Quercetin as a shuttle for labile iron

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1. Introduction

Iron is the most important transition metal for animal metabolism, being involved in redox-active enzymes and oxygen transport. Since it is of value to organisms, biological iron is protected against spurious reactions and accidental mobilization by stable molecular frameworks such as porphyrin complexes in heme proteins, mineral deposits in ferritin and an O-donor-amino acid-enriched coordination environment in transferrin, which stabilizes the metal in its trivalent state. Besides protection against loss to competitive organisms, these chemical safeguards inhibit the generation of deleterious reactive oxygen species (ROS) catalyzed by endogenous substrates such as peroxide, superoxide and ascorbate, one of the most important pathways being the generation of hydroxyl radicals via the Fenton reaction [1].

The iron burden in a healthy subject is controlled by absorption, and there is a significant buffering capacity of circulatory apotransferrin that can deal with momentary episodes of overload as a result of iron supplementation and/or dialysis [1,2]. However, there are instances in which iron overload may occur. Primary (hereditary) iron overload is the result of a primary defect in the regulation of the iron balance, e.g. hereditary hemochromatosis. Secondary iron overload is caused by repeated blood transfusions used to treat certain hematological disorders (e.g. thalassemia, sickle cell disease and myelodysplastic syndromes) or toxic ingestion [2].

Whatever the cause, iron overload gives rise to labile iron pools, which may be cytosolic or in the plasma (NTBI, non-transferrin-bound iron). The chemical composition of NTBI is heterogeneous [3] and involves iron complexes with citrate, amino acids and other serum ligands [4,5]. There is a fraction of NTBI which is redox-active and is termed LPI (labile plasma iron), the arguably toxic component of NTBI which can be controlled by chelation therapy [6]. Increased LPI has been related to neurological damage after ischemia [7], endothelial dysfunction in thalassemic children [8], cognitive defects in Alzheimer’s patients [9], complications of myelodysplastic syndrome [10], especially infection and tissue damage [11], and increased morbidity in transfused [12] or diabetes [13] patients. Treatment of iron overload involves a single or a combination of chelators such as desferrioxamine, deferiprone or deferasirox [14].

However, there are instances in which iron overload may occur without generalized hyperferremia, such as in Friedreich’s ataxia, neurodegeneration with brain iron accumulation, sideroblastic anemia and anemia of chronic disease [15]. In these cases the organism is afflicted by a heterogeneous and unbalanced accumulation of iron within specific compartments, being regionally iron-overloaded even under a general situation of anemia. Therefore, rather than the elimination of excess iron, the therapeutic approach in these cases should aim toward its redistribution [15-18]. Specifically, chelators should be sought that (i) are able to penetrate iron-overloaded tissue, (ii) coordinate iron in order to form stable and redox-inactive species (thus decreasing intracellular labile iron pools), and (iii) transfer it to the circulatory transferrin. Deferiprone, one of the orally-active iron chelators, satisfies all three criteria [18]. EDTA, on the other hand, does not meet the second criteria, forming highly redox-active complexes with...
Polyphenols are low-molecular weight flavanoids present in a great variety of plant foods and pigments [20,21]. Some of them have recognized anti-inflammatory, neuro-regenerative and anti-atherosclerotic activities [22-24]. Most of the beneficial pharmacological activities of polyphenols are believed to result from their antioxidant activity, either by ROS scavenging or iron chelation; catecholate polyphenols have extremely large (logβ = 39–44) stability constants with Fe³⁺ (for a review, see [21]). More specifically, in vitro and in vivo models indicate that supplementation of polyphenols such as quercetin [25,26], catechin, diosmetin [25], rutin [27], baicalin [26,28], myricetin [29] and silybin [30] decreases the deleterious effects associated with iron overload, these therefore being proposed as useful clinical iron chelators [31,32].

In this study, the main objective was to investigate polyphenols as shuttles for labile iron. To this aim, the ability of a series of polyphenols (diosmin, hesperidin, naringin, naringenin, quercetin and rutin; Fig. 1) to decrease the redox activity of both mimetic and natural forms of labile plasma iron (LPI) was assessed in a preliminary trial of the most promising candidate(s) that can remove this important fraction of iron under iron-overload conditions. Our results indicated that only the catechol-containing quercetin and rutin suppressed iron redox activity. Further experiments were conducted with both polyphenols to test whether they are able to mobilize iron from endogenous ligands, to transfer iron to transferrin, and to successfully permeate an iron-overloaded cell and chelate the metal. It was observed that the catechol moiety of quercetin and rutin rendered redox-inactive. However, despite displaying similar abilities to mobilize iron to and from biomimetic ligands, in vitro experiments showed that only quercetin was able to rapidly permeate the cells and suppress intracellular labile iron pools.

2. Materials and methods

2.1. Chemicals

The polyphenols, HEPES, nitrilotriacetic acid (NTA), ferrous ammonium sulfate (FAS), ascorbic acid, calcein, calcein-AM (the cell-permeant form of calcein) and bovine holo-transferrin were obtained from Sigma-Aldrich. Dihydorhodamine hydrochloride (DHR) was obtained from Biotium. Deferiprone was donated by Apotex. HBS (HEPES Buffered Saline) buffer (NaCl 150 mM, HEPES 20 mM; pH 7.4) treated with Chelex-100® (Sigma, 1 g/100 mL) was used throughout the experiments.

2.2. Iron pro-oxidant activity

The pro-oxidant activity of FAS (5 μM) in the presence of ascorbate and the protective effect of the polyphenols was evaluated by a fluorescence method described elsewhere [6] which can be used to assess anti- or pro-oxidant activity under physiologically relevant conditions of salinity, temperature and ascorbate concentration. The kinetic test measures the relative rates of iron-catalyzed oxidation of the fluorescence probe DHR. Aliquots (10 μL) of FAS were transferred to transparent, flat bottom 96-well microplates and treated with 10 μL of the polyphenol solution and 180 μL of a mixture of 40 μM ascorbate and 50 μM DHR in HBS. Assays were performed in duplicate. Fluorescence was measured on a BMG Fluostar Optima instrument for 40 min at 37 °C (λexc/λem = 485/520 nm). The test was repeated with the samples of sera from myelodisplastic patients, some of whom were diagnosed with iron-overload. The samples were furnished by Dr Nelson Hamerschlak, Albert Einstein Hospital, São Paulo.

Due to their ability to suppress iron pro-oxidant activity, rutin (rut) and quercetin (querc) were selected for further experiments.

2.3. Competition studies with calcein

Calcein (2 μM in HBS) fluorescence was recorded at room temperature on a BMG Fluostar Optima instrument (λexc/λem = 485/520 nm) for 10 min and then loaded with FAS (2 μM). After stabilization of fluorescence quenching (~13 min), the solutions were treated with increasing concentrations of rutin and quercetin in 10 μL aliquots and fluorescence was further recorded until stabilization.

2.4. Competition studies with apotransferrin

Iron:rutin and iron:quercetin complexes (1:1 metal:ligand mol ratios) were prepared in methanol (2 mM in Fe) by incubation for 1 h at 37 °C. Fluorescein apotransferrin (FITF) was prepared by a previously described method [33] which involves the conjugation of 5-(4,6-dichlorotriazinyl)aminofluorescein (5-DTAF) with holotransferrin and subsequent dialysis against citrate (pH 5.5) to remove the metal. FITF solutions (2 μM) were treated with the iron complexes described above and FITF fluorescence quenching (indicating iron transfer to transferrin) was recorded (λexc/λem = 485/520 nm).

2.5. Cell studies

Human cervical epithelialoid carcinoma (HeLa) cells were cultivated in DMEM medium supplemented with 10% fetal calf serum and 1% antibiotics, at 37 °C and 5% CO₂. Cells were trypsinized and transferred (5 × 10⁴ cells/well; 2 mL) to the plates of a 24-well microplate and kept for 24 h until complete adherence and confluence. The medium was then removed and the cells were washed with HBS/Chelex buffer and incubated with the cell-permeant iron fluorescent probe acetomethoxycalcein (Cal-AM, 0.32 μM) for 10 min at 37 °C. Cells were washed and treated with 1 mL of a mixture of 10 μM FAS and 100 μM ascorbate in HBS/Chelex (iron-overloaded cells) or 1 mL HBS/Chelex (iron-normal cells) and incubated for 15 min at 37 °C. After washing, iron-overloaded cells were treated with 20 μM chelators (quercetin and rutin) and iron-normal cells were treated with 20 μM (chelator-based) Fe(querc)₂ or Fe(rut)₂ complexes. Fluorescence was recorded on a BMG Fluostar Optima instrument (λexc/λem = 485/520 nm).

In another experiment, iron trafficking in the cells was assessed by fluorescence microscopy. Cells (5 × 10⁴ cells/glass-bottom Petri
were able also to decrease LPI from sera of iron-overloaded patients and the effect of the polyphenols, measured as a decrease in the rate of DHR, is reported in Fig. 2.

The antioxidant activity as assessed by inhibition of iron-catalyzed ascorbate autoxidation in physiologically relevant conditions corroborates previous findings [21] that polyphenols with binding sites with a high affinity for iron are better suited for a polyphenol-based iron chelation therapy.

Maximum protection against DHR oxidation started by Fe(II) was observed at an iron:ruvitin mol ratio of ~1:1 (i.e., when Fe= flavonoid = 5 μM, Fig. 2) and higher for the iron:quercetin system. Polyphenols are molecules with multiple donor atoms, and thus their iron coordination compounds may assume different stoichiometries (from 1:1 to 1:3) which are dependant on the synthetic conditions [21,35,36]. In another experiment we pre-formed iron complexes of several ligands (deferiprone, NTA, querc or rut) at three different metal:ligand ratios by the reaction of FAS with the ligands in a DMSO solution followed by incubation at 37 °C for 2 h. The DHR oxidation rates were then recorded (Fig. 3). Iron nitrilotriacetate is the positive (pro-oxidant) control in the original LPI detection method, and the orally-active iron chelator deferiprone is the negative control [6].

Deferiprone is a bidentate iron chelator which forms maximally Fe (deferiprone)3 complexes. Accordingly, DHR oxidation catalyzed by iron decreases with increasing ligand:metal ratio. This trend can also be noted for iron–flavonoid complexes, where the highest protection against oxidation is observed for the Fe(flavonoid)2 complex, in agreement with the stoichiometry proposed by Afanasev et al. which leads to maximum protection against lipoperoxidation [37]. This is in marked contrast to iron complexes with the aminopolycarboxylates EDTA [11] or NTA [6], where the complexes are known to be redox-active. When metal and ligands interact only during the course of the reaction (Fig. 2), the faster formed 1:1 complexes already exerted anti-LPI activity. Other highly substituted complexes (Fig. 3) present increased anti-LPI activity.

Calcein, a fluorophore containing an EDTA-like binding motif, is extensively used as an iron probe for the assessment of non-transferrin-bound iron (NTBI) in iron overloaded patients [38].

3. Results and discussion

Under physiological conditions, ascorbate and excess iron may generate ROS in both plasma [6] and cytosol [34]. Labile plasma iron (LPI) is conveniently assessed fluorometrically by the oxidation of DHR. Indeed, this characteristic has been used to evaluate the status of iron-overloaded patients and the efficiency of iron chelation therapy [14]. Therefore, control of LPI assessed by suppression of DHR-monitoring ascorbate autoxidation is believed to be an important property of candidate iron chelators. FAS/ascorbate solutions were chosen as a model of oxidative stress induced by LPI, and plasma samples from iron-overloaded patients were also studied. The antioxidant effect of the polyphenols, measured as a decrease in the fluorescence rate of DHR, is reported in Fig. 2.

Only rutin and quercetin were effective inhibitors of the redox activity catalyzed by iron in an FAS solution (2A), and these chelators were able also to decrease LPI from sera of iron-overloaded patients (Fig. 2B). The antioxidant action of polyphenols is a subject of great interest and has been thoroughly reviewed [21]. There is a positive correlation between the number of catechol or gallol moieties in the polyphenol structure (especially at the ring B, but also at ring A) and antioxidant activity, indicating that strong iron chelation is directly associated with this property. Among the polyphenols studied, rutin and quercetin are the only catechol derivatives (Fig. 1, R3=R4=OH). Although other mechanisms have been proposed (such as direct radical scavenging by flavonoids [21]), antioxidant activity as assessed by inhibition of iron-catalyzed ascorbate autoxidation in physiologically relevant conditions corroborates previous findings [21] that polyphenols with binding sites with a high affinity for iron are better suited for a polyphenol-based iron chelation therapy.

![Fig. 2.](image-url)

**Fig. 2.** (A) Effect of the polyphenols on the rate of ascorbate autoxidation catalyzed by (A) FAS (5 μM) in HBS/Chelex buffer (pH 7.4). (B) Sera from iron-overloaded myelodysplastic patients (PAT) were treated with deferiprone (50 μM), quercetin or rutin (100 μM). *P<0.05, significantly different according to Student’s t test employing Origin data analysis software (version 7.0). (control = serum from a normal subject without myelodysplastic syndrome; fu = arbitrary fluorescence units. The y-axis represents the slope of the increase of fluorescence intensity with time. Results are the average of duplicate experiments and representative of at least two separated experiments).

![Fig. 3.](image-url)

**Fig. 3.** Comparison of the effect of different iron chelators on the rate of ascorbate autoxidation catalyzed by pre-formed iron complexes (metal concentration 10 μM in all cases) in HBS/Chelex buffer (pH 7.4). (fu = arbitrary fluorescence units. The y-axis represents the slope of the increase of fluorescence intensity with time).
in order to determine the apparent stability constant ($K_{\text{app}}$) of the respective iron complexes in a physiologically relevant medium (Fig. 4).

Stoichiometric (1:1) quenching of calcein (CA) fluorescence by iron is faster in the presence of Fe(II), where the metal is promptly oxidized by calcein upon coordination [39], so that ferric ions are exchanged between calcein and the flavonoid. Treatment of the calcein-iron complex with increasing amounts of flavonoid chelator (chel; rutin or quercetin) leads to steady recovery of the fluorescence. These competition experiments involved a large excess of ligand over Fe, so that a 1:3 Fe:catechol stoichiometry is assumed. The equilibria involved are (Eqs. (1)–(3)):

$$\text{CAFe} = \text{CA} + \text{Fe}K_{\text{diss}}$$

(1)

$$\text{Fe} + 3\text{chel} = \text{Fe(chel)}_3 \quad K_{\text{app}}$$

(2)

$$\text{CAFe} + 3\text{chel} + \text{CA} = K_{\text{diss}}K_{\text{app}}$$

(3)

The expression for $K$ is given by Eq. (4):

$$K = \frac{[\text{CA}][\text{Fe(chel)}_3]}{[\text{CAFe}][\text{chel}]^3}$$

(4)

A plot of the fluorescence recovery as a function of flavonoid concentration (Fig. 4B) indicates that for [rut] = 158 μM or [querc] = 9.2 μM there is a 50% fluorescence recovery, indicating that half of the calcein is binding with iron and thus [CA] = [CAFe]. Therefore, half (1 μM) of the original iron is complexed by the flavonoid as Fe(chel)$_3$. Substitution of these figures in Eq. (4) for each chelator and each stoichiometry (1:1, 1:2 or 1:3 Fe:L) and assuming the effective $K_{\text{diss}}$ to be $1 \times 10^{-24}$ [40], log$K_{\text{app}}$ can thus be estimated as Fe(querc)$_3$ = 33 and Fe(rut)$_3$ = 29. These apparent formation constants are lower than the thermodynamic constants for both chelators at 1:3 metal:chelator mol ratio (log$K$ = 44 [21]) since they account for salinity and buffering, and assumed an effective dissociation constant for CAFe. Nevertheless they are believed to be a good indicator of thermodynamic stability under a more relevant physiological condition. $K_{\text{app}}$ indicates that quercetin is a particularly good iron scavenger under these conditions, which again supports its primary role as an antioxidant due to immobilization of iron in stable, redox-inactive forms that are of great interest for chelation therapy.
As iron remobilization in the organism has recently gained considerable interest [15], besides forming redox-inactive, stable complexes with iron, the chelators must subsequently be able to deliver it to its due systemic distributor, transferrin, in a timely fashion. It was demonstrated that iron: rutin and iron: quercetin complexes, independently of the metal:ligand mol ratio, were quickly disrupted when challenged with fluorescent apoTf (FlTf; Fig. 5).

FlTf is a very convenient fluorescent probe to assess online iron traffic from both plasma and exogenous chelators in high-throughput systems [33]. Inspection of Fig. 5 shows a quick drop in the fluorescence immediately after Fe(rut) or Fe(querc) addition to the system, with 50% of decrease of FlTf quenchable fluorescence when the metal:transferrin ratio is 1 (half of transferrin saturation) and near total quenching when this ratio is >2. Since these are equilibrium competitions between transferrin and the polyphenols, an excess of the iron complexes is required to fully saturate the protein (as indicated by the ~5× excess required to suppress FlTf quenchable fluorescence). Therefore, both quercetin and rutin form stable, redox-

![Fig. 6](image)

**Fig. 6.** HeLa cells in microplates were loaded with calcein-AM and treated with excess iron (iron-overloaded cells) or buffer (normal-iron cells). Iron-overloaded cells were further treated with quercetin (Q) or rutin (R) 20 μM. Normal-iron cells were treated with 1:2 iron:chelator 20 μM (chelator based). Asterisks indicate differences (P<0.05) relative to the respective controls after Tukey test (one-way ANOVA) (au= arbitrary units).

![Fig. 7](image)

**Fig. 7.** Effect of quercetin, rutin and their iron complexes on the fluorescence of HeLa cells treated with 0.30 μM of calcein-AM. Cells before (A0, B0) and after 10 min of treatment with 20 μM Fe(querc)₂ (chelator based) (A1) or 20 μM Fe(rut)₂ (chelator based) (B1). Iron-overloaded cells (see Materials and Methods) before (C0, D0) and after treatment with 20 μM quercetin (C1) or rutin (D1). Bar diagrams: Fluorescence intensity per area for each treatment was determined for six regions of interest in each picture. *P<0.05, significantly different according to Student’s t test employing Origin data analysis software (version 7.0).
inactive complexes with iron, which are nonetheless sources of the metal to transferrin.

Finally, candidate remobilization chelators must be able to cross biological membranes when loaded with iron. The permeability of a cell membrane model (HeLa) to the iron polyphenols was studied in a microplate reader (Fig. 6) under two different setups. In the first, cells were loaded with iron and then with the probe calcein AM, whose fluorescence is dependent on the cleavage of the acetoxy group by esterases within the cell. Quercetin or rutin (20 μM) were then added to the culture medium and the fluorescence was again registered after a few minutes. We observed that only iron-overloaded cells treated with quercetin displayed a significant fluorescence recovery, indicating that this was the only polyphenol that could cross the biological membrane. In a second experiment, cells were treated only with calcein-AM and then with pre-formed iron–polyphenol derivatives (Fe:polyphenol = 1:2; [polyphenol] = 20 μM). In this case a significant quenching was observed for the two derivatives, indicating that both could cross the biological membrane. These results indicate that quercetin is more efficient than rutin as a shuttle for labile iron, since it can cross the cell membrane in both directions: either loaded or unloaded with iron. Rutin as a free chelator, on the other hand, is prevented from accessing the interior of the cell.

Further confirmation of the above trend was obtained by fluorescence microscopy of Hela cells loaded with cal-AM (Fig. 7). Again, it was observed that cells treated with Fe(querc)2 (A) or Fe(rut)2 (B) exhibit decreased fluorescence, indicating that both complexes are permeable to the cell membrane (although in the case of Fe(rut)2 this trend did not reach statistical significance in this experiment). However, only free quercetin (C) but not free rutin (D) was observed for the two derivatives, indicating that both could cross the same biological membranes and compete for iron in iron-overloaded cells, downregulating the fluorescence of the probe. We were able to observe this dequenching for rutin only at very high concentrations (>250 μM), which are not biologically relevant (data not shown).

The issue of whether or not rutin may cross biological membranes seems to be influenced by the model under study. The hydrophilic nature of the glycosidic substituent of rutin was found to deter its transport into red blood cells [41] (unless at ~0.2 mM concentration, when its absorption may reach 25% [42]) and limit its intestinal absorption rate to ~5% [43]. Also, failure to induce hypoxia-induced-factors (which is enhanced by lipophilic iron chelators such as quercetin [44]) points to the poor absorption of rutin by cells. However, rutin administration to iron-overloaded rats was shown to be beneficial, inhibiting some of the toxic effects of the metal [27], and this activity probably does not involve rutin deglycosylation since the conversion of rutin to quercetin by both human and murine β-glucosidases is not efficient [43]. Quercetin, being more lipophilic than rutin, may cross lipidic bilayers more easily. In addition, it was recently shown that glucose transport proteins allow the traffic of both quercetin and iron–quinone complexes, effectively assisting iron shuttling [45].

4. Conclusions

In this study we demonstrated that the cathecol-containing flavonoids quercetin and rutin are able to decrease LPI in solution and to shuttle iron to transferrin. Quercetin is able also to decrease intracellular iron pools, and iron–quercetin complexes can cross biological membranes. These findings considered together suggest that quercetin could represent a useful chelator for iron-redistribution therapy. The relevance of these findings depends on experiments with in vivo models of iron overload.

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References