-active intracellular iron and could well prove to be a key player in iron-induced oxidative stress (43,44,45). It

was suggested that depletion of LIP by cytosolic increase ferritin could be mechanism that protects cellular structures against oxidative damage and negative correlation between ferritin level and LOOH and AOPP is consistent previous findings (46). The R^2 values for ferritin and transferrin associations with LOOH and AOPP implicate that this relationships have a predictive significance.

In light of the findings of this study, we could conclude that supplementation with antioxidants could contribute to a higher total -SH groups content in plasma and superoxide-dismutase activity in elite athletes. Proteins that regulate iron transport and storage, transferrin and ferritin, were negatively related with oxidative damage in professional athletes regardless of antioxidant supplementation.

ACKNOWLEDGMENTS

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Chapter 6

FERRITIN, MORBID OBESITY AND BARIATRIC SURGERY

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ABSTRACT

The proposal for this chapter is, first, to discuss iron metabolism disorder as a risk factor for obesity-related diseases and what increased serum ferritin means in obese individuals. Later, the effect of surgery on dietary iron absorption, the high prevalence of anemia among operated patients and the impact of the procedure on serum ferritin will be discussed. Ferritin is a nonspecific marker of a great number of disorders. One of the main causes for the increase of serum ferritin is non-alcoholic fatty liver disease (NAFLD). NAFLD is manifested mainly in obese, type-2 diabetic and dyslipidemic individuals. Increased serum ferritin concentration is associated with increased blood pressure, hyperglycemia,

hyperinsulinemia and hypercholesterolemia, representing a marker of poor prognosis in acute stroke and coronary artery disease. High ferritin is associated with the metabolic syndrome and reflects the inflammation process highlighted in fat tissue metabolism, atherosclerosis and diabetes. In this context, ferritin can be involved as a pro-oxidant factor. Increased ferritin can contribute to NAFLD pathogenesis and associated diseases, but the hypothesis that its increase is only a marker of the ongoing inflammatory process cannot be discarded. Retrospective data on the prevalence of anemia and behavior of ferritin before and after bariatric surgery in operated morbidly obese patients will also be presented.

INTRODUCTION

Iron metabolism disorders, characterized by high ferritin and low serum iron, is a common finding in obese patients. The cause is generally attributed to an inflammatory process of moderate intensity, frequently associated with obesity and its comorbidities, involving a functional iron deficiency that can lead to what is known as anemia of chronic disease. If, on the one hand, surgery for controlling obesity reduces inflammation, consequently reducing serum ferritin and increasing serum iron, on the other hand, surgery can lead to restricted dietary iron intake and absorption. The present chapter reviews the prevalence, causes and consequences of high serum ferritin in obese individuals and the effects of bariatric surgery on iron metabolism.

FERRITIN, INFLAMMATION AND OBESITY

Roughly 30% of organic iron is stored in the form of ferritin and the other part is found in heme compounds (Ausk et al., 2008). Serum ferritin concentration does not always reflect the organic stores since the level of this protein increases during inflammatory processes, acting like a nonspecific marker for various disorders (Ausk et al., 2008, Wang et al., 2010). Extracellular ferritin acts like an inflammatory signaling molecule capable of increasing the expression of inflammatory mediators (Runddell et al., 2009, Brien-Ladner et al., 2000).

In obesity, reduction of serum iron and moderate elevation of inflammatory indicators is a very common fact (Tussing-Humphreys et al., 2011). Iron deficiency found in obese individuals has been explained by inadequate intake, reduced absorption secondary to high hepcidin expression,

which regulates iron absorption, and reduced bioavailability of the mineral secondary to inflammation (Zafon et al., 2010).

Epidemiological studies have found a positive association between serum ferritin concentration and overweight and obesity (Broderstad et al., 2011). This usually happens more frequently in males than in females (Emst et al. 2009, Moraes et al., 2011). Men are more susceptible to liver damage (Morais et al., 2011), and elevation in liver enzymes is usually associated with ferritin elevation (Moraes et al., 2011).

One of the main causes of high serum ferritin is non-alcoholic fatty liver disease (NAFLD), which is also more prevalent in men. NAFLD manifests itself mainly in obesity, type-2 diabetes and dyslipidemia (Wang et al., 2010). Half of the patients with high serum ferritin have NAFLD (Pérez-Aguilar, 2004). Increased ferritin could contribute to NAFLD pathogenesis and associated diseases, but one cannot discard the hypothesis that its increase is only a marker of the ongoing inflammatory process.

Obesity-related iron metabolism disorders lead to anemia of chronic disease. They have also been identified in children and adolescents. Furthermore, in this population, increased body weight correlated positively with C-reactive protein levels and negatively with iron serum levels (Richardson et al., 2009). This association was evidenced in children as young as 3 years old in a data analysis of the American population from 1999-2006 (Skinner et al., 2010).

By comparing different body mass index (BMI) categories taken from the American database (NHANES III) with the prevalence of anemia, low serum iron and ferritin, and transferrin saturation, Ausk et al. (2008) found that, for each $1kg/m^2$ gain in BMI, there is an increase of 2.2ng/ml in serum ferritin. Higher BMI categories present a higher proportion of low serum iron and transferrin saturation, but not anemia. Meanwhile, the greater prevalence of anemia in obese women of childbearing age than in normal weight women of the same age range was associated with inflammation, evidenced by high C-reactive protein and transferrin levels (Fanou-Fogny et al., 2010).

Anemia of chronic disease is characterized by impaired mobilization of the iron stores secondary to erythropoietin inactivity and reduced erythrocyte half-life. It is a hypoproliferative anemia, where transferrin is moderately high and serum iron and transferrin saturations are low, despite the adequate reticuloendothelial iron stores (Means, 2004). Hepcidin, a peptide synthesized in the liver and fat tissue and present in blood and urine, mediates this process. Hepcidin expression is induced both by elevated iron stores and inflammation (Bekri et al., 2004). Hepcidin reduces serum ferritin by reducing the intestinal

absorption of iron and increasing iron sequestration by macrophages (Fleming and Sly, 2001). Since iron is a transition metal, the variation between the valence states, accepting and donating electrons, makes this mineral a good catalyzer of reactions that produce free radicals. The iron ion can convert hydrogen superoxide or peroxide into the hydroxyl radical, which is highly deleterious to the body. This occurs through Fenton's reaction (Morris et al., 1995).

Obese children, when compared with normal weight children, present lower transferrin saturation and higher hepcidin levels. Hepcidin correlated positively with the degree of obesity and leptin concentration correlated inversely with transferrin saturation and iron absorption (Giudice et al., 2009). Hepcidin expression was higher in the adipose tissue of morbidly obese individuals submitted to surgery than in tissues of normal weight individuals submitted to lipectomy (Bekri et al., 2004).

The relationship between iron metabolism and insulin resistance has been discussed in epidemiological and clinical studies (Vari et al., 2007, Vallianou et al., 2010, Morais et al., 2011). High ferritin is associated with glucose metabolism disorders and the metabolic syndrome, reflecting the inflammatory process that is going on during the metabolism of fat tissue, atherosclerosis and diabetes (Vallianou et al., 2010, Morais et al., 2011).

Elevation in serum ferritin concentration is associated with elevation in blood pressure, hyperglycemia, hyperinsulinemia and hypercholesterolemia; it is a marker of poor prognosis for acute stroke and coronary artery disease (Pérez-Aguilar, 2004). For instance, a middle-aged man is 2.2 times more likely to experience a myocardial infarction if he has high serum ferritin (Knovich et al., 2008). However, there are still controversies regarding the specific role of ferritin in the disease process (Knovich et al., 2008). One of the mechanisms of action of ferritin involves angiogenesis (Domenico et al., 2009)

Obese patients present higher inflammation and oxidation indices and lower antioxidant defenses than normal weight controls (João Cabrera et al. 2010). The participation of the adipose tissue in the inflammatory process, as it releases a number of pro-inflammatory cytokines, explains the association between obesity and chronic diseases quite well.

It has been shown that type and distribution of adipose tissues are associated with inflammation, which determines insulin resistance and the metabolic syndrome. Since ferritin is associated with obesity-related inflammatory processes and severity of obesity-related comorbidities, Hardy et al., 2011, raised the hypothesis that high ferritin in obese individuals could be

associated with inflammation of the omental adipose tissue (Hardy et al., 2011). Cartier et al., 2009, have found inflammatory markers in the abdominal fat of women regardless of BMI.

The fact is that ferritin elevation is a common finding in obese patients who are on the waiting list for bariatric surgery and, given the important interaction between iron metabolism and inflammation, and the importance of inflammation in the chronic processes that involve obesity, ferritin as a marker of inflammation should be better studied, as also suggested by Drygalski and Andris, 2009.

THE EFFECT OF BARIATRIC SURGERY ON IRON METABOLISM

In order to support the discussion put forth in this chapter, biochemical data were randomly collected from the medical histories of women aged 25 to 50 years before and one year after bariatric surgery performed at the Bariatric Clinic of the *Hospital dos Fornecedores de Cana*, Piracicaba-SP, Brazil. The studied variables were BMI, hemoglobin, ferritin, blood glucose, total cholesterol, HDL and LDL cholesterol fractions, triglycerides, uric acid, aspartate transaminase (AST), alanine transaminase (ALT) and gamma-glutamyl transpeptidase (gamma-GT). The results are shown in Table 1. Weight loss secondary to bariatric surgery, in this case, Roux-en-Y gastric bypass, as seen in other studies, was accompanied by a reduction in many biochemical markers associated with glucose, lipid and iron metabolism. Specifically during the preoperative period, considering that the studied women were of childbearing age, the mean ferritin values were above the expected, as well as mean blood glucose.

In terms of proportions, in the random sample of 45 women before surgery, the prevalence of ferritin was 3% below the expected values and 41% above the expected values. In the postoperative period, 3% of the values were still below and 41% still above the expected values but most women had experienced a reduction in ferritin. In the same group of women of the Bariatric Clinic of the *Hospital dos Fornecedores de Cana*, Piracicaba-SP, Brazil, in preoperative data analysis, ferritin presented a weak but positive correlation with HDL-C and blood glucose. However, from three to twelve months after surgery, this correlation did not hold. One year after surgery, ferritin presented a positive correlation with triglycerides and uric acid.

Table 1. Selected biochemical indicators for women of childbearing age before surgery and one year after surgery

	Before (n=45)		1 year after (n=45)		ρ*
	Med	SD	Med	SD	
Body mass index (kg/m^2)	48.0	7.0	32.0	7.0	0.000
Hemoglobin	13.1	0.8	12.7	0.9	0.026
Ferritin	114.5	106.6	85.6	77.4	0.034
Blood glucose	107.6	41.8	82.5	8.1	0.001
Total cholesterol	189.0	30.3	157.9	26.4	0.000
HDL cholesterol	51.2	12.3	51.4	14.9	0.597
LDL cholesterol	112.2	24.7	93.5	28.3	0.007
Triglycerides	133.1	53.2	85.3	30.9	0.000
Uric acid	5.0	1.3	3.5	1.3	0.000
Aspartate transaminase (AST)	23.9	18.3	22.0	8.8	0.636
Alanine transaminase(ALT)	25.5	16.4	23.6	11.0	0.604
Gamma-glutamyl transpeptidase (gamma-GT)	53.1	77.1	24.1	27.3	0.065

* = Paired t-test.

Source: Bariatric Clinic of the Hospital dos Fornecedores de Cana, Piracicaba-SP, Brazil, 2011.

One year after surgery, there was an improvement in antioxidant protection associated with reduced inflammatory and oxidative stress markers (João Cabrera et al. 2010), which may explain the reduction in serum ferritin and an improvement in ferritin-related biochemical parameters.

Anty et al. (2008) conducted a study with morbidly obese women before and six months after bariatric surgery. Before surgery, the authors found a significant positive correlation between iron depletion and inflammation indices; however, six months after surgery, there was a reduction in inflammation level associated with better iron status, with increased transferrin saturation.

Improvement in the biochemical markers of inflammation after bariatric surgery has been reported by many studies (Tussing-Humphreys et al., 2010 e 2011, Ramalho et al., 2009). In premenopausal women, weight loss 6 months after surgery promoted a reduction in C-reactive protein and hepcidin levels and an increase in hematocrits and hemoglobin (Tussing-Humphreys et al.,

2010). Drygalski et al. (2011) analyzed the frequency of anemia and indicators of iron metabolism in a 4-year retrospective study of bariatric surgery in 1125 patients. The results showed an important reduction in ferritin, an increase in serum iron and no change in serum B_{12} and folate concentrations. The rate of anemia went from 12% preoperatively to 23% 24 to 48 months after surgery. This shows that despite improved inflammatory status and the favorable changes in iron metabolism brought about by this improvement, the incidence of anemia usually happens frequently in people submitted to bariatric surgery. This incidence will depend on the type of surgery and dietary pattern of the patient. Surgeries that restrict acid pH for the solubilization of dietary iron in the ferric form result in less iron absorption. In other words, patients develop food intolerances, such as meat intolerance, more frequently, and meats are good sources of heme iron, which is absorbed as is. Iron-deficiency anemia is common in people who have undergone bariatric surgery (Carrasco et al., 2009). The causes are anatomical, physiological and dietary, considering that the surgery reduces stomach size and modifies gastric pH. It also causes food intolerances, such as intolerance to meat. Furthermore, chronic inflammation helps to reduce the iron stores of the body (Drygalski, 2009), which may improve after surgery, but not enough.

Five or more years after Roux-en-Y gastric bypass, Dalcanale et al. (2009) found a hemoglobin-, iron- and ferritin-deficiency prevalences of 50.8%, 29.8% and 36.8%, respectively. Below-expected values of iron and transferrin were also found preoperatively. Iron deficiency in obese patients waiting for surgery was associated with income, that is, deficiency severity increased as income decreased (Schweiger et al., 2010).

A study done in 2007 with women who had had surgery at the Bariatric Clinic of the *Hospital dos Fornecedores de Cana*, Piracicaba-SP, Brazil, found that 29.8% of the cases presented at least one episode of anemia within the 2 years that followed surgery, and anemia was more common in women who lost greater percentages of excess body weight (Table 2). Vargas-Ruiz et al. (2008) studied 30 patients submitted to Roux-en-Y gastric bypass and found that, despite supplementation, 46.6% of the patients presented episodes of anemia two years after surgery and 63.6% three years after surgery. Serum iron deficiency was 40% and 54.5%, two and three years after surgery, respectively, while vitamin B_{12} deficiency was 33.3% and 27.2%. Folate deficiency was not observed. In addition to restricted nutrient intakes, frequent anemia in women of childbearing age may be related to the hormonal changes stemming from fat tissue loss, with concomitant changes in menstrual frequency and flow.

Table 2. Incidence of anemia (treated episodes) in women submitted to Roux-en-Y gastric bypass, two years after

	≤50% EWL n = 22	50 ┤ 75% EWL n = 68	≥ 75% EWL n = 51	All n = 141
n (%)	4 (18,2)	19 (27.9)	19 (37,2)	42 (29,8)

%EWL = % of the excess weight lost; the data were expressed as sample size and percentage (%).

Source: Novais, 2009.

Type of surgery affects the prevalence of anemia. In laparoscopic sleeve gastrectomy, the prevalence of anemia in 61 patients was 4.9% one year after surgery (Hakeam et al., 2009).

After bariatric surgery, many patients do not consume the recommended iron intake. Table 3 shows the intake of some nutrients before and two years after bariatric surgery. According to the American Dietary References Intakes (DRI) from the Institute of Medicine, women of childbearing age should take from 8.1mg (EAR) to 18mg (RDA) of dietary iron daily (Institute of Medicine, 2001). The EAR (Estimated Average Requirements) corresponds to the mean requirement of a reference population, while the RDA (Recommended Dietary Allowances) corresponds to the mean plus two standard deviations or 2 variation coefficients of 10% when the standard deviation cannot be calculated.

Table 3. Comparison of the estimated intake of minerals and vitamins by women before bariatric surgery and two years after

Nutrient	Before surgery (n=40) Median	Minimum	Maximum	After surgery (n=35) Median	Minimum	Maximum	*p-value**
Vitamin C (mg)	41.41	13.74	98.41	56.27	3.45	363.47	0.388
Pyridoxine (mg)	1.26	0.65	1.77	0.84	0.26	1.95	0.000
Iron (mg)	10.13	4.50	12.42	7.77	1.87	332.40	0.002
Zinc (mg)	8.36	3.36	10.17	5.17	0.93	10.67	0.000

* Mann-Whitney test.

Source: Bariatric Clinic of the Hospital dos Fornecedores de Cana, Piracicaba-SP, Brazil, 2011.

Intake adequacy should be assessed by the inter- and intra-individual intake variance. Other nutrients in addition to iron related to hematopoiesis are under-consumed by the women studied in Piracicaba-SP-Brazil. For women of childbearing age, the EAR for vitamin C is 60mg, pyridoxine is 1.1mg and zinc is 6.8mg.

The food habits of obese individuals are usually chaotic, with low consumption of vegetables and preference for high-energy foods that are low in vitamins and minerals. An example of this is the low consumption of vitamin C of women hospitalized at the Bariatric Clinic of the *Hospital dos Fornecedores de Cana*, Piracicaba-SP, Brazil.

A common problem that makes a substantial contribution to anemia after bariatric surgery regards food intolerances that many patients develop after surgery. Table 4 shows food aversions to dietary iron sources, especially heme iron, in patients submitted to Roux-en-Y gastric bypass. Aversion rates are high shortly after surgery but tend to improve over time.

Iron absorption was investigated before and 6, 12 and 18 months after Roux-en-Y gastric bypass. It was found that, under a standard diet, intake decreased by 32.7% (Carrasco et al., 2009).

Table 4. Incidence of food aversions before surgery and six, twelve and twenty-four months after bariatric surgery (in percentages), (n=141)

Foods	BS (n=141)		0.5 year (n=141)		1 year (n=141)		2 years (n=141)		X^2
	yes	no	yes	no	yes	no	yes	no	*p*
Red meat	0	141	65	76	45	96	23	118	<0.0001
Chicken	0	141	28	113	23	118	14	127	0.0009
Fish	0	141	16	125	10	131	5	136	0.0017
Egg	2	139	22	119	17	124	13	128	0.0100
Beans	2	139	12	129	9	132	5	136	0.1593
Sausages*	13	128	15	126	14	127	10	131	0.0042

BS = before surgery; * includes bologna sausage, hot dogs, sausages and salami.
Source: Novais, 2009.

Dietary iron may be in the form of heme, derived from hemoglobin and myoglobin, and in the form of non-heme. Although heme iron is ingested in much lower amounts, its bioavailability is much greater (López and Martos, 2004).

The absorption of non-heme iron requires the release of hydrochloric acid since this substance increases its solubility and keeps it in the ferrous form. The proportion of absorbed dietary iron is low, from 5 to 10%. In conditions of deficiency, this value may reach 30%. There is an inverse correlation between iron absorption and serum ferritin (López and Martos, 2004). Vitamin-A deficiency has a negative impact on iron absorption and mobilization (López and Martos, 2004). Vitamin C increases iron absorption by facilitating the reduction of Fe^{3+} to Fe^{2+} (López and Martos, 2004). Absorption of heme iron is little affected by diet composition and even less influenced by nutritional status (López and Martos, 2004). In relation to minerals, iron competes with cobalt, nickel, manganese and zinc at the absorption sites. The absorption of non-heme iron is inhibited by calcium, dietary fiber, alcohol, phosphates, amino acids, soy protein and egg protein. It is facilitated by ascorbic acid, oxalates, organic acids, polyphenols and animal protein (Hunt, 2005).

Finally, based on the knowledge currently available, supplementation of iron and other vitamins associated with anemia should be routinely recommended for bariatric patients, and specifically in relation to iron, it is important that the supplement presents goof bioavailability, such as, for example, chelate iron, which is absorbed in the same way heme iron is absorbed, that is, unchanged.

CONCLUSION

Ferritin elevation in obese individuals is associated with reduced serum iron and can be accompanied by anemia. In obese individuals, elevation of ferritin mediated by hepcidin results from an inflammatory process and is related to insulin resistance and other components of the metabolic syndrome. Weight loss reduces inflammation, which is indicated by reduced serum ferritin and increased serum iron, stemming from better absorption of the mineral. Among patients submitted to surgery, the incidence of anemia is high, because of anatomical and physiological changes that most often lead to low iron intake, reduced iron absorption by mechanisms that occur in the digestive lumen or eventual blood losses, since the cellular absorption mechanisms are improved with weight loss and reduced inflammation. Special attention should be paid to the type of iron supplement that is prescribed to the patient, to ensure adequate absorption and prevention of anemia.

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Chapter 7

ASSOCIATION OF ELEVATED SERUM FERRITIN CONCENTRATION AND RISK OF GESTATIONAL DIABETES MELLITUS

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ABSTRACT

Serum ferritin level has been used as a standard measurement of body iron stores, and is the most reliable marker of iron status. The demand for iron is particularly critical in pregnant women, who need to expand their erythrocyte mass and generate the iron supply of the growing fetus. The amount of iron passing through the placenta increases as gestation progresses. This transfer of iron occurs against a concentration gradient from the placenta to the fetus, especially during the third trimester. During pregnancy, serum ferritin levels display a gradual decline, with a nadir at 35-38 weeks of gestation, followed by a slight increase in late pregnancy. In pregnant women, a favorable iron status is a prerequisite for a good perinatal outcome for both mother and infant.

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An elevated serum ferritin level can be a biomarker of body fat mass before and during pregnancy, especially in obese women. Some studies have shown an association between significantly increased ferritin levels during pregnancy and impaired glucose tolerance and gestational diabetes mellitus (GDM). However, these findings have been inconsistent. This article reviews the evidence for an association of elevated serum ferritin level and the risk of gestational diabetes mellitus. Its aim is to highlight the importance of maintaining good health because both GDM and iron deficiency anemia are enormous public health problems among reproductive age women.

INTRODUCTION

Serum ferritin level is a standard measurement of body iron stores [1], and is the most reliable marker of iron status [2]. It estimates the risk of subsequent iron deficiency or overload. In body tissues, ferritin exists as a 24-unit polymer consisting of two subunits, the heavier acidic subunits and the lighter basic subunits [3]. In adults, a serum ferritin reading of 1 μg/L is equivalent to 8-10 mg of storage iron [4].

During pregnancy, the daily demand for iron is enormously increased due to the expansion of blood volume by ~35% and growth of the fetus, placenta, and other maternal tissues. Between the second and third trimesters, this increased demand for iron intake progresses from about 1.0 ~1.5 mg to 5.0 mg per day. The total increase in plasma volume is about 42%, whereas the total red cell volume is less than one-third of the increase in plasma volume [5]. This leads to hemodilution, and indicates that estimation of hemoglobin alone might not be a good indicator of iron status.

The placenta serves as the regulatable conduit for maternal-fetal iron transport, and the amount of iron passing through the placenta increases with gestation. Iron is transferred against a concentration gradient from the placenta to the fetus, especially during the third trimester [6]. Maternal serum ferritin levels display a gradual decline, with a nadir at 35-38 weeks of gestation; this is followed by a slight increase in late pregnancy. Immediately after delivery, serum ferritin is influenced by the acute blood losses associated with childbirth. Later in the postpartum period, when the circulation has become stabilized, an increase in serum ferritin, as well as hemoglobin, occurs (Fig. 1) [7,8]. Iron absorption increases during pregnancy, but not to levels sufficient for prevention of iron deficiency anemia in 20% of women not taking supplementary iron. Iron-treated pregnant women have greater iron reserves,

higher hemoglobin levels, and a lower prevalence of iron deficiency anemia compared to placebo-treated women, both during pregnancy and postpartum [9].

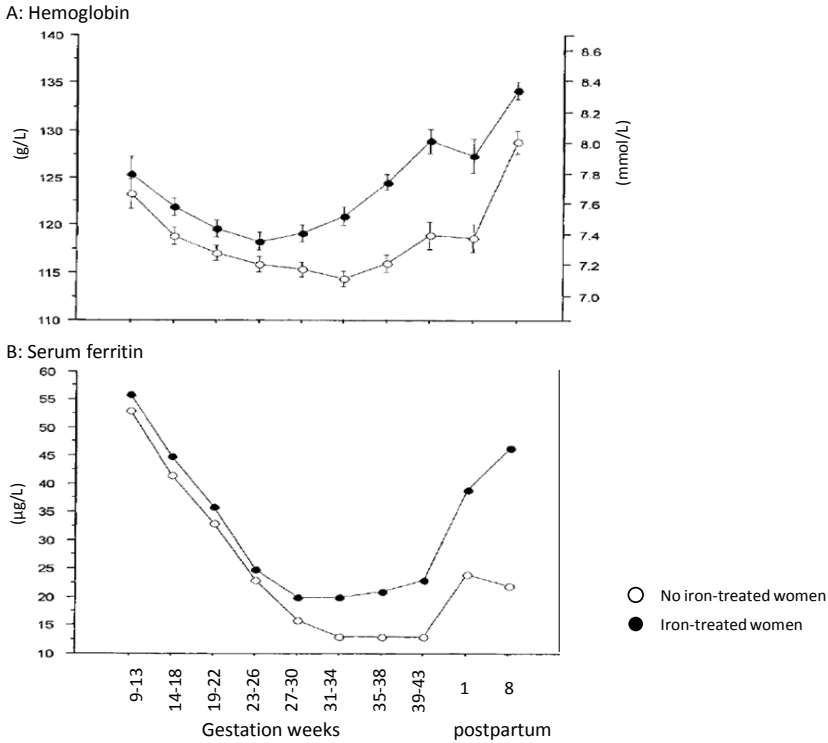

Figure 1. Variations in hemoglobin and serum ferritin levels in normal pregnancy and postpartum women. Iron-treated women; taking 66 mg ferrous iron daily. Based on ref. 7,8

IRON STATUS AND PERINATAL OUTCOMES

The World Health Organization has reported that iron deficiency anemia during pregnancy is a significant problem throughout the world. The prevalence is 15% in industrialized countries, but is substantially higher (35-75%) in developing countries [10] due to low socioeconomic conditions and a lack of knowledge of good dietary habits. The US Centers for Disease Control and Prevention examined the distribution of total body iron and the prevalence

of iron deficiency, based on total body iron, in 1,171 pregnant US women in the National Health and Nutrition Examinations Survey (NHANES), 1999-2006 [11]. An abnormal value for ferritin and the ratio of soluble transferring receptor (sTfR) concentrations were defined as <12.0 μg/L and >4.4 mg/L, respectively [12]. The prevalence of iron deficiency, based on low serum ferritin concentrations, increased significantly with each trimester (7.3%, 23.7%, and 39.2% in the first, second, and third trimesters, respectively) [11]. A similar pattern was observed based on high sTfR concentrations.

Iron is an essential micronutrient that plays a significant role in critical cellular function in all organ systems. In pregnant women, a favorable iron status is a prerequisite for a good perinatal outcome for both mother and infant. Nevertheless, thirty percent of pregnant women have low serum ferritin concentrations at the end of pregnancy [13]. Iron deficiency in the fetus during the fetal or postnatal periods can alter brain structure, neurochemistry, and cognitive functioning, and can lead to long-term cognitive and motor impairment that cannot be corrected by later iron supplementation [14,15]. Infants of diabetic mothers (IDMs) who are born with low neonatal ferritin concentrations (≤35 μg/l) have impaired auditory recognition memory processing at birth compared with iron-sufficient IDMs (neonatal ferritin>35 μg/l) [16].

On the other hand, elevated iron stores during pregnancy have been also associated with maternal and neonatal morbidity. Elevated maternal ferritin levels were related to an increased risk of intra-uterine growth retardation (IUGR), preterm delivery [17,18], and pregnancy–induced hypertension and eclampsia [19]. In addition, high serum ferritin levels have been linked with type 2 diabetes and the development of GDM in pregnant women [20,21]. Scholl et al. observed that women with high levels of ferritin (107 ng/ml) were nearly three times more likely to develop type 2 diabetes over a 10-year interval [17].

SERUM FERRITIN AND THE INSULIN RESISTANCE SYNDROME IN THE GENERAL POPULATION

Iron is a transitional metal and a potential catalyst in many cellular reactions that produce reactive oxygen species. These types of reactions contribute to tissue damage and increase oxidative stress, thereby potentially increasing the risk of development of insulin resistance syndrome [22].

Ferritin, the major iron storage protein, plays a key role in iron metabolism [23]. Positive associations have been reported between mildly increased serum ferritin levels and indexes of insulin resistance in both healthy subjects and patients with type 2 diabetes [20, 24, 25].

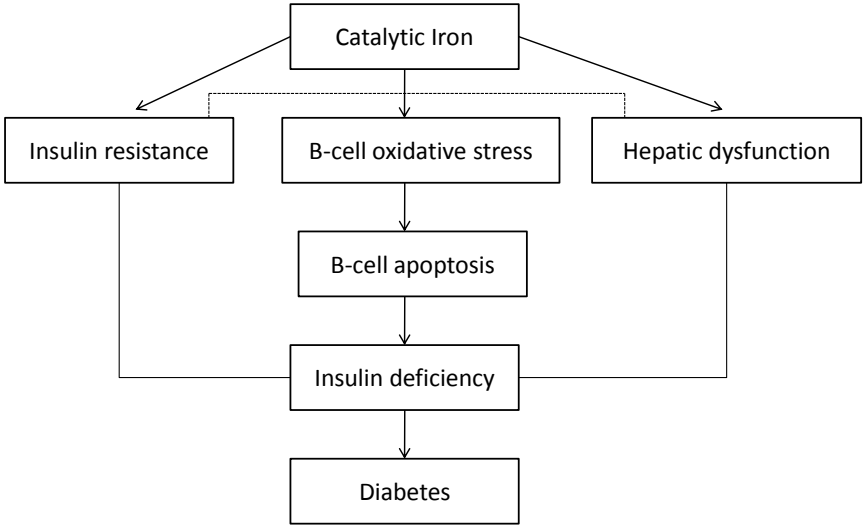

Figure 2. Pathogenic pathways for iron in induction of diabetes. Based on ref. 26.

Increased serum ferritin can reflect hepatic iron overload due to hemochromatosis or blood transfusions and is often associated with insulin resistance [26, 27]. Bozzini et al. [28] reported an increased prevalence of body iron excess in patients with Metabolic Syndrome identified within the Verona Heart Project. Wrede et al., [25] investigated the association between insulin resistance syndrome and serum ferritin among 516 women and 554 men in an adult German population (mean age 44.8 ±14.4 years). They found a significant association between a body mass index (BMI) value >25 kg/m^2 and hypertension, increased serum ferritin, and diabetes, regardless of gender. Women with diabetes had significantly higher ferritin levels (Fig. 2). Similar findings were reported in other studies [24, 25]; for example, Tuomainen et al. examined whether body iron stores were associated with serum insulin and blood glucose concentrations among 1,013 middle-aged men in eastern Finland [24]. They reported 21.6% higher serum insulin levels in the 5th quintile of serum ferritin values (>216 µg/l) when compared with the 1st quintile (<57 µg/l).

Serum ferritin levels were also associated with elevated serum insulin, blood glucose, and serum fructosamine levels.

PATHOGENIC PATHWAYS FOR IRON IN INDUCTION OF DIABETES

The mechanisms for insulin resistance include the possibility of iron overload causing resistance, either directly or through hepatic dysfunction [26]. Jiang et al. [29] have reported increases in hydroxyl radicals during iron overload and resultant cell damage. This damage leads to initial insulin resistance-hyperinsulinemia, followed by decreased insulin secretion and diabetes. Although the exact mechanism of iron-induced diabetes is unclear, it is likely to be mediated by three key mechanisms: 1) insulin deficiency, 2) insulin resistance, and 3) hepatic dysfunction (Fig. 3)[25].

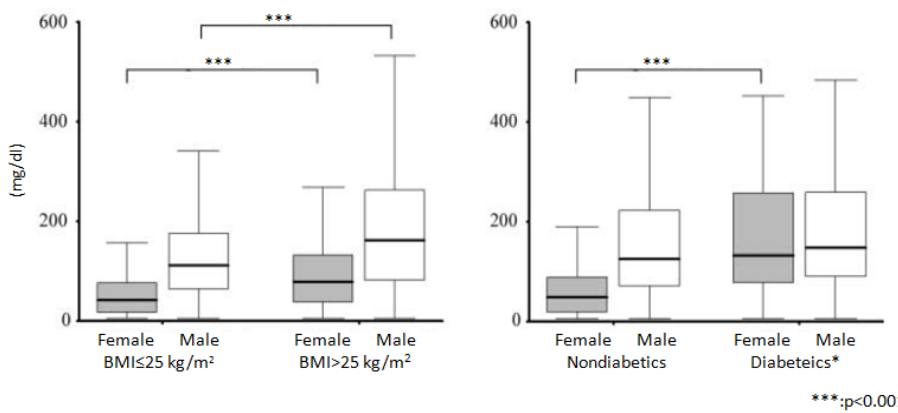

Figure 3. Serum ferritin levels in male and female according to the BMI and insulin resistance syndromes *Diabetic; insulin resistance syndromes. Based on ref. 25.

Available evidence suggests that the main defect in the pathogenesis of GDM is a relatively diminished insulin secretion coupled with pregnancy-induced insulin resistance [30]. In a study of patients with unexplained hepatic iron overload, most were found to be insulin resistant, which suggests that hepatic iron, hepatic dysfunction, and insulin resistance share a common

etiological link [31]. Iron, a redox-active transitional metal, is a strong prooxidant. A number of experimental studies have demonstrated that iron overload can lead to β-cell toxicity, β-cell dysfunction, and impaired glucose metabolism [32].

Iron accumulation can affect glucose metabolism in a number of ways. Iron promotes the formation of hydroxyl radicals that can attack cell membranes, thereby affecting insulin synthesis and secretion by the pancreas and interfering with the insulin-extracting capacity of the liver. Insulin also stimulates cellular iron uptake, creating a vicious cycle that leads ultimately to insulin resistance and diabetes [33]. However, the mechanism underlying the development of iron-induced diabetes is not yet completely understood.

ELEVATED SERUM FERRITIN LEVELS AND RISK OF GESTATIONAL DIABETES MELLITUS

Gestational Diabetes Mellitus

Gestational diabetes mellitus is defined as glucose intolerance that first occurs, or that is first identified, during pregnancy [34]. GDM is one of the most common pregnancy complications and affects approximately 14% of all pregnancies [35]. In 30-70% of GDM patients, type 2 diabetes mellitus may develop at a later age [36]. The International Association of Diabetes in Pregnancy Study Group recently published a consensus derived from the Hyperglycemia Adverse Pregnancy Outcome (HAPO) study data, which suggested that all pregnant women without known diabetes should have a 75 g oral glucose tolerance test at 24-28 weeks gestation. GDM would be diagnosed if one or more blood glucose values met or exceeded the following levels: fasting, 5.1 mmol/l; 1 h post glucose, 10.0 mmol/l; and 2 h post glucose, 8.5 mmol/l (Table 1) [37]. The HAPO study was a basic epidemiological investigation that conclusively identified, for the first time, a strong continuous association of maternal glucose levels below those diagnostic of diabetes with several perinatal outcomes. A significant obstetrical complication of GDM is the birth of a large-for-gestational-age infant (LGA) [38-40].

The neonatal complications of GDM, including hypoglycemia and hypocalcaemia, are caused by fetal hyperinsulinemia, which results from

maternal hyperglycemia. The long-term complications of GDM include an association with diabetes in the mother and diabetes and obesity in their offspring [41-43].

Table 1. Diagnosis of gestational diabetes mellitus in pregnancy.

Glucose measure	Glucose concentration threshold*	
	mmol/l	mg/dl
Fasting plasma glucose	5.1	92
1-h plasma glucose	10	180
2-h plasma glucose	8.5	153

*One or more of these values from a 75g oral glucose tolerance test must be equaled or exceeded for the diagnosis of gestational diabetes mellitus. Based on ref. 38

Overweight and Obesity

Overweight and obesity are the major modifiable risk factors of GDM. The high prevalence of and increasing trend toward obesity worldwide, and especially in Western societies, is of great concern. The NHANES reported a continued high prevalence of obesity (BMI $\geq$30 kg/m^2) in women aged 20-49 years, with a value that exceeded 30% after 1999 [44]. The latest NHANES data from 2007-2008 showed a prevalence of overweight (BMI $\geq$25 kg/m^2) and obesity of 59.5% and 34.0%, respectively.

A direct association between maternal pre-pregnancy BMI and the risk of developing GDM is becoming evident [26]. The odds ratios of overweight, moderately obese, and morbidly obese women developing GDM are 1.97, 3.01, and 5.55, respectively. Obese women are generally more insulin resistant when compared with non-obese women, whether pregravid or during pregnancy, and significant decreases are seen in maternal insulin sensitivity by the end of a pregnancy [45]. Decreased insulin sensitivity limits the ability of insulin to transport glucose from the intravascular into peripheral tissues such as skeletal muscle and adipose, which suggests that obese women are more likely to be at a greater risk for the development of GDM.

SERUM FERRITIN LEVELS IN WOMEN WITH GESTATIONAL DIABETES MELLITUS

An association has been reported between increased serum ferritin levels and insulin resistance and diabetes in pregnant women. High serum ferritin levels have been linked both with type 2 diabetes and with the development of GDM [20,21]. Lao et al., conducted a prospective observational study of 762 nondiabetic Chinese women with singleton pregnancies, recruited at 28-30 weeks. They found that the group in the highest hemoglobin quartile (> 13 g/dL) had a significantly higher incidence of GDM (18.7% vs. 10.9%, p=.007), as well as greater age, weight, and serum ferritin and iron concentrations. They found that high haemoglobin (Hb) (>13 g/dL) was an independent risk for GDM in pregnant women and that women with iron deficiency anemia had a reduced risk of GDM [46].

Soubasi et al. [18] reported that high maternal ferritin levels (>60 μg/L) were significantly associated with a higher rate of GDM and IUGR. However, maternal serum ferritin was not correlated with sTfR, Hb, or mean corpuscular volume levels. Thus, elevated maternal ferritin did not appear to reflect an excess iron storage, but was related to an increased risk of GDM or IUGR.

Chen et al. [47] examined the relationship between elevated serum ferritin levels and the risk of GDM in a cohort of 1,456 healthy pregnant women in Camden, New Jersey. Elevated serum ferritin level (highest quintile; >131.8 pmol/l) was significantly and positively correlated with prepregnancy BMI. Women with the highest levels of serum ferritin had a 2-fold increased risk of developing GDM, when adjusted for confounding factors. In addition, obese women with high ferritin levels had a 3.5 fold increased risk of developing GDM, whereas non-obese women did not. Therefore, the impact of high serum ferritin on the risk of GDM appeared to be at least partially mediated by obesity.

Consistent findings were reported for non-diabetic Turkish women with singleton pregnancies. Women who presented with serum hemoglobin values above the 50^{th} percentile (12.2 g/dL) in the first trimester of pregnancy were at increased risk for developing GDM. No similar association was found for serum ferritin and GDM [48]. In pregnant Chinese women, serum ferritin level was higher in women with impaired glucose tolerance and GDM, but whether this increase reflected inflammation or excess iron stores is unclear.

Although the Centers for Disease Control and Prevention has defined an abnormal value for below-range ferritin concentrations as one less than 12.0 μg/L [12], an upper range has not been identified. Conflicting findings and the different incidence rates of GDM in pregnant women may therefore arise due to differences in the cutoff values used to define a high serum ferritin concentration. This greatly complicates the determination of whether a high serum ferritin level is a risk factor for type 2 diabetes or GDM.

ELEVATED SERUM FERRITIN AND INFLAMMATION IN GESTATIONAL DIABETES MELLITUS

Ferritin has been identified as a marker of inflammation. Pregnancy is also considered an inflammatory status; serum C-reactive protein (CRP) levels are raised as early as 4 weeks into gestation [49]. A systemic inflammation is also increasingly being recognized in GDM, as indicated by higher levels of serum CRP and /or interleukin-6 [50,51]. Inflammation is also associated with obesity as adipocytes from adipose tissue can secrete proinflammatory cytokines [52]. These inflammatory cytokines, including tumor necrosis factor-α and interleukin-1α, have been shown to induce ferritin synthesis in experimental models [23].

Studies suggested that adipose tissue is an important determinant of a low level of chronic inflammations, as indicated by a strong positive correlation between serum CRP levels and body fat mass in both non-pregnant and pregnant women [52,53]. Some studies reported that the higher serum CRP levels associated with a risk of GDM were mediated by increased BMI [50,53]. In contrast, the increased risk of GDM associated with higher CRP levels was independent of maternal adiposity [51]. Chen et al. [47] reported that women who had both high CRP and serum ferritin had a greater than twofold increased risk of developing GDM. However, the effect was reduced after adjusting for prepregnant BMI. The authors suggest that a systemic inflammation might be involved in the pathophysiology of GDM, and that elevations of both serum ferritin and CRP levels are indicators. Whether elevated serum ferritin in GDM is a consequence of inflammation is not yet clear.

CONCLUSION

Accumulating evidence suggests a link between elevated serum ferritin levels and insulin metabolism both in the general population and especially in pregnant women. Excess iron can affect insulin synthesis and secretion, and enhance oxidation of lipids, which in turn can decrease glucose utilization in muscles and increase gluconeogenesis in the liver. GDM is one of the most common complications of pregnancy. The incidence of GDM has been on the increase over the last decade, and may reflect the increase in numbers of obese women of reproductive age.

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