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Advantages of the Supplementation with both a Protein and Heme Hydrolyzate and Ionic Iron during Iron Deficiency Anemia

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Abstract **Background:** nutritional anemia caused by iron (Fe) deficiency is considered a major public health problem. Interventions to address nutritional anemia have been traditionally focused on supplementation with ionic Fe that causes gastrointestinal adverse effects. On the other hand, some nutritional studies have demonstrated the advantages of supplementation with both, a protein and heme hydrolyzate and ionic Fe. However there are few experimental and clinical evidences to conclude on the efficacy of this supplementation strategy to treat the Fe deficiency and anemia. **Aim:** is to know about the physiological and biochemical events proposed to explain the anemia recovery during a treatment with both a protein and heme hydrolyzate and ionic Fe during Fe deficiency anemia recovery. **Results:** some aspects related to the most recent events elucidated about the metabolism of both chemical forms of Fe were included in this work. Nutritional supplements that exist from both, a protein hydrolyzate with heme and ionic Fe, with some results that demonstrate the efficacy of this treatment in humans and a rat model of anemia, are also discuss. **Conclusion:** supplementation with both Fe sources allows, simultaneously, the anemia recovery and the decreased oxidative damage caused by traditional Fe therapies to treat nutritional anemia by Fe deficiency.

Keywords: *anemia, heme, ionic Fe, supplementation*

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1. Introduction

Fe deficiency anemia is extremely common, particularly in the developing world, reaching a state of global epidemic. Fe deficiency during pregnancy is one of the leading causes of anemia in infants and young children [1]. The World Health Organization estimates actually that in developing countries every second pregnant woman and about 40% of preschool children are estimated to be anemic. In many developing countries, iron deficiency anemia is aggravated by worm infections, malaria and other infectious diseases such as HIV and tuberculosis [2]. Main causes of Fe deficiency are inadequate Fe source intake (quantitatively and qualitatively), low Fe absorption and increased Fe requirements during growth and pregnancy [3]. Anemia is defined as a low hemoglobin concentration in blood, under the normal physiological range (110 g/L for preschool aged children and pregnant women, 120 g/L for nonpregnant women) [4]. Anemia condition is the final stage of Fe deficiency and is established due to body depletion of Fe storage, low plasma Fe concentration and inadequate Fe supply to the bone medulla for hemoglobin synthesis during erythropoiesis [5].

Interventions to address nutritional anemia have been traditionally focused on supplementation with ionic Fe as ferrous sulphate or ferrous fumarate [6]. However, these preparations have generally failed to show significant reductions in anemia prevalence [7], due to the gastrointestinal adverse reactions in the 30 to 50 % of patients, and this situation leads to a low treatment adherence and compliance [8]. Adverse reactions of ionic Fe formulations are in relation with their low bioavailability [9,10]. Fe administration during long periods could produce intestinal Fe accumulation and the increase of oxidative damage [8]. The disadvantages of the ionic Fe administration lead to the development of heme preparations, which are obtained from hemoglobin of animal blood [11,12,13]. However, the utilization of heme preparations had been limited, due to the low Fe content in the hemoglobin that represents only the 0.34 % of its molecular weight [14].

Hydrophobic peptides from enzymatic hydrolyzate of bovine hemoglobin and pepsin, and free amino acids as cysteine, lysine and histidine enhanced heme and ionic Fe

absorption in experimental models [15,16]. The presence of these components at proximal intestinal level leads to the formation of chelates with ionic Fe and this situation facilitate the conservation of the ferrous state [17]. In the case of the heme, they also favor its absorption, since the peptides and amino acids from the hydrolysis of the bovine hemoglobin chelate the molecule [14], and this prevents the formation of dimers that could precipitate the heme [18]. All these events could facilitate intestinal heme absorption [14]. On the other hand, the high bioavailability of Fe from heme [19], makes possible to obtain heme supplements containing low Fe doses [20,21], thus minimizing the adverse effects of the mineral.

Nutritional studies have demonstrated the advantages of combining both chemical forms of Fe, ionic and heme. Some of these studies were performed in humans [20,22], and some others were conducted in anemic rat models [23,24]. They obtained similar hematological parameters in response to the supplementation with different Fe sources and doses. However there are not enough experimental and clinical evidences to conclude on the efficacy in anemia recovery of the supplementation with heme preparations from a protein hydrolyzate when they were combined with ionic Fe. The aim of this review is to deepen in the biochemical and physiological events taking place during a supplementation with both a protein and heme hydrolyzate and ionic Fe to explain their efficacy during anemia recovery process.

2. Methods

A transversal descriptive study was made of the papers retrieved from September of 2013 until September 2016. The data used in this study were obtained from the Google search engine, and scientific data bases as Scielo and PubMed. We used as keywords: Fe absorption, anemia, heme supplements, ionic Fe supplements and oxidation, according to the objective of each heading. We summarize the papers used in each topic in Table 1.

Table 1. List of articles according to the topics addressed at work

Topic	Reference
Fe absorption	[25-75]
Distribution and utilization of Fe	[76-90]
Oxidative effect of Fe supplementation	[44,91-101]
Fe supplements with both heme and ionic Fe	[11,12,102,103,104]
Beneficial effect by the supplementation with both heme and ionic Fe	[20,22,23,24,105,106,107,108]
Oxidative effect by the supplementation with both heme and ionic Fe	[99,108,110,114]

3. Absorption of Fe

Dietary Fe is broadly classified into two categories based on its chemical form [25]. The majority of Fe is ionic, which is present in most foods, including cereal, vegetables, and meat. It is found in ferritin and other proteins, as well as several nonprotein-bound forms [26], while Fe from heme molecule is mainly found in the hemoglobin and myoglobin from meat [27]. Fe absorption occurs mainly in the proximal small intestine or duodenum, and involves the uptake and transfer of Fe across the enterocyte into the systemic circulation [28,29]. Ionic Fe absorption is impaired by alkaline pH and by interactions with food components such as tannins, phytates, polyphenols and other components from the diet [30,31,32]. In contrast to the ionic form of the mineral, heme is soluble at duodenal pH and avoids food interactions [33]; except for calcium [34].

Ionic Fe could found at intestinal lumen as Fe^{+2} or Fe^{+3} and the divalent metal transporter 1 (DMT1) transports only the ferrous form [28] (Figure 1). Due to absorption of ionic Fe requires reduction to the ferrous state by dietary components such as ascorbic acid, and duodenal cytochrome b561 (Dcytb), a diheme-containing protein, and ascorbate dependent ferric enzyme, has been proposed to mediate this reductive event in the duodenum [35]. While Dcytb knockout mice display no overt defects in Fe homeostasis when subjected to Fe-rich or Fe deficient diets [36]. Ferric reductases belonging to the Steap family also have been proposed to act at the brush border membrane too [37]. The incorporation of this reduction step for Fe in the process of absorption of ionic Fe, explains that this chemical form of Fe is only absorbed between 2 to 10 % [19]. In case of Fe from heme, a small proportion of dietary Fe is in this chemical form and the quantity of absorption depends of level of meat consumption [33], that's why the percentage of absorption are in the range between 5 and 35 % [19]. On the other hand absorption of Fe from heme does not compete with inorganic Fe [16].

Apical heme uptakes from the lumen have been proposed by different mechanisms as receptor-mediated endocytosis, passive diffusion, or active transport mediated by a transporter [16,38]. In all of these cases, heme is taken up as an intact metalloporphyrin [39,40]. The hypothesis of Fe from heme uptake by receptor mediated endocytosis is originated in 1979, thanks to the discovery of a heme binding protein on the microvillus membrane of the upper small intestine of both pigs and humans [38]. In the past decade a mammalian heme transporter has been discovered, namely heme carrier protein 1 (HCP1) [41], which functions as an active carrier, saturable and temperature dependent [16]. However, this protein is acting mainly as a folate-proton cotransporter in the acidic microclimate of the upper small intestine and was called proton-coupled folate transporter (PCFT) [42]. The function of this carrier in relation to the heme has been clarified in the cellular line Caco2, which has been obtained evidence that HCP1/PCFT transporter is not constrained to folate transport but also is involved in heme transport and could play a physiological role in Fe nutrition and metabolism as a low-affinity transporter for heme [43]. Other authors consider that the role of HCP1/PCFT as a heme importer could be relevant only in some cell types or in particular physiologic or pathologic situations [44]. During Fe deficiency, heme uptake by rat duodenal enterocytes increases and correlates with the increase in heme binding capacity, suggesting that the amount of receptors present on the microvillus membrane contributes to regulation of Fe from heme absorption [38].

Additionally, it was described the heme response gen 1 transporter (HRG-1), that is expressed in the brain, heart, kidney, and small intestine [45]. Especially in duodenum this protein is located in the basolateral membrane and transport heme from body fluids into the cell [46], but the significance of this molecule as a heme transporter in enterocytes is not clear yet. Too HRG-1 plays an essential role for macrophage heme recycling [47].

Feline leukemia virus subgroup C cellular receptor (FLVCR) is a transmembrane protein that plays a key role in erythropoiesis by exporting excess heme from maturing erythroblasts in humans, as an overflow valve for excess heme that would otherwise result in cellular toxicity by producing oxidative stress before it can bind to globins for hemoglobin production [48]. FLVCR is abundantly expressed too at potential sites of heme trafficking, such as the liver and small intestine. Protein expression is also noted in the brain, kidney, lung, spleen, uterus, and placenta [49]. In the human enterocyte–like cell line, Caco-2, which has very high expression of the heme exporter FLVCR, heme is capable of being acquired and transported in both absorptive and secretory directions [50]. This protein exists in different isoforms in mammalian cells as FLVCR1a and FLVCR1b, expressed on the plasma membrane and mitochondria respectively, and function as plasma heme exporters [51]. Also was described FLVCR2, unlike FLVCR1 isoforms was unable to export heme, and was postulated to be a heme importer [48]. The assumption that FLVCR2 is a heme importer is not definitive and further studies are needed to fully address the substrate specificity of this transporter. By the other hand, heme export by FLVCR1 requires the presence of an extracellular heme-binding protein, such as albumin or hemopexin (Hpx) in the media [52]. However the role of protein binding on intestinal heme uptake has not been described and Hpx receptors have not been reported in intestinal epithelial cells [50]. Yang *et al.* (2010) [52], suggested that Hpx has a role in assuring systemic Fe balance during homeostasis, in addition to its established role as a scavenger during internal bleeding or hemolysis.

On the other hand, the ATP-Binding Cassette, subfamily G, a member 2 protein (ABCG2) is a member of transporter family that was originally found to confer drug resistance in breast cancer cell [53]. ABCG2 is localized at the plasma membrane and it is expressed in several tissues including hepatic canalicular membranes, renal proximal tubules, intestinal epithelium and placenta [54]. ABCG2 was found to prevent heme accumulation in erythroid progenitor K562 cells submitted to hypoxic conditions of growth [55]. Too ABCG2 appears to act principally as a safety valve regulating porphyrin levels during the early stages of erythropoiesis and its role in systemic heme metabolism and erythrophagocytosis [56]. Further studies are necessary to define the exact histological localization of FLVCR in the gut and whether both, ABCG2 and FLVCR are regulated by Fe [57,58].

Heme oxygenase (HO) is an enzyme that catalyses the mixed function oxidation of heme, using nicotinamide-adenine dinucleotide phosphate (NADPH), NADPH-cytochrome P-450 reductase, and molecular oxygen (O_2), producing carbon monoxide (CO), Fe and biliverdin IX-α, which is rapidly reduced to bilirubin IX-α [59]. This enzymatic activity was identified of several mammalian tissues, include small intestine [60]. There are two well characterized isoforms of heme oxygenase, referred to as HO-1 and HO-2 [61]. HO-1 is mainly located in the smooth endoplasmic reticulum (SER) fraction, and their expression is induced by numerous factors including oxidative stress [62], and Fe deficiency [63]. By the other hand, the isoform HO-2, is not inducible [66], and appears to function as a sensor for O_2, CO, and NO. In the intestine these functions are relevant in the interstitial cells, where HO-2 regulates levels of CO, which in turn affects potassium currents and resting membrane potential of intestinal smooth muscle, and thus intestinal motility. The subcellular location of HO-2 is consistent with the by receptor-mediated endocytosis hypothesis for heme uptake and may suggest a possible role for this enzyme in heme degradation [65] (Figure 1).

The absorption of Fe is a process highly regulated and in general, intestinal Fe transport is known to be the "checkpoint" of body Fe homeostasis [66]. Absorption of mineral is increased during Fe deficiency and decreased upon Fe loading [67,68]. On the other hand, Fe can be lost into the lumen of the gut when the mucosal cells of duodenum are sloughed from the villus in a selective manner, so that needed Fe is conserved and the excess is eliminated [70]. Mechanism of Fe absorption depends on a network of signals, which reflect systemic body Fe requirements and the interaction of these signals with the enterocyte Fe sensing and transport machinery [72]. Fe homeostasis is regulated systemically by the hormone hepcidin, a 25-aminoacid peptide produced mainly by the hepatocytes. Hepcidin expression is increased by Fe loading and inflammatory signals and is attenuated by Fe deficiency, anemia, increased erythropoietic activity, and hypoxia [71,72]. Hepcidin is thought to affect Fe absorption by inducing degradation of the basolateral Fe transporter ferroportin (FPN) thus limiting Fe export into the circulation [72].

The absorption of ionic Fe is tightly controlled by hepcidin [73]. However, recent studies in mice and Caco-2 cells further suggest that DMT1, not FPN, is the primary target of hepcidin regulation [74]. *In vivo* experiments to elucidate regulation of heme absorption and the interrelations with hepcidin to modulate the absorption of dietary are not enough yet. One of the few studies reported in a rat model of Fe overload obtained that elevated hepcidin significantly decreased heme and ionic Fe absorption, but had a greater impact on ionic Fe absorption [75]. This work also showed that DMT1 and not FPN is a primary target for hepcidin regulation in the duodenum. On the other hand, this work showed that the expression of protein relation with heme transport and metabolism in duodenum, as HPC1 and HO-1 neither was changed by Fe injection or hepcidin.

4. Distribution and Utilization of Fe

Once within the enterocyte, Fe has two basic pathways depending on Fe requirements. When Fe demand is low, it will remain in the enterocyte sequestered by the Fe storage protein ferritin and it will be lost when the enterocytes are sloughed from the villus tip several days later [76]. If Fe is required by the body, it will cross the basolateral

membrane through the Fe export protein FPN [77] (Figure 1). FPN is a multi-pass membrane protein that has been demonstrated to require a ferroxidase to function. The Fe release from FPN is coupled to the actions of copper-dependent ferroxidases, the transmembrane protein hephaestin (Heph), that catalyzes the oxidation of Fe^{+2} to Fe^{+3} [78,79]. However, recent data have suggested that, while Heph increased the efficiency of Fe absorption, it is not essential due to, at least in mice, Fe absorption can occur in knout out Heph animals [80]. This works indicated that Heph is not essential and that other mechanisms, multicopper ferroxidase-dependent or not, must compensate for Heph deficiency. This catalyzed oxidation step also ensures that adequate Fe is available to bind to its carrier in the blood, which under physiological conditions, only binds ferric Fe only [81].

In normal individuals, extracellular Fe circulates in the serum bound to transferrin (Tf), which also delivers it, to cells using an endocytotic pathway involving the transferrin receptor (TfR) [81,82]. Tf is a protein synthesized at liver that has two binding Fe sites [83]. There are two differents transferrin receptor, called 1 and 2 (TfR1 and TfR2) [84]. TfR1 is ubiquitously expressed and Tf-mediated Fe uptake is thought to occur in most cell types. TfR-2- is restricted to hepatocytes, duodenal crypt cells, and erythroid cells, suggesting a more specialized role [85]. Tf-mediated Fe uptake pathway appears to be most important in developing, erythroid precursors because of their enormous need for Fe for hemoglobin synthesis [66]. On the other hand, HPx is a 60-kDa serum b1-glycoprotein and a positive acute phase reactant protein that binds heme that is released into the blood as the result of intra and extra-vascular hemolysis or rhabodomyolysis, and transports it principally to the liver [86].

Heme synthesis takes place in all metazoans cellules and tissues, but requirements of this molecule are highest in the erythroid cells [87]. This process includes eight steps using glycine, succinyl-Coa and Fe^{+2} as substrates [88]. On the other hand, the erythroid cells can uptake heme via the transporter HRG-1[89]. Finally, Fe is recycled and thus stored in the body. When senescent erythrocytes die, Fe hemoglobin content is uptake by phagocytosis in the reticuloendothelial system of macrophages and is stored in ferritin form [90].

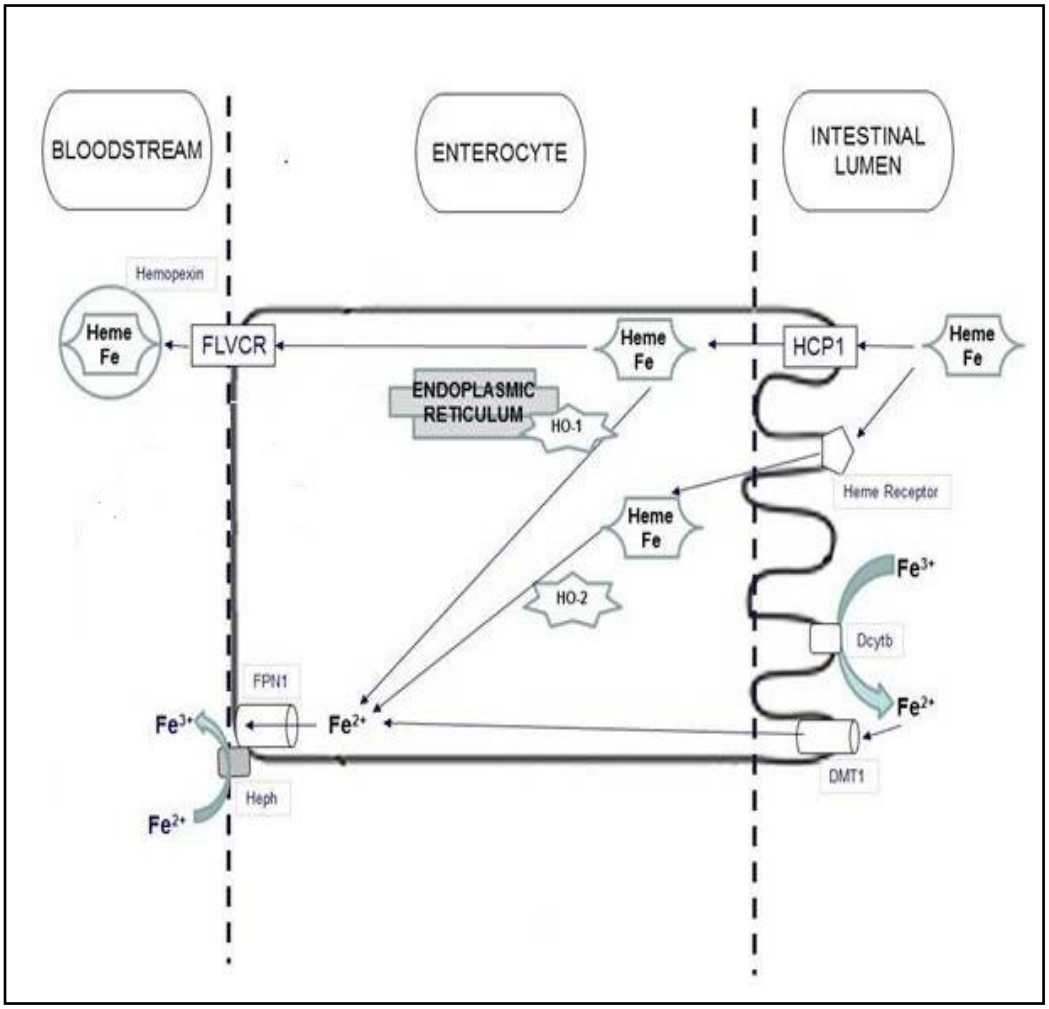

Figure 1. Intestinal Fe absorption pathways according to chemical structure. At intestinal lumen, especially in duodenum level, ionic Fe in ferrous state (Fe^{+2}) is absorbed mediated by the divalent metal transporter 1 (DMT1). When ionic Fe is in ferric form (Fe^{3+}), is reduced to Fe^{2+} by some ferric reductases as Dcytb, because it can only be absorbed in form of Fe^{2+} by DMT1. Heme can be absorbed via a heme transporter namely heme carrier protein 1 (HCP1), or the membrane receptor-mediated endocytosis. Heme once absorbed via the heme receptor HCP1, is released as Fe^{3+} by heme oxygenase (HO-1), or can be transported intact across the basolateral membrane by the heme exporter Feline leukemia virus subgroup C cellular receptor (FLVCR), and binds to the hemopexin. On the other hand heme transporter by membrane receptor-mediated endocytosis is degraded by HO-2 to released Fe+3 too, that constitutes an intracellular "pool" within the enterocyte. In this chemical form, the mineral diffuses through the basolateral membrane, mediated by ferroportin 1 (FPN1) and hephaestin (Heph)

5. Oxidative Effect of Fe Supplementation

Daily oral Fe supplement has shown improvement of hematological indices but with frequent gastrointestinal symptoms [91] and increases oxidative damage at intestinal level [92]. Oxidative effect of mineral is because Fe has the ability to gain and lose electrons i.e. ($Fe^{2+} \leftrightarrow Fe^{3+}$) very easily. The interaction of Fe^{+2} ion with H_2O_2 generating highly reactive hydroxyl radicals (•OH) and this reaction is known as Fenton chemistry [93]. The generation of •OH can react at diffusion-limited rates with various biomolecules, including lipids, proteins, and DNA. Attack by •OH on a membrane lipid can cause a series of radical chain reactions that can severely damage the membranes. The hydroxyl radical can also add to DNA bases leading to generation of a variety of oxidative products [94].

Organisms have evolved to protect themselves from the Fe-mediated damage by several different mechanisms: by sequestering proteins, increasing intestinal Fe absorption or with the combined action of intracellular enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-px)) with glutathione (GSH) as a major partner. On the other hand, low molecular weight antioxidants, especially vitamin E and vitamin C, also provide significant protection [95]. There is also a response at DNA level called phase II. The transcriptional activation of phase II detoxifying and antioxidant enzymes are quinine oxidoreductase, g-GCS, GST, GRed, GPx, sulfotransferases, epoxide hydrolases and other enzyme superfamilies, and/or antioxidant genes, has been related to cis-acting elements, detected in the promoter region of those genes. They regulate either or both constitutive and inducible gene expressions and are called Antioxidant Response Elements (AREs) [96]. Transcription of antioxidant enzymes as GPx, SOD, and the proteins of Fe metabolism as ferritin and HO-1 are regulated by ARE [97]. Activation of ARE regions is mediated by different transcriptional factors that are specific of each tissue and each antioxidant response [98,99].

On the other hand, heme is another source of essential Fe, and like free Fe, their release into extracellular fluids has potentially severe consequences for health. Heme is highly toxic because of its ability to catalyze free radical formation, resulting in oxidative stress [44]. Heme that is not bound to proteins is considered the labile heme pool; this portion of heme is derived from newly synthesized heme that has not yet been incorporated into hemo- proteins, or heme that has been released from hemoproteins under oxidative conditions [100]. “Free heme” is an abundant source of redox-active Fe that can also participate in the Fenton reaction to produce the •OH. Due to its lipophilic nature, heme may initially lodge within the hydrophobic phospholipid bilayer. Within this highly oxidizable matrix, Fe catalyzes the oxidation of cell membrane and promotes the formation of cytotoxic lipid peroxide, which enhances membrane permeability, thus promoting cell lysis and death. Additionally, heme is a potent hemolytic agent. It affects erythrocyte membrane stability as a result of the increase of free radicals and oxidative membrane damage. Finally, heme is strongly pro-inflammatory since it induces the recruitment of leukocytes, platelets and red blood cells to the vascular endothelium, it oxidizes low-density lipoproteins and it consumes nitric oxide, thus impairing vascular function [101].

6. Current Fe Supplements with both Heme and Ionic Fe

Most oral Fe supplements contained ionic Fe and these preparations have the advantage of generally lower cost than products containing Fe from heme. However, disadvantages of ionic Fe supplements may include poorer tolerability and affect food absorption when dosed with meals. Supplements that contained ionic Fe can be further divided into ionic forms of Fe (ferrous sulfate, ferrous gluconate and ferrous fumarate) and non ionic forms of Fe (polysaccharide iron complex and carbonyl iron) [12]. The use of supplements of Fe from heme source as a concentrate has been customary since the publication in 1981, describing the enzymatic hydrolysis of pig hemoglobin by means of proteases (subtilisins) obtained from *Bacillus subtilis*. According to the information available, the japanese government has approved the use of a heme concentrate, known as Heme Iron Polypeptide (HIP), which is obtained from pig hemoglobin digested with proteolytic enzymes, which produced a highly soluble Fe from heme concentrate, with small globin polypeptide chains and Fe content in excess of 1%. HIP in Japon is marketed as a FOSHU food that is the Japanese term for a functional food or a food with a specific health application. It is recognized in this FOSHU designation that heme is a highly bioavailable source of Fe in human nutrition [102]. Heme iron supplements available in the United States are the dietary supplement Proferrin® ES (HIP) and the medical food Proferrin® Forte (HIP plus folic acid). Each tablet of Proferrin® ES contains 12 mg of elemental Fe as HIP and Proferrin® Forte additionally includes 1 mg of folic acid. Bifera® is the only dietary supplement made of a combination of heme Fe polypeptide and polysaccharide iron complex. Each tablet of Bifera®, contains 6 mg of Fe as heme Fe polypeptide as Proferrin® and 22 mg of Fe as polysaccharide Fe complex. Bifera® recommended dosing is one tablet daily with or without food, or as directed by a physician [12]. International regulations require that an antianemic preparation to treat Fe deficiency anemia, aport at least 30 mg of Fe [103]. The low Fe concentration in heme natural source, could explain that there isn´t in the market around the world an antianemic formulation with heme as Fe source, or the combination of both, heme and ionic Fe.

In Cuba, since the past century, there have been developed different proteins hydrolyzates as heme Fe source from bovine blood to prevent Fe deficiency anemia. As results of this work, there are in cuban market different products in oral solution as Ferrical® and Trofin® to prevent Fe deficiency anemia [11]. Other groups of preparations are in solid pharmaceutical forms, as NeoTrofin® and NeoTrofinCF® which are supplements to prevent Fe deficiency anemia in pregnancy [104]. These products contain as Fe source, a protein and heme hydrolyzate from whole bovine blood that is obtained by incubation with an extract of bovine pepsin from the ruminal abomasum. In other step of the manufacture

process of the protein and heme hydrolyzate, it is added honey bee as sweetener and a quantity of alcoholic extract of Cuban propolis having antimicrobial activity and ensures the microbiological quality prepared so that it can be used in the production of nutritional supplements for human use [11].

7. Beneficial Effect by the Supplementation with both Heme and Ionic Fe

Several nutritional studies have demonstrated the advantages of the supplementation with both heme and ionic Fe, to prevent and treat Fe deficiency anemia. These studies include basically two different experimental designers. In some studies a total quantity of Fe is given (heme and ionic) enough to complete the nutritional requirements of the mineral in animals or humans. Experimental studies that use the first strategy were reported by Campos *et al.* (1996) and Lisbona *et al.* (1999) in an animal model of anemia [23,24]. In these works they employed for the recovery of anemic rats a combination of corpuscles of bovine hemoglobin as source of Fe from heme and ferric citrate as ionic Fe, and a group was treated with Fe salt only. In these studies it was obtained that the bioavailability of Fe was increased when both chemical forms of mineral were used, and that it was dependent on the proportions used of either chemical form of Fe. This same experimental design was employed in healthy volunteers who were given the combination of bovine hemoglobin dehydrated and Fe bis-glycinate as a source of ionic Fe. This study demonstrated that the bioavailability of Fe was higher than when they were treated with bovine hemoglobin only [22].

Another group of studies to evaluate the effect of the supplementation with both heme and ionic Fe, used a lower amount of Fe to the nutritional requirements of the mineral. For instance, reported a double-blind study that treated healthy volunteers with a supplement containing a mixture of Fe from heme that was obtained from pig hemoglobin containing 1.2 mg of Fe and 8 mg ferrous fumarate [20]. This group was compared with a control group who were given a supplement containing 60 mg of Fe as ferrous fumarate only. After two months of supplementation, the serum ferritin and hemoglobin levels were similar in both supplemented groups, showing that less Fe as heme was required to maintain blood Fe levels.

In an anemic rat model, García *et al.* (2013) compared the efficiency of the treatment with combinations of a protein hydrolyzate from bovine blood, whose composition was described in the previous section, and ionic Fe from ferrous sulphate [105]. The total content of Fe supplement in this group represents 60 % of the nutritional requirements of Fe for rats (35 mg per kg of diet) [106]. Another anemic group and the control group received a normal Fe diet from ferrous sulphate. At the end of the experimental period of 14 days, the hemoglobin concentration in the group supplemented with both, a protein and heme hydrolyzate and ionic Fe, was higher than in the anemic group treated with ferrous sulphate only. Another interesting result in the group supplemented with both chemical forms of iron and proteins, and ionic Fe, was that serum ferritin concentrations showed no differences with regard to the control group. This result confirms the efficacy of this treatment in relation to the recovery of mineral reserves, which is the final stage in the physiological events to anemic recovery condition [107]. The hemoglobin concentration and Fe storage recovery obtained in the anemic group treated with the protein and heme hydrolyzate and ferrous sulphate, suggested that in anemic rat treated with both Fe source, absorption of Fe is facilitated by different pathways in both chemical forms of the mineral [67].

In order to clarify the results described above, the same group reported another research to evaluate the antianemic efficacy of the supplementation with both chemical forms of Fe, in anemic rat model. In this work they employed the same experimental design in relation to types and quantity of Fe, but in this case nutritional balance method was used, to analyze the mineral utilization and finally, and was measured indirectly, the amount of Fe absorbed at intestinal level [108]. This result showed that the high antianemic efficacy of the supplementation with both, the protein and heme hydrolyzate and ferrous sulphate, during anemia recovery, in relation to anemic group that was treated with ionic Fe source only, was not explained by the increased Fe absorption. This findings could be explained considering the pathway for heme absorption described in Caco-2 cells and in the intestine of other mammals, through the membrane transporters as FLCVR and ABCG2, which could take place in the basolateral membrane of rat enterocytes and thus enhancing the fully absorption of this molecule [88,109] (Figure 2). The assumption of the existence in rat of different absorption pathways for heme molecule, at basolateral enterocyte faces, are in agreement with the results reported by Cao *et al.* (2014) [75]. Due to in this work, the FPN1 activity is low by the elevated hepcidin levels, they suggested that the increase in absorption of Fe from heme, occurs from other mechanisms involving the transport of the whole molecule.

Another element that which contribute to the efficacy in the anemia recovery of the treatment with both, the protein and heme hydrolyzate and ionic Fe, is the lipidic nature of heme molecule, allowing direct absorption through the basolateral membrane of the enterocyte into the blood stream by diffusion [58]. Moreover, it could also be expected that the administration of heme at low concentrations, resulted in a use of the most efficient mineral regarding recovery from anemia, because it has been demonstrated that this molecule can be absorbed directly into the blood stream and also be used in the synthesis of hemoglobin by the erythroid cells [57,110].

8. Oxidative Effect by the Supplementation with both Heme and Ionic Fe

Most of the studies that reported the oxidative effects of Fe supplementation used ionic Fe source only [10]. The higher oxidative damage of these preparations is in correspondence with high doses of mineral administered

[6,8], due to the low mineral bioavailability of ionic Fe [19]. In addition, studies reported in relation to the assessment of oxidative effect of nutritional interventions that use sources of both heme and ionic Fe, have been assumed in the rat model of anemia by the same group of researchers mentioned above [108,110]. In these studies authors evaluated the oxidative effect during anemia recovery in rat treated with both, a protein and heme hydrolyzate and ferrous sulphate as ionic Fe source too. These studies showed that the oxidative damage was lower in duodenum, revealing that small amounts of Fe to treat anemia are able to reduce the oxidative effect [108], [110], even with dosages bellow reference normal levels of 35 mg for Fe per kg of diet [106]. There is a correlation between the low oxidative damage to proteins and lipids and the low activity of SOD and GPx (Díaz-Castro et al. 2013; García et al. 2013a [108,110]. These results indicate that a lower oxidative imbalance is recorded in the duodenum mucosa, compared with the anemic group treated with ferrous sulphate only. On the other hand, these results support the hypothesis proposed previously by Vaghefi *et al.* (2005) and Thomas *et al.* (2013) [14,111], reporting the contribution of amino acids and peptides from the protein and heme hydrolyzate from hemoglobin and other blood proteins, to facilitate ionic Fe chelation provided by the ionic Fe source (Figure 2) [15,16,17]. These conditions could enhance intestinal Fe absorption and could explain the low oxidative damage produced by the treatment with the combination of both heme and ionic Fe sources [108,110].

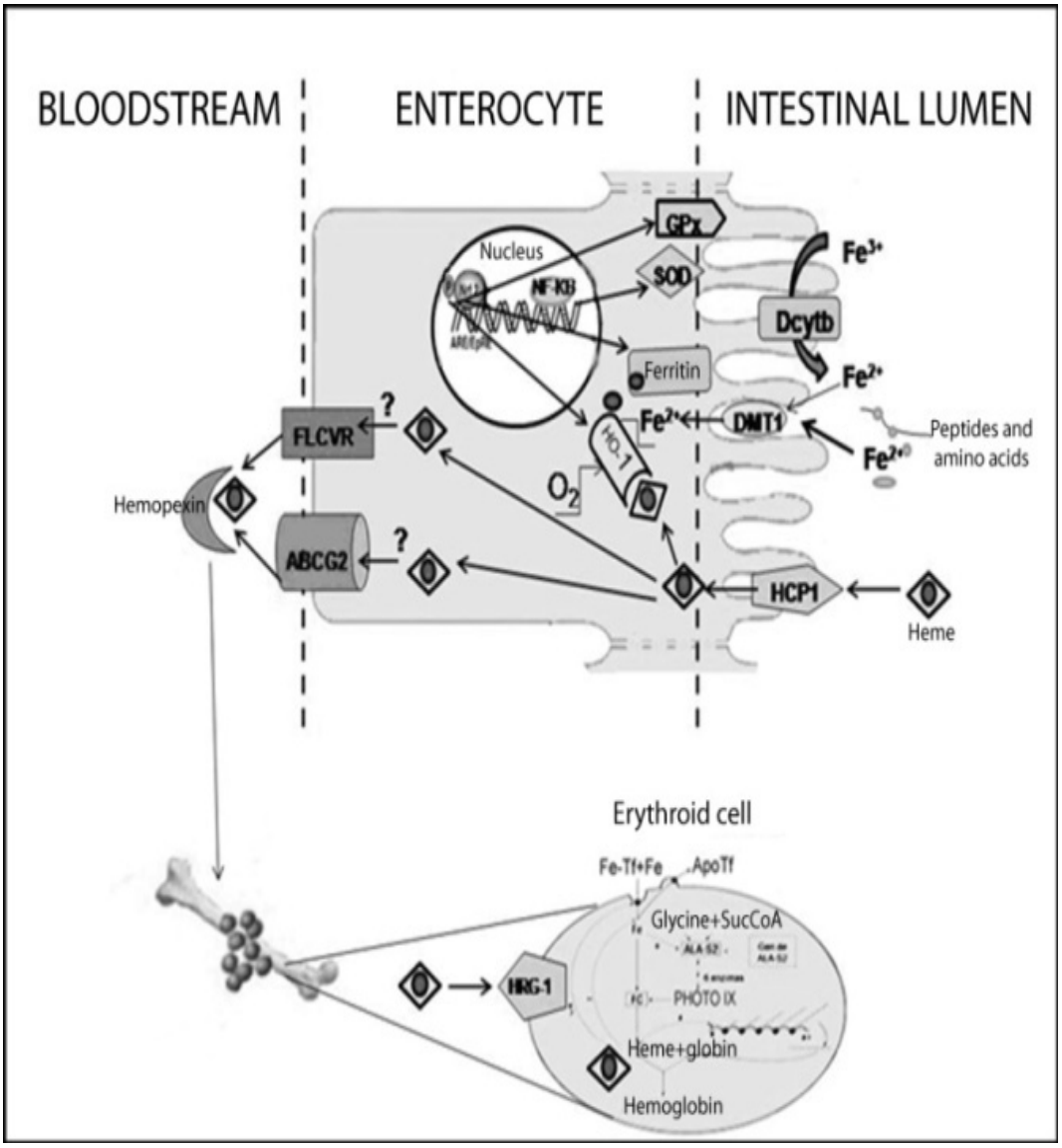

Figure 2. Physiological and biochemical events proposed to explain the anemia recovery during a treatment with both a protein and heme hydrolyzate and ionic Fe. The presence of peptides and amino acids (aa) released from the intestinal hydrolysis of bovine blood proteins decrease intestinal ionic Fe adverse effects and promote intestinal absorption mediated by DMT1. Oxidative effect generated at intestinal lumen by the presence of ionic Fe as Fe^{+2} decreased, and is less stimulated antioxidant response regulated at the level of DNA regions called the nucleus ARE, which is mediated by activating transcription factors as Nrf2 and NF-kß. This situation conduces to decreased activation of antioxidant enzymes as SOD, GPx and HO-1. The low activities of the antioxidant enzymes, coupled with the decreasing availability of O_2 as a consequence of anemia, stimulated that heme absorbed from the intestinal lumen by the transporter HCP1 not be degraded at enterocyte level by HO-1, and it is exported to plasma as an intact metalloporphyrin. This process may be mediated by membrane transporters as FLCVR or the ATP-Binding Cassette, subfamily G, a member 2 protein (ABCG2). In plasma, the heme is carried by hemopexin to the bone marrow where is transported to erythroid cells by heme response gen 1 transporter (HRG-1). Inside erythroid cell, heme molecule can be incorporated into hemoglobin synthesis process and this would be a more efficient way to recover from anemia situation that when erythroid cell only depends of heme synthesis

In these two works, authors also used in anemic rats a period of Fe supplementation of 30 days [108,110], which it is sufficiently long to occur as a cellular response to oxidative effect of Fe supplementation regulated at the DNA level of the intestinal cell, which stimulated the expression of endogenous enzymes responsible antioxidant response to oxidant stimulus of the continuous mineral supplementation [99,114]. They supposed that SOD and GPx activity increased in the post-mitochondrial fraction of the duodenal mucosa in anemic animals treated with the source of ionic Fe only, were mediated by the activation of transcription factors in the nucleus such as nuclear factor kappa B (NF-kB) and nuclear factor erythroid-2 related factor-2 (Nrf2), which have been described in several mammals [99]. NF-kB that is a transcriptional activator of the three isoforms of SOD [112], and Nrf2 can activate transcription of GPx, other GSH-dependent enzymes, in addition to ferritin and HO-1 [97].

The role of transcriptional activation of ferritin and HO-1, as cellular response to oxidative effect of Fe treatment at intestinal level is focused to reduce cellular toxicity of both heme and ionic Fe [58,88,97]. In relation to ferritin, this protein allows that the Fe stored in oxidized form as Fe^{3+} into the intestinal cell, being this chemical state less reactive than the reduced form Fe^{2+} [68]. On the other hand, the activity of HO-1 is induced by the presence of heme in the enterocytes and serves to separate the ion Fe^{2+} of the carbon backbone of this molecule, when it is absorbed by the intestinal receptor HCP1 [65]. Considering the results obtained in the Caco-2 cell line, in which heme transporter FLVCR cooperates with the HO-1 [16], it is possible to deduce that in the anemic group treated with the combination of both chemical forms of the mineral, the HO-1 activity was stimulated to a lower extent. It is also known that the activity of HO-1 is dependent on NADPH and O_2 (West and Oates 2008) [65]. Because of, during Fe deficiency anemia condition it's low the production of red blood cells to carry O_2 to all tissues [113], it's highly probable that at intestinal level the availability of O_2 should be low too. This situation could also affect the activation of HO-1. All these elements favored heme efflux into the blood stream, before their degradation in the enterocytes. The possible relationship among cell responses to the stimulus that produces oxidative treatment with the combination of the protein and heme hydrolyzate and ionic Fe, and the stimulation of intestinal absorption heme to stimulate recovery from anemia is showed in Figure 2.

Moreover, it is important to notice that when heme is circulating in the bloodstream, in situations where there is no Fe deficiency, it is a highly toxic molecule and it is eliminated by liver macrophages [44,88]. However, in severe ferropenic anemia condition, the possibility to be used in the hemoglobin synthesis is greater, in order to facilitate the anemia recovery. In this regard, it has been shown in biopsies of erythroid cells from bone marrow of human, as well as rabbit erythrocytes, that these cells are capable to uptake the heme administered as hemin or hematin [114]. These molecules derived from heme are obtained from human blood since the last century for the treatment of porphyria [115,116,117]. In addition, it is also known in the erythroid cells the transporter HRG-1 which acts as an importer of heme [88,109,117]. All these elements indicate that in anemic rats treated with the protein hydrolysate as source of heme and ferrous sulphate could also enhance heme uptake, which was absorbed completely, to the erythroid cell and increases hemoglobin synthesis process (Figure 2). This form of heme supplied to the erythroid cell is much more efficient to stimulate anemia recovery than the process that only depends on endogenous synthesis of this molecule, which is highly complex, because it requires several steps and it is energetically costly.

9. Conclusions

Supplementation with both heme and ionic Fe, during anemic recovery condition, allows the optimization of Fe sources to stimulate, simultaneously, two beneficial events for human's life: the anemia recovery, and the decreased oxidative damage caused by traditional Fe therapies from ionic source, to treat nutritional anemia by Fe deficiency.

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