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Dendritic Cells Modulate Iron Homeostasis and Inflammatory Abilities Following Quercetin Exposure

Vanessa Galleggiante[1]*, Stefania De Santis[1,2]*, Elisabetta Cavalcanti[1], Aurelia Scarano[2], Maria De Benedictis[2], Grazia Serino[1], Maria Lucia Caruso[1], Mauro Mastronardi[1], Aldo Pinto[3], Pietro Campiglia[3,4], Dale Kunde[5], Angelo Santino[2]*, Marcello Chieppa[1,3,4]*

*these authors equally contributed

1 National Institute of Gastroenterology "S. de Bellis", Institute of Research, Castellana Grotte (BA), Italy.

2 Institute of Sciences of Food Production C.N.R., Unit of Lecce, via Monteroni, 73100, Lecce, Italy.

3 Department of Pharmacy, School of Pharmacy, University of Salerno, Fisciano (SA), Italy.

4 European Biomedical Research Institute of Salerno (EBRIS), Salerno, Italy.

5 University of Tasmania, School of Health Sciences, Launceston, Tasmania, Australia.

Correspondence to: Marcello Chieppa, **email:** transmed@irccsdebellis.it

ABSTRACT

Background: Fruit and vegetables are rich in plant polyphenols, whose consumption is encouraged in healthy dietary regimes due to their antioxidants and anti-inflammatory effects. These organic molecules possess numerous properties including phylochelation; the ability to create complex with metal ions. Among polyphenols, we focused our attention to quercetin that previously demonstrated its ability to reduce dendritic cells (DCs) inflammatory cytokine secretion and antigen presentation following LPS exposure. Dendritic cell inflammatory response is also associated with modulation of several iron metabolism related genes.

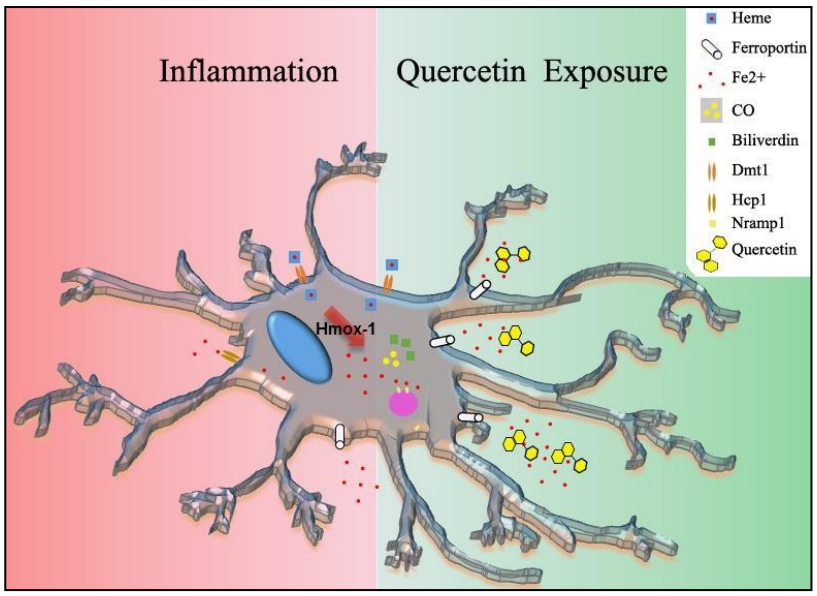
Objective: characterize the axis between quercetin exposure and iron extracellular transport that may explain polyphenol anti-inflammatory abilities.

Method: Bone marrow derived DCs were exposed to 25µM of quercetin on day 7 and treated with 1 µg/mL of LPS on day 8. The relation between quercetin exposure and the expression level of genes involved in iron homeostasis was addressed by qPCR. The axis between iron export and quercetin exposure was confirmed in vitro and in vivo using quercetin gavage and quercetin enriched diet.

Results: Here we demonstrate that DCs, exposed to quercetin, activate a pattern of genes that increase extracellular iron export, resulting in an overall decrease in the intracellular iron content and consequent diminished inflammatory abilities. This DCs phenotype is consistent with anti-inflammatory phenotype of the mucosal resident DCs, the ones most commonly exposed to polyphenols.

Conclusions: Iron balance is a crucial checkpoint for DCs inflammatory abilities. Quercetin-enriched nutritional regimes that favor DCs extracellular iron transport could reduce the incidence of chronic inflammatory syndromes.

GRAPHICAL ABSTRACT



Keywords: Dendritic Cells; Iron; Inflammation; Mucosal Immunology, Quercetin; Nutrition

INTRODUCTION

Among the numerous metals required for life, iron plays a major in many biochemical reactions of nearly all living organisms from eukaryotes to archaea and bacteria. Humans primarily require iron to deliver and store

oxygen, as a reactive element in the mitochondrial electron transport chain and as well as a co-factor in a number of enzymes [1, 2]. In humans, hemoglobin synthesis requires approximately 20mg/day of iron but only 10% of it could be absorbed from the diet, mainly compensating for constitutive losses due to bleeding or desquamation [3]. Since dietary iron availability is very low, many iron-balancing systems are in place to conserve and recycle it. New erythrocytes mature every day while old ones are destroyed in the spleen where macrophages degrade heme and release iron excess for recycling. Because of its chemical property an excess to free reactive iron is toxic therefore it requires sequestration on a variety of carrier and storage proteins resulting in no free iron present in biological fluids. Tight control of iron/heme intracellular transport, heme degradation and iron export is required to regulate the extra- intracellular iron concertation [4]. In this context, the divalent-metal transporter 1 (Dmt1) and heme carrier protein (Hcp1) are involved in iron and heme uptake [5]. Heme catabolism requires heme oxygenase (Hmox1), a cytosolic enzyme able to produce ferrous iron, biliverdin and carbon monoxide [6]. Ferrous ions are then exported extracellularly by ferroportin-1 (Fpn1) [7]. Finally, phagosomal membrane also contribute to the iron transport via the Natural resistance-associated macrophage protein 1 (Nramp1) that exports iron from the phagosome into the cytoplasm [8]. The balance between intracellular influx and extracellular export is also associated with the activation of immune cells, particularly macrophages and DCs. These cells can be activated to support inflammation or tissue healing and tolerance. Inflammation favors iron influx while tolerance favors heme degradation and iron export [9]. Further supporting this concept, recent data demonstrated that iron depletion inhibits DCs maturation [10]. As mentioned, iron is also a crucial element for bacterial growth, for this reason, inflammation and proinflammatory cytokines suppress ferroportin-1 dependent iron export to withhold iron from the extracellular compartment [11-12]. At the same time, during inflammation, iron intake is favored by Dmt1 expression. We recently described the anti-inflammatory effects of quercetin administration to bone marrow derived murine DCs [13,14, 15]. Keeping in mind that quercetin is also a well described phytochelator able to bind Fe^{2+} with high affinity [16, 17] we investigated the effect of quercetin administration to in vitro cultured murine DCs in the context of iron metabolism pathway. Confirming previous observation, we noticed that quercetin supplementation in the culture media suppress inflammatory cytokines production, but the effect is lost if an excess of iron is present in the media. In line with the observed anti-inflammatory effects, quercetin administration was able to induce strong upregulation of Hmox-1 and ferroportin-1 (Fpn-1) transcription that result in an extracellular iron release and reduced cytoplasmic iron. Hcp-1 transcription was also induced favoring heme intake that serves as substrate for Hmox-1. Furthermore, the molecular pattern that defines iron metabolism of alternatively activated macrophages (or M2) [18] was characterized in the intestinal $CD11c^{+}$ enriched cells of mice that received quercetin supplementation in the diet. In particular, differently from M1 macrophages, M2 show elevated level of Fpn-1and Hmox-1 leading to increased ferrous iron export [19].

Overall, we characterized a new hallmark of the quercetin induced gene pattern that may contribute to explain polyphenol anti-inflammatory abilities.

MATERIALS AND METHOD

Mice

Ethics Statement: investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines and has been approved by the authors' institutional review board.

6- to 8-week-old male mice were purchased from Jackson Laboratories: Wild-type C57BL/6 (Stock No: 000664; weight: approximately 20gr).

All animal experiments were carried out in accordance with Directive 86/609 EEC enforced by Italian D.L. n. 116 1992, and approved by the Committee on the Ethics of Animal Experiments of Ministero della Salute - Direzione Generale Sanità Animale (Prot. 2012/00000923 A00:Eo_GINRC) and the official RBM veterinarian. Animals were sacrificed if found in severe clinical condition in order to avoid undue suffering.

Generation and culture of murine DCs

Bone marrow derived DCs (BMDCs) were obtained from C57BL/6. Briefly, single cell suspension of BM cells from the tibiae and femurs of 6- to 8-week-old male C57BL/6 mice were flushed with 0.5mM EDTA (Thermo Fisher Scientific, MA, USA), and depleted of red blood cells with ACK lysing buffer (Thermo Fisher Scientific, MA, USA). Cells were plated in a 10 ml dish ($1x10^6$ cells/mL) in RPMI 1640 (Thermo Fisher Scientific, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Thermo Fisher Scientific, MA, USA), 100 U/mL penicillin (Thermo Fisher Scientific, MA, USA), 100 mg/mL streptomycin (Thermo Fisher Scientific, MA, USA), 25 µg/mL rmGM-CSF (Miltenyi Biotec, Bergisch Gladbach, Germany), and 25 µg/mL rmIL-4 (Miltenyi Biotec, Bergisch Gladbach, Germany) at 37°C in a humidified 5% CO_2 atmosphere. On day 5 BMDCs were differentiated, with new growth factors and plated at $1x10^6$ cells/mL on 24-well culture plate. To evaluate the iron-induced inflammatory cytokine secretion, differentiating cells (at day 7) were treated with quercetin (Sigma-Aldrich, St Louis, MO, USA) or OH-piridone used as control chelator. Immediately after, FeCl3 (Sigma-Aldrich, St Louis, MO, USA) and Ascorbic Acid for 24 hours were additioned in the culture media. On day 8 BMDCs were stimulated with 1 µg/mL of LPS (Sigma-Aldrich, St Louis, MO, USA) for 24 hours. For mRNA expression differentiating cells were treated with quercetin on day 5 and day 7. On day 8 BMDCs were stimulated with 1 µg/mL of LPS (Sigma-Aldrich, St Louis, MO, USA) for 6 hours.

RNA extraction and qPCR analysis

Total RNA was isolated from BMDCs, and tissue using TRIzol® (Thermo Fisher Scientific, MA, USA) according to manufacturer's instructions. 500ng of total RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, MA, USA) by using random primers for cDNA synthesis. Gene expression of Dmt1, Hcp1, Nramo1, Fnp1, Hmox1 and GAPDH was performed with TaqMan Gene Expression Assays (Thermo Fisher Scientific MA, USA) - murine probes: Mm00435363, Mm00546630_m1, Mm00443045_m1, Mm01254822_m1, Mm00516005_m1 and Mm00484668_m1,

respectively. Real-time analysis were run on CFX96 System (Biorad, CA, USA) and the expression of all target genes was calculated relative to GAPDH expression using ΔΔCt method.

ELISA

Cell culture supernatants were analyzed for IL-6 and IL-12p70 release in triplicate, using an ELISA kit (R&D Systems, Minneapolis, MN, USA) following manufacturer' instructions.

Western blotting

The amount of Hmox1, after stimulation of quercetin, was determined by Western blot analysis. Total protein extract were prepared with lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8), 1% NonidetP-40, 0.1% sodium deoxycholate, 0.1% SDS, plus proteinase inhibitors. The protein concentration was determined by the Bradford assay (BioRad). 30 □g of each protein lysate was separated on a 10% SDS-PAGE andtransferred to polyvinyldene difluoride (PVDF) membrane (Millipore). The membranes were incubated in 5% non-fat milk powder diluted in PBS containing 0.1% Tween-20 (T-PBS) for 2h at room temperature (RT) and probed with a rabbit polyclonal anti–HMOX1 antibody (PA5-27338 ThermoFischer scientific MA, USA) in blocking buffer overnight at 4°C at a final dilution of 1:1000. Finally, membranes were incubated with secondary antibody of horseradish peroxidase conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) at a final dilution of 1:5000. Immunocomplexes were detected with the ECL method (GE Healthcare). The same membranes were stripped and re-probed with anti-□□-tubulin monoclonal antibody (Santa Cruz Biotechnology) at a final dilution of 1:2000. Images of Western-blots were acquired and quantified using a ChemiDoc MP (Biorad) apparatus. Ratio between intensities of Hmox1 and □-tubulin bands was used to normalize Hmox1 in each sample.

Iron staining

BMDCs, were plated for 24h into Glass Bottom Cell Culture Dishes (MatTek corporation P35G-1.5-14.C), and treated with and without quercetin and FeCl3 **(**day 7). Following BMDCs cultures were exposed to 1 µg/mL of LPS and stained with The Iron Stain Kit (GENTAUR Molecular Products; IRN-2 Belgium) for the determination ferric iron deposits in tissue samples. This product is based on the Prussian Blue reaction in which ionic iron reacts with acid ferrocyanide producing a blue color. BMDCs, were fixed in 1% paraformaldehyde and then stained following manufacturer instructions.

Intestinal DCs analysis following quercetin administration

Mice were injected intragastrically with quercetin [0.5 µM/g] or vehicle at day 0, 3, 5 and 7. Seven days following the first injection, mice were sacrificed, duodenum, jejunum, ileum and colon were explanted, gently washed to remove fecal material. Intestinal segments (1cm long) were washed with EDTA 2.5mM to remove the epithelial cells and digested with Collagenase and DNase (Sigma Aldrich St Louis, MO, USA) using the GentleMacs suggested protocol. Single cells suspensions were used for CD11c magnetic sorting using CD11c

MicroBeads (Miltenyi Biotec 130-097-059) accordingly with the manufacturer protocol. Cells were then stored in RNA later (ThermoFischer Scientific MA, USA), lysed with TRIzol® (Thermo Fisher Scientific MA, USA) and used for total RNA extraction.

Statistical Analysis

Statistical analysis was performed using the Graphpad Prism. statistical software release 5.0 for Windows XP. All data were expressed as means ± SEM or SD of data obtained from at least three independent experiments. We evaluated statistical significance with two-tailed Student's t test. Results were considered statistically significant at $P < 0.05$.

RESULTS

Quercetin exposure affects iron metabolism in murine DCs.

We recently described the possibility to obtain inflammatory-impaired DCs providing 25μM of quercetin into reconstituted oil bodies [13, 14] or 25μM of free quercetin [15] during DCs maturation. In the microarray data obtained from ROBs-QP treated DCs, the expression of genes related with the iron metabolism pattern was modulated compared to vehicle treated DCs (Figure 1). Significant difference could be observed in the expression levels of Fpn1 involved in iron efflux from DCs. Also the expression of heme-inducible enzyme (Homx1) was induced by ROBs-QP exposure. Hmox1 is an enzyme responsible for heme digestion into equimolar amounts of iron, carbon monoxide and biliverdin, and plays a non-redundant role for iron recycling. Same patter was observed for Nramp1 expression, a crucial protein that exports iron from the phagosome into the cytoplasm. Polyphenols, including quercetin, are potent phytochelators, thus iron balance could be perturbed by quercetin administration and in turn produce a cascade of events leading to the observed inflammatory suppression.

Quercetin exposure skews DCs towards iron export pathway.

Based on our previous results we evaluated the expression level of a panel of genes related with iron transport and metabolism. We compared the expression level of control and LPS-matured in vitro cultured BMDCs. LPS administration induced a significant increase in the expression of the divalent-metal transporter 1 gene (Dmt1) (Slc11a2) and Hmox1, responsible for the production of heme oxygenase (Figure 2). The expression of the heme carrier protein (Hcp1) (Slc46a1), natural resistance-associated macrophage protein 1 (Nramp1) (Slc11a1) and the Ferroportin1 (Fpn1) (Slc40a1) did not change following LPS induced maturation. In line with the quercetin-induced anti-inflammatory phenotype previously described [12-15], quercetin exposure, strongly induced DCs expression of Fpn1 and Nramp1 while Dmt1 and Hcp1 expression level did not change (Figure 2). A consistent but not significant tendency to Hmox1 induction was observed in quercetin treated DCs before or after LPS administration (Figure 2). The overall analysis of the gene expression indicates that quercetin treatment induces a pathway that favors iron extracellular secretion.

Quercetin effects are lost in presence of high doses of iron in the culture media

As quercetin exposure induced Fpn1 expression we expected reduced intracellular iron concentration of iron in quercetin-treated DCs. DCs were plated into glass bottom cell Culture dishes, at day 7, 1µM of iron was additioned in the culture media, with quercetin (25µM) or same volume of vehicle (1µl DMSO/1ml media). LPS was then additioned 24 hours later and 6 hour later, cells were stained with the Iron Stain Kit. Figure 3A shows strong cytoplasmic blue color into LPS treated cells, while quercetin presence severely reduced cells positivity. We quantified the blue signal intensity in 30 different images obtained from 3 separated experiments (Figure 3B). We then compared Hmox1 protein expression in DCs lysate 24h after LPS administration. Figure 3C shows the immunoblot of vehicle or quercetin exposed DCs in presence or absence of LPS. Vehicle and quercetin treated DCs expressed similar level of Hmox1, while LPS administration significantly reduced it. Importantly, when LPS was administered to quercetin-exposed DCs, Hmox1 signal was higher than control. To investigate the axis between iron chelation and quercetin, we additioned different concentration of iron in the DCs culture media. Figure 3D shows the LPS induced secretion of IL-6 and IL-12p70 by DCs treated with quercetin or vehicle. Cells were treated with quercetin at day 7 of culture and immediately after with iron at the indicated doses. Confirming previous observations, the left side of the figure 3D shows the quercetininduced reduction of LPS-induced IL-6 and IL-12p70 secretion. Quercetin efficiency is gradually lost in presence of increasing doses of iron. Indeed, administration of 2µM of iron was able to revert quercetinmediated IL-6 and IL-12p70 suppression. Of notice, we administered the synthetic iron chelator hydroxypyridinones (2.4 mM) using the same protocol used for quercetin. IL-6 and IL-12p70 secretion was reduced but with lower efficiency if compared with quercetin (data not shown).

Oral administration of quercetin induces the expression of Hmox1 in CD11c enriched lamina propria cells.

Based on our data, iron metabolism appears to be strongly modulated by quercetin exposure. The gastrointestinal tract is exposed to nutritional derived polyphenols. For this reason, we investigated if the intestinal resident DCs-iron metabolism pathway was affected by oral quercetin administration. Mice were treated at day 0 and 3 with 100µl of quercetin (50 µM) by gavage. At day 4 mice were sacrificed, duodenum, jejunum, ileum and colon were explanted. The luminal side was exposed and gently washed to remove fecal material, and then the epithelial cells monolayer stripped, the tissue digested and the $CD11c^+$ cells magnetically sorted. Quantitative PCR analysis of the different tract revealed differential expression of the iron pathway along the intestinal tract. Figure 4 shows $CD11c^+$ enriched cells expression of Dmt1, Hcp1, Nramp1, Hmox1 and Fpn1 in the different areas of the gastrointestinal tract. In line with what observed in vitro, following quercetin exposure we observed a substantial increase in the Fpn1 expression, particularly in the terminal part of the intestine. Dmt1, Hcp1, Nramp1 and Hmox1 were also upregulated in the colon, while in the small intestine the upregulation, when present, was not significant. All together, these data indicate that quercetin

oral administration significantly affects iron metabolism of intestinal dendritic cells in line with the observed reduction of their inflammatory ability.

Quercetin enriched diet modifies iron metabolism pathway in the colon.

We finally analyzed the iron expression profile in the colon of mice that received a quercetin-enriched diet. Mice received conventional or quercetin-enriched diet (6mg/g pellet) for two weeks were than sacrificed, the colon explanted, measured and studied by immunohistochemistry (not shown). No significant differences were found among the groups in term of colon length or inflammation hallmarks (data not shown). The central region of the colon of each mouse was used to extract the mRNA, which was later used for our qPCR analysis. Figure 5 shows the quantitative gene expression in the aforementioned colon samples. Similarly to what observed when administered by gavage, quercetin enriched diet was able to induce the expression of Hcp1, Nramp1, Hmox and Fpn1. Surprisingly, Dmt1 expression was reduced, nonetheless, the overall result suggest a decrease iron intracellular uptake, and an increase in iron export.

DISCUSSION

Quercetin is among the best known phytochemical able to induce a variety of physiological effects. We recently contributed to better describe the tolerogenic effects of quercetin demonstrating that dendritic cells, exposed to a combination of quercetin and piperine reconstituted oil bodies (ROBs-QP), become unresponsive to LPS administration. Our results were obtained starting from the mRNA pathway analysis of DCs exposed or not to ROBs-QP before LPS administration [12-15]. Further analysis of previous dataset revealed that ROBs-QP exposure imprinted a specific phenotype related with iron metabolism. Iron intracellular and extracellular balance is known to affect immune-mediated inflammatory responses [12]. In iron homeostatic conditions, macrophage correctly respond to bacterial products and release inflammatory cytokine, wile, iron depletion suppress TLR4-mediated NF-kB activation [18-22]. Furthermore, during bacterial invasion, macrophages accumulate intracellular iron to reduce its availability and consequently, impair bacterial growth [23 -24]. Our recent data demonstrated that quercetin administration was able to suppress LPS mediated inflammatory cytokine secretion [15], similarly to what observed using ROBs-QP administration [13, 14]. Knowing that quercetin is able to bind iron [15], we decided to investigate the axis between quercetin administration and iron homeostasis in DCs. We first confirmed the panel of genes previously investigeted by microarray analysis, using quercetin instead of ROBs-QP. DCs exposed to quercetin upregulate the expression of Fpn1, Nramp1 and Hcp1. Nonetheless, the effects to quercetin exposure were more pronounced following LPS administration. Indeed, quercetin-treated DCs respond to LPS up-regulating Fpn1, Nramp1 and Dmt1 gene expression. Hmox1 expression is reduced during inflammation as confirmed by its protein expression level following LPS administration. Hmox1 increase in quercetin-exposed DCs treated with LPS perfectly fits with our previous observations [13-15] as we demonstrated that these cells fail to become inflammatory. Finally, Hcp1 expression is suppressed following LPS, both in control and quercetin treated DCs. The overall result

of the quercetin-induced gene pathway is reduced iron accumulation following LPS administration. This effect appears evident by staining cytoplasmic iron content in LPS vs quercetin+LPS treated DCs. Furthermore, iron enriched supernatant was able to antagonize quercetin-mediated IL-6 and IL-12p70 suppression in a dose dependent manner. In line with what observed in vitro, following quercetin exposure we observed a substantial increase in the Fpn1 expression, particularly in the CD11c+ enriched population obtained from the colon. Dmt1, Hcp1, Nramp1 and Hmox1 were also upregulated in the colon of quercetin treated mice. The absence or limited results observed in the small intestine were surprising, but most likely due to the administration by gavage. Nutrition is the major source of quercetin, for this reason we explored the possibility of a switch in the iron metabolism and iron-dependent inflammatory response using a quercetinenriched diet.

We analyzed by gene expression the colon mRNA expression in mice that received a quercetin enriched diet. The results clearly demonstrated that different diets induced distinct gene expression profiling in the host colon. Among the genes differently expressed, Hmox1 was significantly up-regulated by quercetin enriched diet. Hmox1 expression is among the most important genes induced in the M2 type of anti-inflammatory macrophages [25]. Indeed, hemoglobin degradation is promoted by the expression Hmox1 (Figure 5) thus promoting further iron delivery in the extracellular compartment. Iron homeostasis shows distinct features in pro or anti-inflammatory macrophages. Inflammatory conditions favor a macrophage profile called M1, associated to the immunological response towards pathogen invasion [26] while a second profile called M2 is characteristic of the resolution phase of an inflammation [27, 28, 29]. M1 macrophages maximize their ability to take up iron and decrease export by reducing ferroportin expression [18]. Doing so, macrophages retain iron dampening bacteria and parasites growth and enhance their inflammatory ability. In an opposite manner, M2 release it into tissues to facilitate tissue repair [19]. Moreover, anti-inflammatory macrophages are also characterized by increased heme uptake and heme-oxygenase-dependent heme catabolism, thus increasing the iron efflux into the tissues. Here we described for the first time the possibility to bend dendritic cells gene pathway towards a tolerogenic Hmox1^{+} phenotype using dietary products. We also demonstrate that quercetin can up-regulate Fpn1 gene expression and increase dendritic cells uptake of heme and non-heme-associated iron (Figure 5). Furthermore, Hmox1 catabolic action produces CO and biliverdin, products with potent antiinflammatory effects [29, 30], thus, quercetin-enriched diet [31] can promote anti-inflammatory effects in a direct (suppressing NF-kB translocation, inflammatory cytokines secretion and antigen presentation) and indirect manner (catabolic products from iron metabolism pathway). All together, these data indicate that the exposure to a quercetin-enriched diet bends the DCs molecular signature towards an M2-like profile that favors tissue repair rather than inflammation.

CONCLUSION

Quercetin exposure affects dendritic cells cytoplasmic iron content favoring its extracellular export. Quercetin is part of a family of natural bio-chelates whose anti-inflammatory effects are well recognized, but never associated to their iron-chelating properties. Quercetin-enriched nutritional regimes may contribute to reduce

chronic inflammatory syndromes dampening inflammation and favoring tissue healing immune cells polarization.

LIST OF ABBREVIATIONS

LPS: lipopolysaccharide

ROBs-QP: reconstituted oil bodies

CO: carbon monoxide

DCs: Dendritic Cells

BMDCs: Bone Marrow Dendritic Cells

Dmt1: Divalent-metal transporter 1

Hcp1: Heme carrier protein

Hmox1: Heme Oxygenase

Nramp1: Natural resistance-associated macrophage protein 1

Fpn1: Ferroportin1

M1: type 1 inflammatory macrophages

M2: type 2 anti-inflammatory macrophages

DMSO: Dimethyl sulfoxide

DISCLOSURE OF POTENTIAL CONFLICTS OF INTERESTS

The authors declare no financial or commercial conflict of interest.

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Figure 1: Representative genes of iron metabolism from DCs 6 h following LPS administration is different in ROBs-QP-treated cells versus vehicle-treated cells. Dmt1, Hmox1, Nramp1 and Fpn1 expression from the microarray data of DCs exposed to vehicle (round dots) or ROBs-QP (square dots) at day 5 and 7 and treated with 1 μg/mL of LPS for 6 hours. Each dot represents the mean ± SEM of 3 independent experiments $^{**}P<0.01$;$^{***}P<0.001$

Figure 2: Quercetin exposure skews the expression of genes involved in iron transport towards extracellular secretion. (A) Dmt1, Hcp1, Nramp1, Hmox and Fpn1 mRNA expression in an independent set of 3 BMDCs exposed to quercetin (25 μM;) and treated with LPS [1 μg/mL] for 6h. Expression levels were quantified using pRT-PCR. RNA expression was normalized to the expression of GAPDH. (The histogram represents the mean ± SEM. *P<0.05;**P<0.01;***P<0.001.

Figure 3: **Quercetin effects are lost in presence of high doses of iron in the culture media**. Histological staining for the determination of ferrous pigment in BMDCs, fixed paraformaldehyde 1% (A). LPS treated dendritic cells (right panel) and quercetin-exposed dendritic celle (left panel) cultured in FeCl3 (1μM) enriched media. Blue signal intensity was quantified in 30 different images obtained from 3 separeted experiments. Scale bar is 50μm. The histogram (B) represents the mean blue signal intensity for the iron staining ± SEM. C) Western blot analysis of BMDCs lysate 24h post LPS administration. The histogram represents the ratio between Hmox1 and tubulin bar quantification obtained from 3 different experiments. Panel D shows the LPS-induced secretion of IL-6 and IL-12p70. Quercetin (white bars) or vehicle (black bars) DCs were treated with $FeCl_3$ and LPS at the indicated doses, IL-6 and IL-12p70 were determined by ELISA. Bars represent mean cytokine concentration ± SEM of 3 independent experiments. *P<0.05; **P<0.01;***P<0.001.

Figure 4: Oral administration of quercetin affects iron gene pathway in the intestinal lamina propria.
Relative expression of Dmt1, Hcp1, Nramp1, Hmox and Fpn1 in different areas of the gastrointestinal tract after treatment at day 0 and 3 with 100μl of quercetin (50 μM) by gavage. Data refers to 3 independent experiments. Expression levels were quantified using pRT-PCR. RNA expression was normalized to the expression of GAPDH. The histogram represents the mean ± SEM. *P<0.05;**P<0.01;***P<0.001.

Figure 5: Quercetin enriched diet modulates iron metabolism. Relative expression of Dmt1, Hcp1, Nramp1, Hmox and Fpn1 in mice feeded with control diet (black bars) or quercetin enriched food (white bars). The results represent the expression level of 3 animals per group. Expression levels were quantified using pRT-PCR. RNA expression was normalized to the expression of GAPDH. The histogram represents the mean ± SEM. *P<0.05;***P<0.001.

Figure 6. DCs modulate intracellular iron following quercetin exposure. In inflamed conditions, DCs sequester extracellular iron and accumulate it in the cytoplasm (red side). Quercetin chelate iron in the extracellular compartment and induces Fe^{2+} secretion by DCs. Hmox-1 catabolic activity produces Fe^{2+} starting from the heme transported by Dmt1 and releases CO and biliverdin with potent anti-inflammatory properties. The overall result of quercetin exposure is bending DCs towards an anti-inflammatory state (green side).

Figure 1

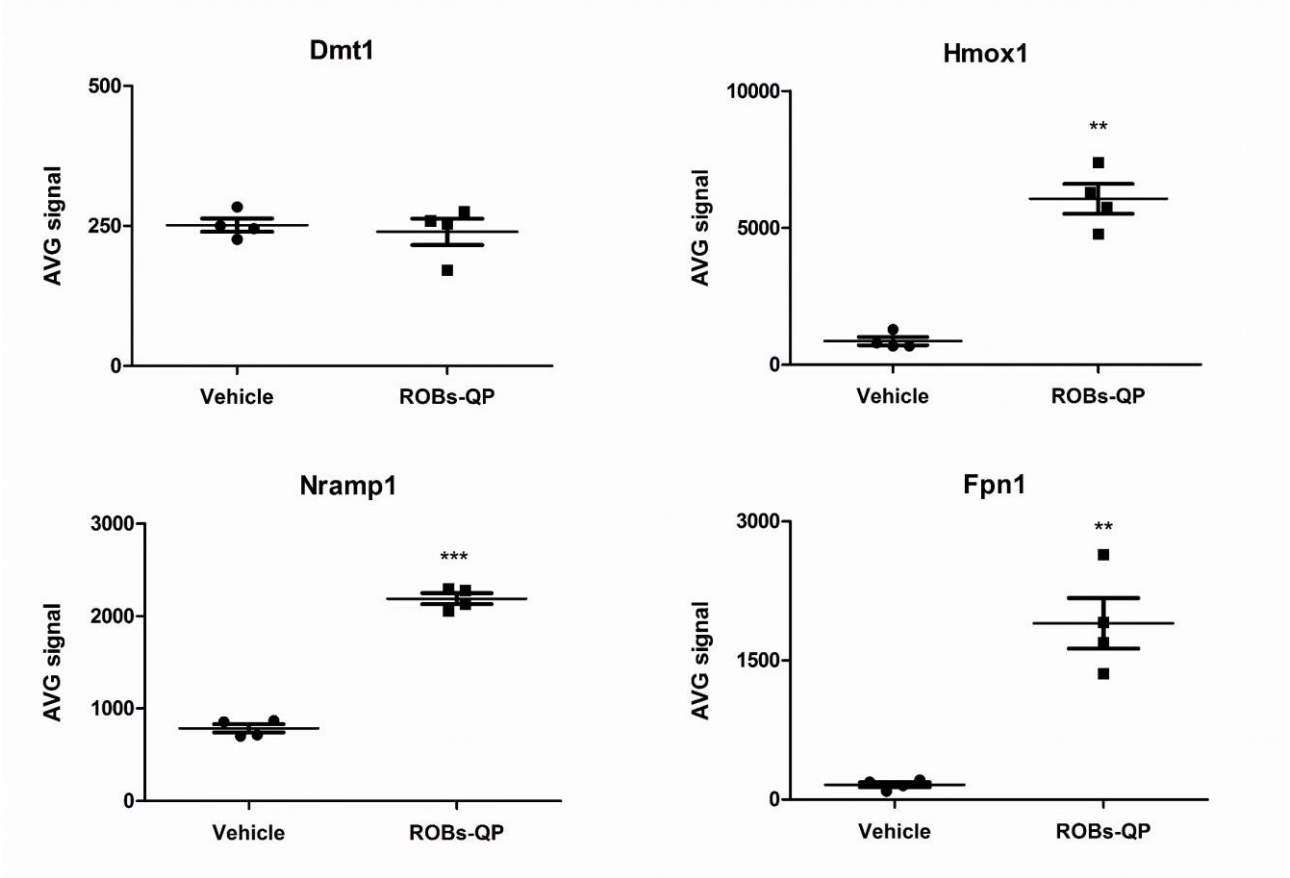

Dmt1
AVG signal
500
250
0
Vehicle
ROBs-QP
Hmox1
AVG signal
10000
5000
0
**
Vehicle
ROBs-QP
Nramp1
AVG signal
3000
2000
1000
0
***
Vehicle
ROBs-QP
Fpn1
AVG signal
3000
1500
0
**
Vehicle
ROBs-QP

Figure 2

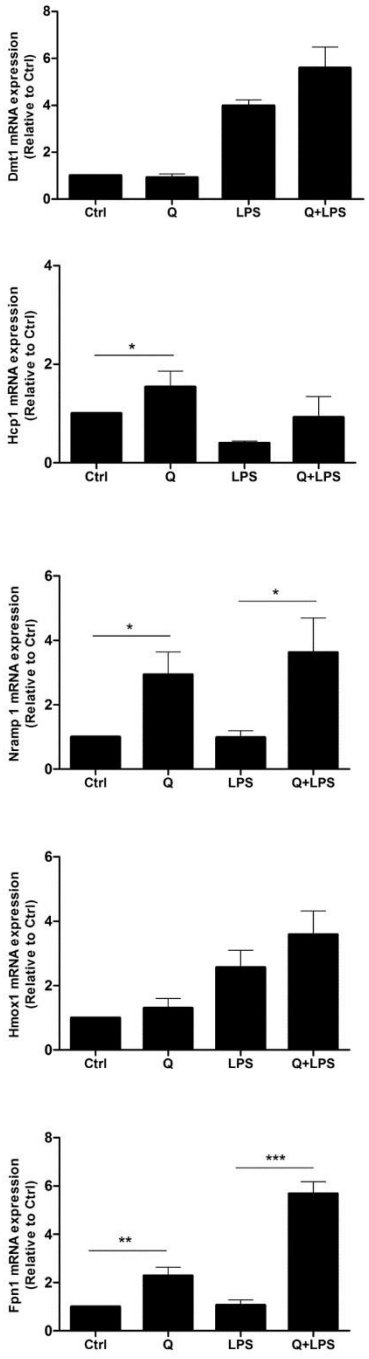

Dmt1 mRNA expression (Relative to Ctrl)
Ctrl
Q
LPS
Q+LPS
Hcp1 mRNA expression (Relative to Ctrl)
*
Nramp 1 mRNA expression (Relative to Ctrl)
*
*
Hmox1 mRNA expression (Relative to Ctrl)
Fpn1 mRNA expression (Relative to Ctrl)
**
***

Figure 3

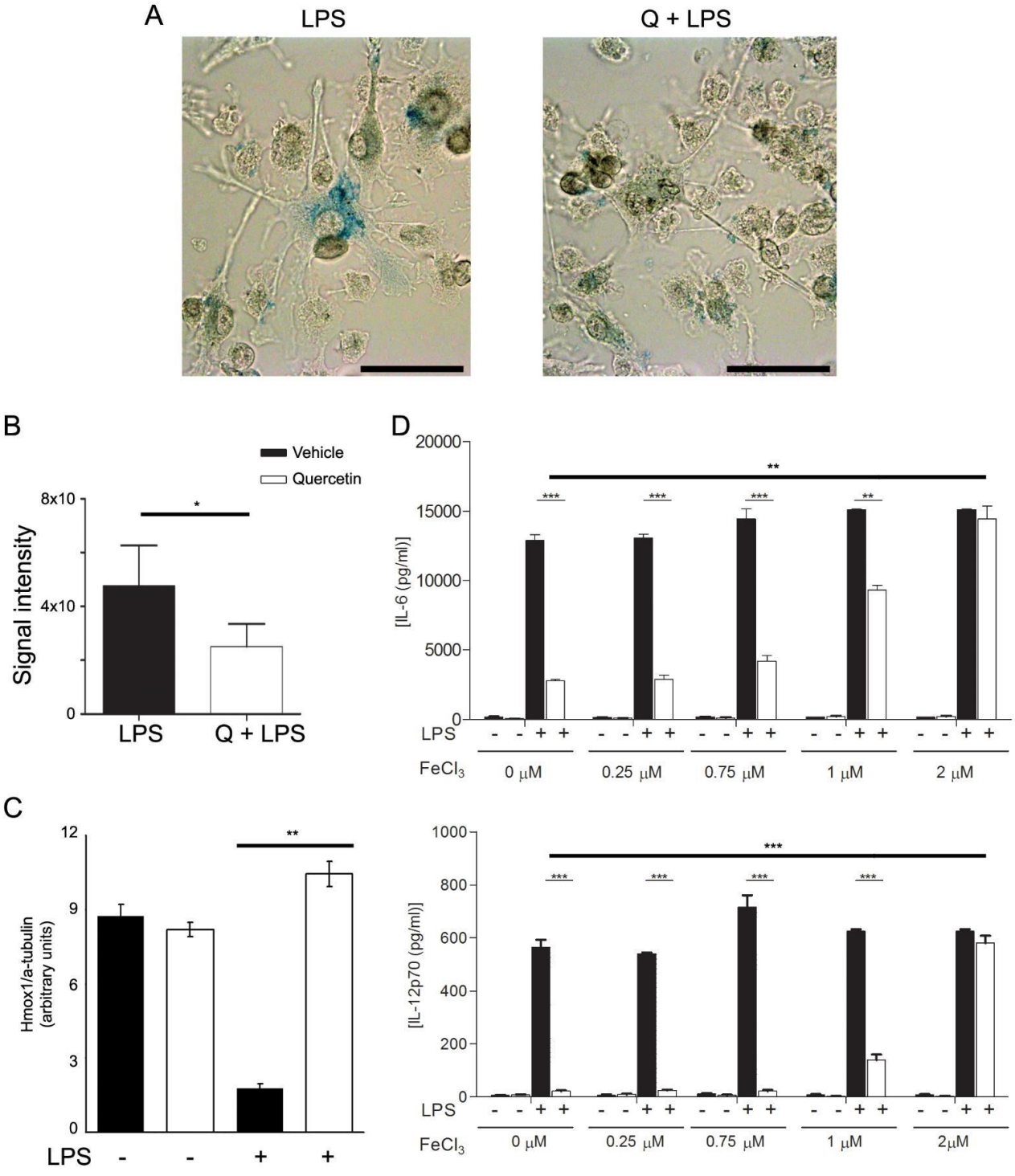

A
LPS
Q + LPS
B
Vehicle
Quercetin
Signal intensity
8x10
4x10
0
LPS
Q + LPS
C
Hmox1/a-tubulin (arbitrary units)
12
9
6
3
0
LPS
D
[IL-6 (pg/ml)]
20000
15000
10000
5000
0
[IL-12p70 (pg/ml)]
1000
800
600
400
200
0
FeCl3
0 μM
0.25 μM
0.75 μM
1 μM
2 μM
2μM

Figure 4

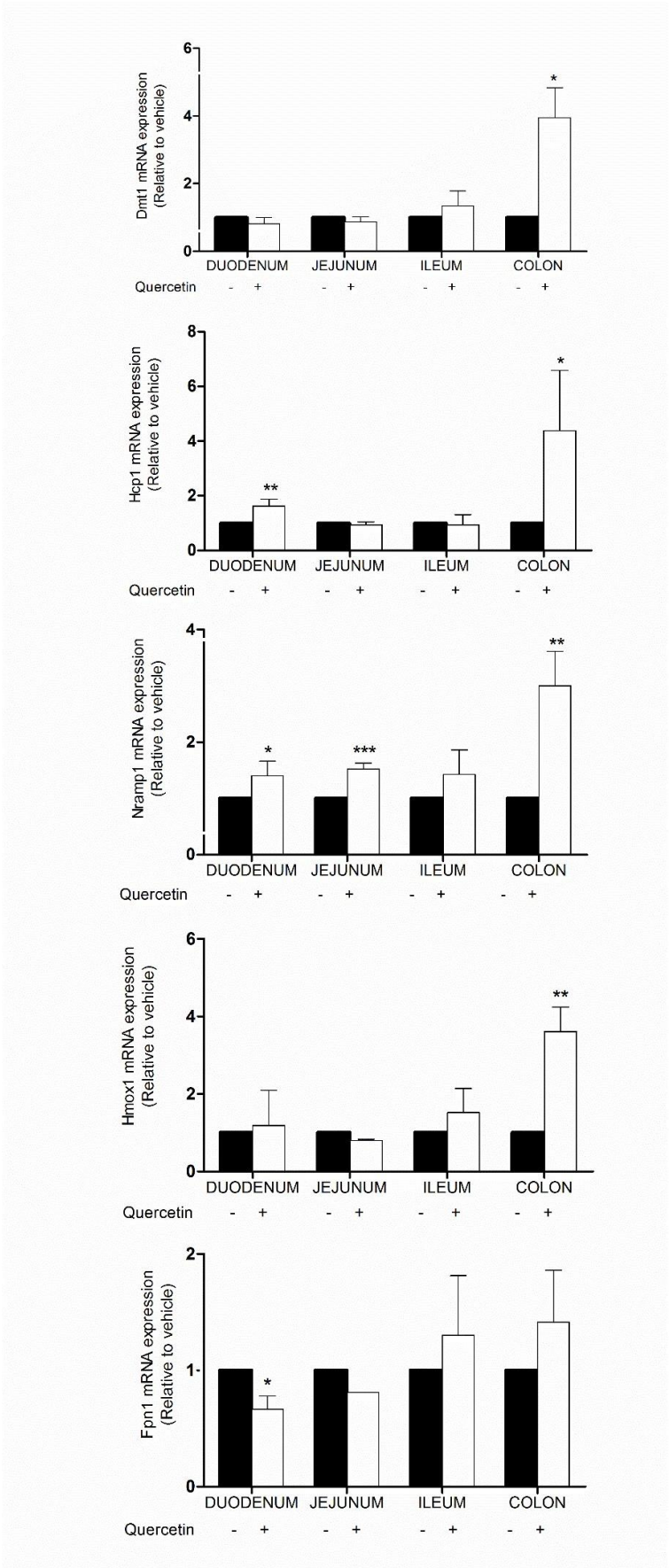

Dmt1 mRNA expression (Relative to vehicle)
6
4
2
0
*
DUODENUM
JEJUNUM
ILEUM
COLON
Quercetin - + - + - + - +
Hcp1 mRNA expression (Relative to vehicle)
8
6
4
2
0
**
*
DUODENUM
JEJUNUM
ILEUM
COLON
Quercetin - + - + - + - +
Nramp1 mRNA expression (Relative to vehicle)
4
2
0
*
***
**
DUODENUM
JEJUNUM
ILEUM
COLON
Quercetin - + - + - + - +
Hmox1 mRNA expression (Relative to vehicle)
6
4
2
0
**
DUODENUM
JEJUNUM
ILEUM
COLON
Quercetin - + - + - + - +
Fpn1 mRNA expression (Relative to vehicle)
2
1
0
*
DUODENUM
JEJUNUM
ILEUM
COLON
Quercetin - + - + - + - +

Figure 5

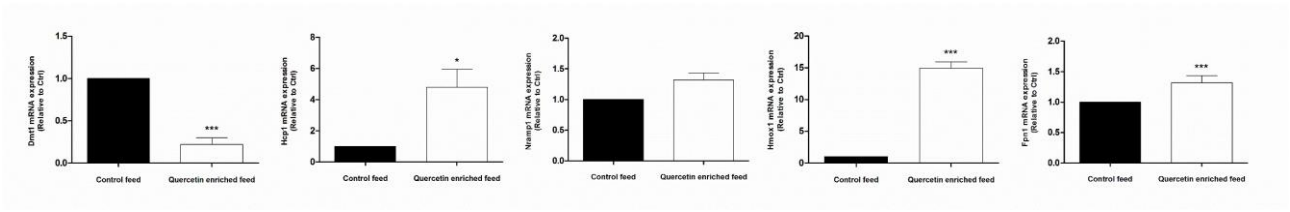
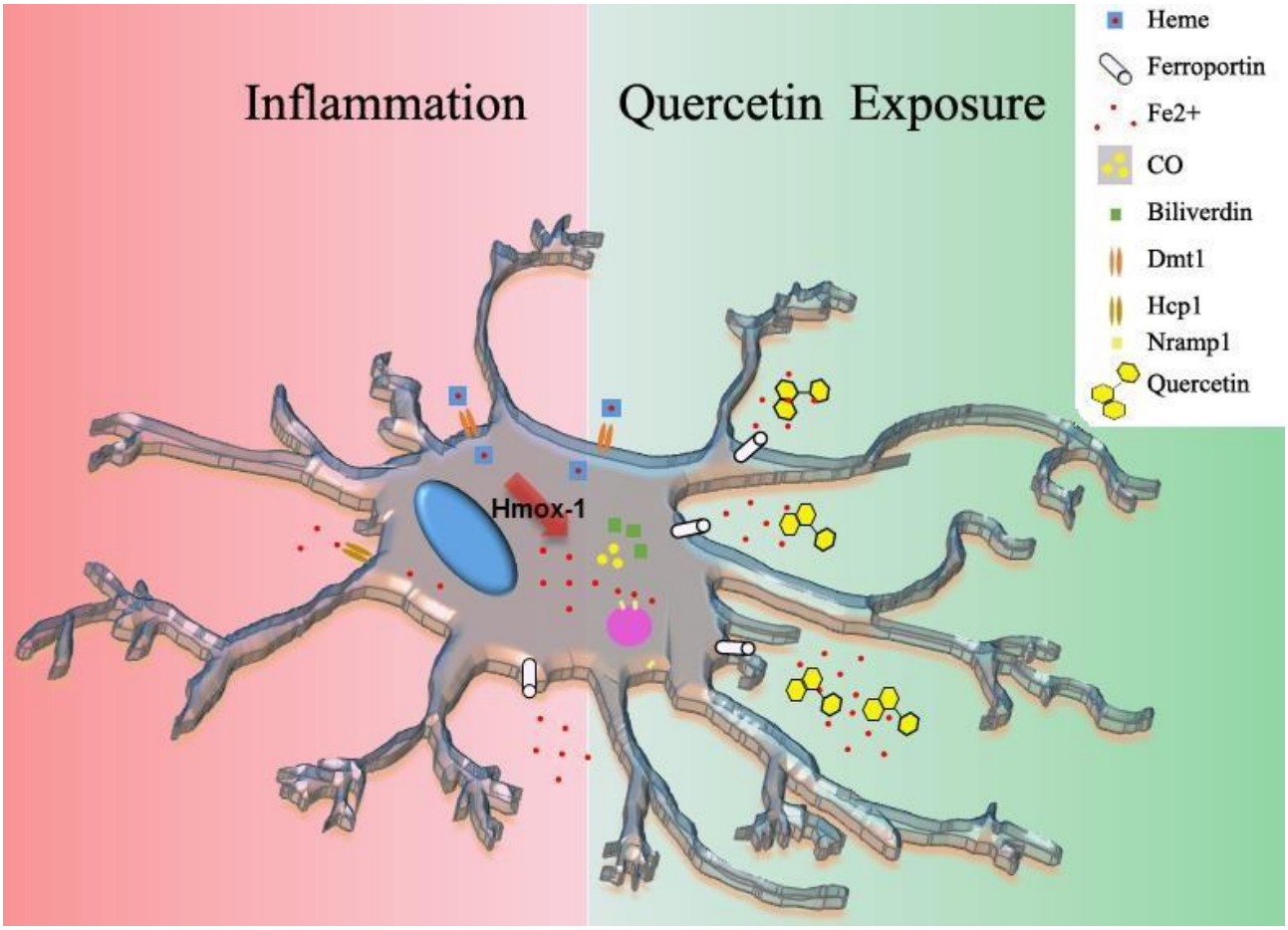

Dmt1 mRNA expression (Relative to Ctrl)
1.5
1.0
0.5
0.0
***
Control feed
Quercetin enriched feed
Hcp1 mRNA expression (Relative to Ctrl)
8
6
4
2
0
*
Control feed
Quercetin enriched feed
Nramp1 mRNA expression (Relative to Ctrl)
2.0
1.5
1.0
0.5
0.0
Control feed
Quercetin enriched feed
Hmox1 mRNA expression (Relative to Ctrl)
20
15
10
5
0
***
Control feed
Quercetin enriched feed
Fpn1 mRNA expression (Relative to Ctrl)
2.0
1.5
1.0
0.5
0.0
***
Control feed
Quercetin enriched feed

Figure 6


Inflammation
Quercetin Exposure
Heme
Ferroportin
Fe2+
CO
Biliverdin
Dmt1
Hcp1
Nramp1
Quercetin
Hmox-1