FINAL REPORT

FUNGAL SIDEROPHORES FUNCTION AS PROPHYLACTIC AND PROTECTIVE AGENTS AGAINST VASCULAR DISORDERS AND CANCER AND PROMISING DRUGS FOR TREATMENT OF ATHEROSCLEROSIS AND LEAD-POISONING

1. THE ROLE OF IRON AND IRON CHELATION IN ATHEROSCLEROTIC PLAQUE PROGRESSION

Iron accumulates in human atherosclerotic lesions but whether it is a cause or simply a downstream consequence of the atheroma formation is still an open question. According to the so called "iron hypothesis", iron is believed to be detrimental for the cardiovascular system, thus promoting atherosclerosis development and progression. Many lines of evidence suggest that intraplaque hemorrhage is a relevant mechanism via which iron can enter the plaque area. Intraplaque hemorrhage is thought to occur from leaky neovessels invading the intima from vasa vasorum as the intima thicken, and contributes significantly to the enlargement of the necrotic core. Increasing evidence indicates that plaque neovascularization and vasa vasorum density accompanied by intraplaque hemorrhage is a strong marker for plaque vulnerability.

Following intraplaque hemorrhage red cells can be taken up by macrophages or burst extracellularly releasing free hemoglobin (Hb). Hb is prone to oxidation, especially in the highly oxidative milieu of the atheroma leading to the formation of metHb and higher oxidation states such as ferryl Hb, those can release heme. Intraplaque hemorrhage not only drives iron into the plaque but these Hb oxidation products and heme exert unique pro-oxidant and pro-inflammatory effects targeting different cellular, *i.e.* endothelial cells, smooth muscle cells, macrophages, and acellular *i.e.* low-density lipoprotein (LDL) components of the atherosclerotic vessel wall.

1.1. Oxidized hemoglobin species induce oxidative modification of LDL, degrade and release iron.

To model the possible interactions that could take place inside a complicated atherosclerotic lesion between lipids and different Hb species, we purified Hb from human blood and generated metHb and ferrylHb. When incubated with human plasma, heme, metHb and ferrylHb intercalated into LDL and caused LDL oxidation with parallel degradation of heme and release of iron (Jeney et al. 2013, Potor et al. 2013).

1.2. Oxidized Hb species trigger EC death.

Heme sensitizes various cells to oxidant-mediated killing because it can increase labile iron pool inside the cells and thus amplifying the generation of reactive oxygen species. We demonstrated that metHb and ferrylHb, species produced during Hb oxidation are also able to sensitize endothelial cells to oxidative stress (Jeney et al. 2013, Potor et al. 2013, Belcher et al. 2010).

1.3. Oxidized Hb species induce HO-1 and ferritin expression in HUVECs.

Cells response to free heme by the induction of heme oxygenase-1 (HO-1) and ferritin, molecules those assure degradation of heme and safe storage of liberated iron, respectively. We showed that oxidized Hb species also induce HO-1 and ferritin, proving that heme transfer readily occurs from metHb and ferrylHb towards endothelial cells (Jeney et al. 2013, Potor et al. 2013, Belcher et al. 2010).

1.4. OxLDL and reactive lipid mediators derived from complicated atherosclerotic lesions initiate Hb oxidation and globin-globin crosslinking.

Lipid hydroperoxides, such as those found in oxLDL as well as in lipids derived from atheromatous lesions, can initiate Hb oxidation resulting in metHb formation and subsequent heme release. Oxidized lipids and ferrylHb coexist in advanced atherosclerotic lesions but the role of reactive lipid mediators in the formation of ferrylHb and the subsequent crosslinking of Hb subunits has not been addressed. We showed that oxLDL and plaque lipids induce Hb crosslinking, a surrogate marker of ferrylHb formation (Nagy et al. 2010, Potor et al. 2013, reviewed in Belcher et al. 2010, Jeney et al. 2013).

1.5. FerrylHb increases EC monolayer permeability and enhances monocyte adhesion.

Chronic inflammation and endothelial activation are characteristic of the atherosclerotic vessel wall. Whether Hb oxidation products play any pro-inflammatory role has not been addressed. We showed that ferrylHb disrupts EC monolayer integrity and induces expression of adhesion molecules, increases monolayer permeability and enhance monocyte adhesion (Silva et al. 2009, Potor et al. 2013, reviewed in Jeney et al. 2013,).

1.7. Chronic administration of Hb accelerate atherosclerosis in apoE deficient mice

After we proved that Hb oxidation can trigger deleterious events *in vitro* targeting LDL and endothelial cells, those are two crucial components of the atherosclerotic vessel wall, our next question was whether chronic administration of Hb can facilitate atherosclerosis in apoE deficient mice. We injected apoE deficient mice with Hb and heme at a dose that provided 80 μmol/L of heme concentration in the plasma every other day for 8 weeks. Parallel with the Hb/heme injections mice were fed with high-cholesterol diet (HCD: 15.8% fat, 1.25% cholesterol). After 8 weeks of treatment aortas were dissected and cleaned from adventitial fat, and plaques were stained with oil o red and quantified (Fig 1). We found that administration of Hb increased plaque size in the aorta of apoE deficient mice significantly, whereas heme did not have atherogenic effect (Fig. 1) (unpublished).

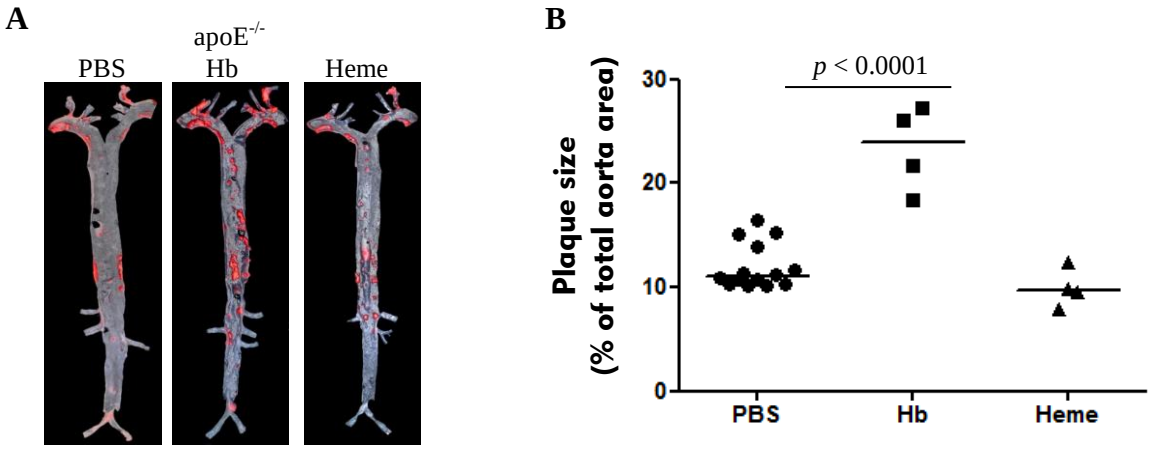


Figure 1. Chronic Hb administration accelerates atherosclerotic lesion progression in ApoE deficient mice.
(A) ApoE$^{-/-}$ mice were treated with Hb, heme or vehicle (PBS) every other day for 8 weeks. Parallel with Hb/heme/vehicle treatement all mice were fed with HCD. After 8 weeks of treatment aortas were dissected, cleaned and plaques were stained with oil O red. Representative images of aortas prepared for plaque size quantification are shown. Quantification was carried out by using ImageJ software. (B) Plaque size were determined in control mice (n=13), Hb-treated (n=4) and heme-treated (n=4) mice. Two-way Anova test was used for multiply comparisons.

8. Hemolysis accelerates atherosclerosis in apoE deficient mice.

Next we tested whether hemolysis can trigger atherosclerosis in apoE deficient mice. Hemolysis was induced by intraperitoneal injections of phenyl-hydrazine (30 mg/kg) in every other day for 8 weeks starting at the age of 8 weeks (n=5). This treatment induced about a 50-60 % of RBC lysis that could recover within two days. Parallel with the PHZ treatment ApoE mice received HCD to facilitate plaque formation. Control mice received PBS and HCD (n=14). After 8 weeks of treatment aortas were dissected and cleaned from adventitial fat, and plaques were stained with oil o red and quantified. Chronic hemolysis significantly increased plaque size (Fig. 2 A and B), and decreased collagen content (stained in blue) of the atherosclerotic vessel wall that was assessed by Trichrome staining of the aortic root (Fig. 2C) (unpublished).

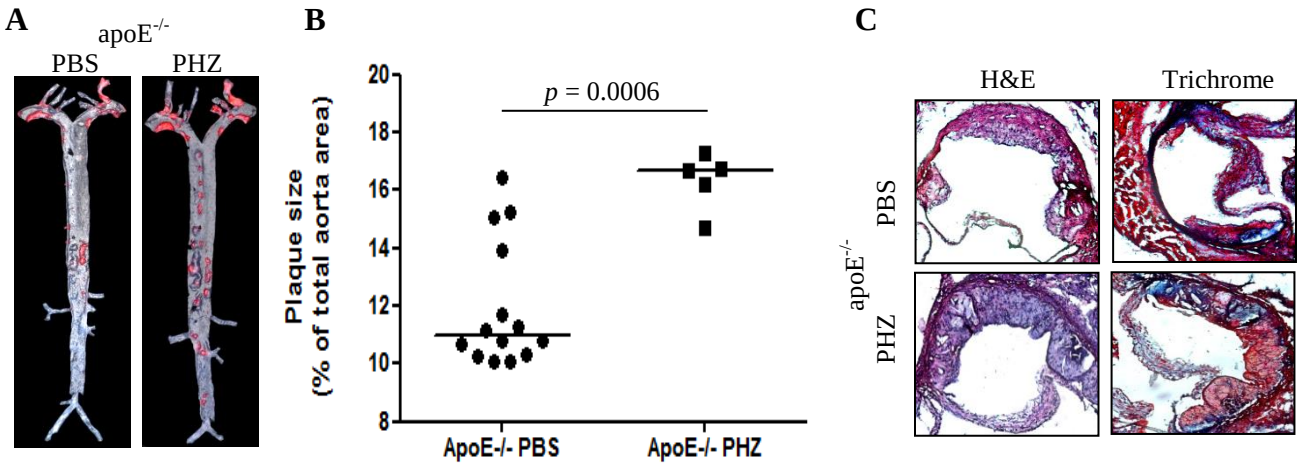


Figure 2. Hemolysis accelerates atherosclerotic lesion progression in apoE deficient mice.
(A) ApoE$^{-/-}$ mice were treated with PHZ or vehicle every other day and parallel were fed with HCD for 8 weeks. Aortas were dissected, cleaned and plaques were stained with oil O red. Representative images of aortas prepared for plaque size quantification. Quantification was carried out by using ImageJ software. (B) Plaque size were determined in control mice (n=16) and PHZ-terated mice (n=5). Two-tailed unpaired T-test was used to calculate p value. (C) Aortic root samples were embedded in TissueTek and were cryosectioned into 6 μm thick slices. Representative images of hematoxylin-eosin (H&E) and Masson trichrome stainings (nuclei: black, cytoplasm: red, muscle fiber: red, collagen:

8. Desferricoprogen (DFC) inhibits atherosclerosis in apoE deficient mice

Previously we found fungal siderophores in Camembert- and Roquefort-type cheeses and also some sausages. These siderophores, bind redox active iron, via which they inhibit LDL oxidation, and prevent iron-mediated endothelial cytotoxicity. Among the tested siderophores we found that DFC is the most biologically active compound with potential in vivo effects. We administered DFC (2 mg/kg) intraperitonealy into apoE deficient mice (n= 17) in every other day for 8 weeks. Parallel with the DFC treatment ApoE mice received HCD to facilitate plaque formation. Control mice received PBS and HCD. After 8 weeks of treatment aortas were dissected and cleaned from adventitial fat, and plaques were stained with oil o red and quantified. DFC treatment significantly reduced plaque size (Fig. 3) (unpublished).

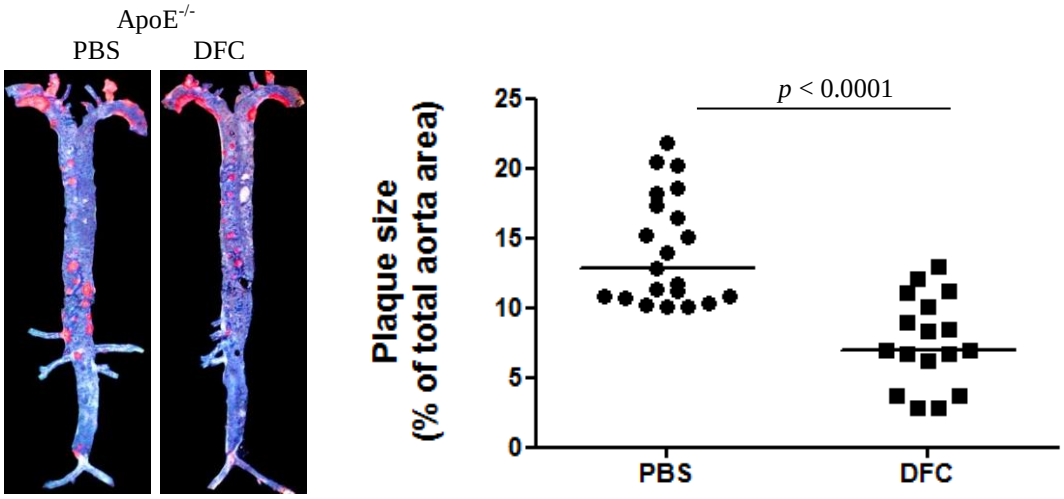


Figure 3. Desferricoprogen inhibits atherosclerotic lesion progression in apoE$^{-/-}$ mice.
(A) ApoE$^{-/-}$ mice were treated with DFC or vehicle every other day and parallel were fed with HCD for 8 weeks. Aortas were dissected, cleaned and plaques were stained with oil O red. Representative images of aortas prepared for plaque size quantification. Quantification was carried out by using ImageJ software. (B) Plaque size were determined in ApoE$^{-/-}$ vehicle-injected mice (n=21), ApoE$^{-/-}$ DFC-terated mice (n=17). Two-tailed unpaired T-test was used to calculate p

2. FUNGAL SIDEROPHORE SYNTHESIS, PURIFICATION, BIOTECHNOLOGICAL OPTIMIZATION TOWARDS EXPERIMENTAL AND CLINICAL UTILIZATION

2.1. Optimization of desferrioxamine E production by *Streptomyces Coelicolor*

Siderophores are iron chelators produced by a number of microorganisms to sequester iron from the environment. The siderophores bind iron with high affinity and are vital for growth of microbes and required for the virulence of certain pathogens. Iron is an abundant element on Earth, its bioavailability in environment is considerably limited by the solubility of Fe^{3+} (~10−24 mol/L). *Streptomyces* spp. is Gram positive soil bacteria which produce commercially important antibiotics and other metabolites like iron chelator siderophores.
Glycerol gave the highest desferrioxamine E yield. L-aspartic acid is a suitable nitrogen sources for ferrioxamine E production. *Streptomyces coelicolor* was able to produce desferrioxamine E in detectable quantity. Carbon and nitrogen sources and additives need to be further analyzed and optimized to improve the desferrioxamine E yield.

	Flask 1	**Flask 2**	**Flask 3**	**Flask 4**	**Flask 5**
Carbon source	**8% glycerol**	**8% galactose**	**8% Na-citrate**	**8% lactose**	**8% sorbitol**
Nitrogen source	**5 g/L aspartic acid**	**5 g/L aspartic acid**	**5 g/L aspartic acid**	**5 g/L aspartic acid**	**5 g/L aspartic acid**

Flask 6	**Flask 7**	**Flask 8**	**Flask 9**	**Flask 10**
8% glycerol	**8% galactose**	**8% Na-citrate**	**8% lactose**	**8% sorbitol**
5 g/L NaNO3	**5 g/L NaNO3**	**5 g/L NaNO3**	**5 g/L NaNO3**	**5 g/L NaNO3**

2.2. Optimization of triacetylfusarasine c and ferricrocin productions in *Aspergillus fumigatus.*

Iron is an essential element for all microorganisms. Bacteria and fungi produce versatile siderophores for binding and storing this essential transition metal when its availability is limited in the environment. The aim of the study was to optimize the fermentation medium of *Aspergillus fumigatus* for siderophore production. Triacetylfusarinine C and ferricrocin yields were dependent on glucose and glycine supplementations as well as the initial pH of the culture media.

The optimal fermentation medium for triacetylfusarinine C production contained 8 % glucose, 0.4 % glycine and the initial pH was set to 5.9. Meanwhile, maximal ferricrocin yields were recorded in the presence of 10 % glucose, 0.5 % glycine and at an initial pH of 7.4. Under optimized fermentation conditions, the yields for triacetylfusarinine C and ferricrocin increased up to 2.9 g/l culture medium and 18.9 mg/g mycelium, respectively.

This work is accepted for publication in the *Acta Microbiologica et Immunologica Hungarica*

2.3. Lanthanide(III) complexes of some natural siderophores: a thermodynamic, kinetic and relaxometric study.

Stability constants of the complexes formed between the natural trihydroxamic acids desferrioxamine B (DFB) and desferricoprogen (DFC) with Nd^{III}, Gd^{III} and Yb^{III} ions were determined using pH-potentiometry. The equilibrium in these systems can be described by models containing mononuclear protonated (Ln(HL), $Ln(H_2L)$ and $Ln(H_3L)$), deprotonated (LnL) and ternary hydroxo $Ln(H_{-1}L)$ complexes, but for both ligands dinuclear complexes of low stability were also detected. The stability constants for the $Ln(HDFB)^+$ complexes are 11.95 (Nd^{III}), 13.16 (Gd^{III}) and 14.67 (Yb^{III}), while these values of the Ln(DFC) complexes are considerably higher (14.42 (Nd^{III}), 15.14 (Gd^{III}) and 16.49 (Yb^{III})). The stability constants of the complexes of DFB and DFC are much lower than those of the $Ln(L)_3$ complexes formed with some aromatic hydroxamic acids indicating that the relatively long spacer between the hydroxamic acid moieties in DFB and DFC is unfavorable for Ln^{III} complexation. The relaxometric study conducted for the $Gd(HDFB)^+$ species revealed an interesting pH dependence of the relaxivity associated with a large hydration number (bishydrated complex) and fast water exchange ($k_{ex} = (29.9 \pm 0.4) \times 10^6\ s^{-1}$), which would be favorable for CA use. However the dissociation of $Gd(HDFB)^+$ is fairly fast (b2 ms) under all conditions employed in the present work thus the kinetically labile $Gd(HDFB)^+$ is not suitable for in vivo CA applications. Some low stability ternary complexes were also detected with $K(Gd(HDFB)(HCO_3)) = 17.5 \pm 1.9$ and $K(Gd(HDFB)(Lactate)) = 8.4 \pm 3.2$ but in the presence of citrate and phosphate ions the $Gd(HDFB)^+$ complex was found to dissociate. *(J. Inorg. Biochem.* **127,** 53-61, 2013.)

2.4. In vitro susceptibility testing of *Aspergillus fumigatus* to flucanazol, amphotericin B, PAF and different siderophores.

This study aimed at the investigation of antifungal drug interactions between siderophores (coprogen, triacetil fusarinin C, ferricrocin), amphotericin B, fluconazole and PAF in *Aspergillus fumigatus* cultures. We attempted to trigger iron homeostatic imbalance in the fungus by adding desferri-siderophores to the culture medium. Desferri-siderophores employed in combination with antifungal agents may lead to apoptosis in *Aspergillus fumigatus* cultures due to the disturbances in the biosynthesis of membrane lipids like ergosterol, which are iron-dependent processes.

Aspergillus fumigatus was more susceptible to the antimycotics PAF, fluconazole and amphotericin B. Importantly only PAF shows no toxic side-effects on mammalian cells *in vitro* and *in vivo* in mice. Consequently, interaction studies have a great significance to improve antifungal therapy especially using azoles and polyens. There were not any interactions between either PAF and amphotericin B or PAF and siderophores,

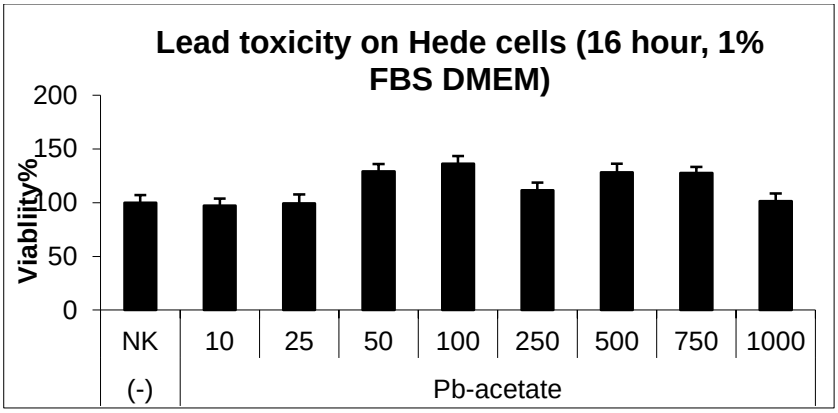
however, antagonism was observed between PAF and fluconazole. The antagonistic behavior of these two agents has already been demonstrated in zygomycetes. In our experiments, fluconazole showed a moderate antagonistic activity with all three siderophores suggesting that fluconazole is an inadequate antifungal agent to treat aspergillosis, a severe disease caused by siderophore producing *Aspergillus* species. Despite the fact that amphotericin B acted additively with desferri-ferricrocin and desferri-coprogen, its combination with desferri-triacetil-fusarinin C was synergistic. Which suggests that an effective inhibition of fungal growth could be achieved using this combination. Recently, antifungal activities of iron chelators in combination with fluconazole and amphotericin B have already been demonstrated. But there were synergistic and antagonistic combinations as well. For example, lactoferrin interacted synergistically with amphotericin B and antagonistically with fluconazole. In contrast, deferiprone showed antagonism with amphotericin B and synergism with fluconazole. These findings support our assumption that iron acquisition in fungal cells may provide an alternative drug target. Our data clearly show that iron chelation therapy with desferri-triacetil-fusarinin C or desferri-ferricrocin alone or in combination with amphotericin B and PAF should be tested in a murine aspergillosis model *in vivo*.

2.5. Antitumor effect of desferri-coprogen

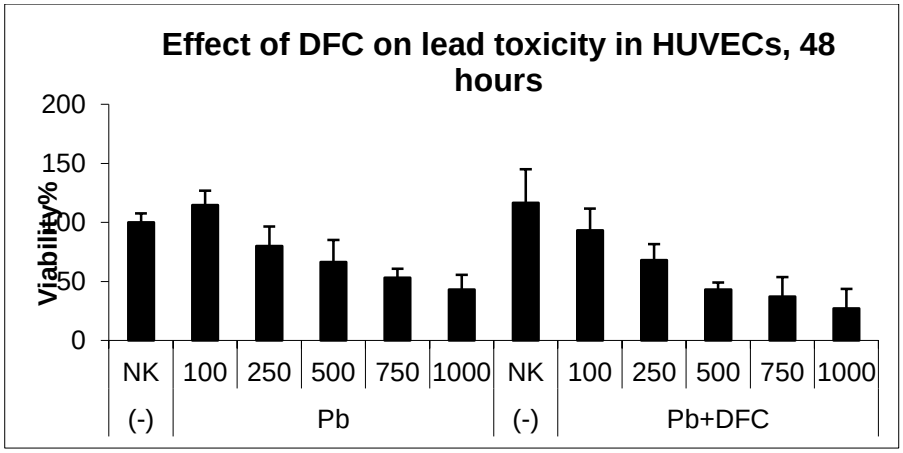
Ovarian cancer (OC) is a very aggressive tumor that represents a great burden on the health system. Ovarian cancer cell (OCC) line was established to study the effect of iron and iron chelation on the growth of OCC cells alone or in combination with doxorubicin, a drug used widely in chemotherapy of OC. Iron chelation can have opposing effects on tumor growth: (i) it can limit growth by iron restriction, or (ii) it can induce neovascularization and therefore support tumor growth via the induction and secretion of vascular endothelial growth factor (VEGF). We found that (i) VEGF is highly expressed in OCC, (ii) doxorubicin treatment decrease VEGF expression of OCC significantly, (iii) desferri-coprogen augment the cytostatic effect of doxorubicin toward OCC.

2.6. Treatment of lead-poisoning with desferri-coprogen

To access the protective effect of DFC in lead poisoning, mouse hepatocyte cell line (HeDe) was challenged by Pb-acetate at the concentration range of 10-1000 μmol/L. we observed that Hede cells were extremely resistant to Pb.



Then we tested the cytotoxic effect of Pb on human umbilical vein endothelial cells. Confluent HUVECs were treated with Pb-acetate or Pb-acetate complexed with DFC at the concentration of 100-1000 μmol/L for 24 or 48 hours. Cell viability was measured by MTT assay. DFC could not prevent Pb-mediated endothelial cell death.



2.7. Towards high-siderophore content foods – optimization of coprogen production in submerged cultures *of Penicillium nalgiovense.*

Fungal siderophores are likely to possess atheroprotective effects in humans and, therefore, studies are needed to develop siderophore-rich food additives or functional foods to increase the siderophore uptake in people prone to cardiovascular diseases. In this study, the siderophore contents of mold-ripened cheeses and meat products were analyzed, and the coprogen production of *Penicillium nalgiovense* was characterized.

High concentrations of hexadentate fungal siderophores were detected in penicillia-ripened Camembert-type and Roquefort-type cheeses and also in some sausages. In one sausage fermented by *P. nalgiovense*, the siderophore concentration was comparable to those found in cheeses. *P. nalgiovense* produced high concentrations of coprogen in submerged cultures, which were affected predominantly by the available carbon and nitrogen sources under iron starvation. Considerable coprogen yields were still detectable in the presence of iron when the fermentation medium was supplemented with the iron chelator Na_2-EDTA or when *P. nalgiovense* was co-cultivated with *Saccharomyces cerevisiae*.

These data may be exploitable in the future development of high-siderophore-content foods and/or food additives. Nevertheless, the use of *P. nalgiovense* fermentation broths for these purposes may be limited by the instability of coprogen in fermentation media and by the β-lactam production of the fungus. (*J. Sci. Food Agr.* **93,** 2221-2228, 2013.)

3. SEMINAL RESEARCH OF VASCULAR AND HEART DISORDERS, ATHEROSCLEROSIS, ATHEROGENESIS AND VASCULAR CALCIFICATION.

3.1. Ferritin prevents calcification and osteoblastic differentiation of vascular smooth muscle cells.

Vascular calcification plays a role in the pathogenesis of atherosclerosis, diabetes, and chronic kidney disease. Human aortic smooth muscle cells (HSMCs) undergo mineralization in response to elevated levels of inorganic phosphate (Pi) in an active and well-regulated process. This process involves increased activity of alkaline phosphatase and increased expression of core binding factor alpha-1, a bone-specific transcription factor, with the subsequent induction of osteocalcin. Mounting evidence suggests an essential role for the heme oxygenase 1 (HO-1)/ferritin system to maintain homeostasis of vascular function.

The aim of this study was to examine whether induction of HO-1 and ferritin alters mineralization of HSMCs provoked by high Pi. We found that upregulation of the HO-1/ferritin system inhibited HSMC calcification and osteoblastic differentiation. Of the products of the system, only ferritin and, to a lesser extent, biliverdin were

responsible for the inhibition. Ferritin heavy chain and ceruloplasmin, which both possess ferroxidase activity, inhibited calcification; a site-directed mutant of ferritin heavy chain, which lacked ferroxidase activity, failed to inhibit calcification. In addition, osteoblastic transformation of HSMCs provoked by elevated Pi (assessed by upregulation of core binding factor alpha-1, osteocalcin, and alkaline phosphatase activity) was diminished by ferritin/ferroxidase activity. We concluded that induction of the HO-1/ferritin system prevents Pi-mediated calcification and osteoblastic differentiation of human smooth muscle cells mainly via the ferroxidase activity of ferritin. (*J Am Soc Nephrol*. 2009 Jun;20(6):1254-63.)

3.2. Heme degradation and vascular injury

In this review article we summerized the role of heme and heme degradation in vacular injury. Heme is an essential molecule in aerobic organisms. Heme consists of protoporphyrin IX and a ferrous (Fe(2+)) iron atom, which has high affinity for oxygen (O(2)). Hemoglobin, the major oxygen-carrying protein in blood, is the most abundant heme-protein in animals and humans. Hemoglobin consists of four globin subunits (alpha(2)beta(2)), with each subunit carrying a heme group. Ferrous (Fe(2+)) hemoglobin is easily oxidized in circulation to ferric (Fe(3+)) hemoglobin, which readily releases free hemin. Hemin is hydrophobic and intercalates into cell membranes. Hydrogen peroxide can split the heme ring and release "free" redox-active iron, which catalytically amplifies the production of reactive oxygen species. These oxidants can oxidize lipids, proteins, and DNA; activate cell-signaling pathways and oxidant-sensitive, proinflammatory transcription factors; alter protein expression; perturb membrane channels; and induce apoptosis and cell death. Heme-derived oxidants induce recruitment of leukocytes, platelets, and red blood cells to the vessel wall; oxidize low-density lipoproteins; and consume nitric oxide. Heme metabolism, extracellular and intracellular defenses against heme, and cellular cytoprotective adaptations are emphasized. Sickle cell disease, an archetypal example of hemolysis, heme-induced oxidative stress, and cytoprotective adaptation, is reviewed. (*Antioxid Redox Signal*. 2010 Feb;12(2):233-48.)

3.3. Ferritin ferroxidase activity: a potent inhibitor of osteogenesis.

After we observed the marked regulatory effect of iron and ferritin on osteoblastic differentiation of vascular smooth muscle cells, we speculated whether ferritin and iron regulates normal bone metabolism. Hemochromatosis is a known cause of osteoporosis, and iron overload has deleterious effects on bone. Although iron overload and its association with osteoporosis has long been recognized, the pathogenesis and exact role of iron have been undefined. Bone is an active tissue with constant remodelling capacity. Osteoblast (OB) development and maturation are under the influence of core binding factor alpha-1 (CBF-alpha1), which induces expression of OB-specific genes, including alkaline phosphatase, an important enzyme in early osteogenesis, and osteocalcin, a noncollagenous protein deposited within the osteoid. This study investigates the mechanism by which iron inhibits human OB activity, which in vivo may lead to decreased mineralization, osteopenia, and osteoporosis. We demonstrate that iron-provoked inhibition of OB activity is mediated by ferritin and its ferroxidase activity. We confirm this notion by using purified ferritin H-chain and ceruloplasmin, both known to possess ferroxidase activity that inhibited calcification, whereas a site-directed mutant of ferritin H-chain lacking ferroxidase activity failed to provide any inhibition. Furthermore, we are reporting that such suppression is not restricted to inhibition of calcification, but OB-specific genes such as alkaline phosphatase, osteocalcin, and CBF-alpha1 are all down-regulated by ferritin in a dose-responsive manner. This study corroborates that iron decreases mineralization and demonstrates that this suppression is provided by iron-induced up-regulation of ferritin. In addition, we conclude that inhibition of OB activity, mineralization, and specific gene expression is attributed to the ferroxidase activity of ferritin.

3.4. Hydrogen sulfide inhibits the calcification and osteoblastic differentiation of vascular smooth muscle cells.

Osteoblastic differentiation of vascular smooth muscle cells (VSMCs) is involved in the pathogenesis of vascular calcification. Hydrogen sulfide (H(2)S) is a gas endogenously produced by cystathionine γ-lyase in VSMC. In this work we determined whether H(2)S plays a role in phosphate-induced osteoblastic transformation and mineralization of VSMC. Hydrogen sulfide was found to inhibit calcium deposition in the extracellular matrix and to suppress the induction of the genes involved in osteoblastic transformation of VSMC: alkaline phosphatase, osteocalcin, and Cbfa1. Moreover, phosphate uptake and phosphate-triggered upregulation of the sodium-dependent phosphate cotransporter (Pit-1) were also prevented by H(2)S. Reduction of endogenous production of H(2)S by inhibition of cystathionine γ-lyase activity resulted in increased osteoblastic transformation and mineralization. Low plasma levels of H(2)S, associated with decreased cystathionine γ-lyase enzyme activity, were found in patients with chronic kidney disease receiving hemodialysis. Thus, H(2)S is a potent inhibitor of phosphate-induced calcification and osteoblastic differentiation of VSMC. This mechanism might contribute to accelerated vascular calcification in chronic kidney disease. (*Kidney Int*. 2011 Oct;80(7):731-9.)

3.5. Ethanol increases phosphate-mediated mineralization and osteoblastic transformation of vascular smooth muscle cells.

Vascular calcification is implicated in the pathogenesis of atherosclerosis, diabetes and chronic kidney disease. Human vascular smooth muscle cells (HSMCs) undergo mineralization in response to elevated levels of inorganic phosphate (Pi) in an active and well-regulated process. This process involves increased activity of alkaline phosphatase and increased expression of core binding factor α-1 (CBF-α1), a bone-specific transcription factor, with the subsequent induction of osteocalcin. It has been shown that heavy alcohol consumption is associated with greater calcification in coronary arteries. The goal of our study was to examine whether ethanol alters mineralization of HSMCs provoked by high Pi. Exposure of HSMCs to ethanol increased extracellular matrix calcification in a dose responsive manner, providing a significant additional calcium deposition at concentrations of ≥60 mmol/l. HSMC calcification was accompanied by further enhancement in alkaline phosphatase activity. Ethanol also provoked a significant increase in the synthesis of osteocalcin. Moreover, in cells challenged with ethanol the expression of CBF-α1, a transcription factor involved in the regulation of osteoblastic transformation of HSMCs, was elevated. The observed effects of ethanol were not due to alterations of phosphate uptake by HSMCs. We conclude that ethanol enhances Pi-mediated human vascular smooth muscle calcification and transition of these cells into osteoblast-like cells. (J Cell Mol Med. 2012 Sep;16(9):2219-26.)

3.6. Cardioprotective effects of sour cherry seed extract (SCSE) on the hypercholesterolemic rabbit heart.

In this work we evaluated the hypothesis that sour cherry seed extract (SCSE) protects against cardiovascular disease and inflammation in hypercholesterolemic rabbits, and that this protection correlates with SCSE-induced activity of heme oxygenase- 1 (HO-1), a cytoprotective enzyme contributing to oxidative stress responses. We found that relative to cholesterol-treated animals not receiving SCSE, SCSE-treated animals exhibited significantly improved cardiac function and improved peak early diastolic velocity to peak atrial velocity ratio, along with decreased atherosclerotic plaque formation and infarct size. Increased HO-1 and cytochrome c oxidase III protein expression and COX activity were also noted in hearts from SCSE-treated rabbits. We concluded that SCSE acts in a cardioprotective manner on hypercholesterolemic hearts. Correlation of these

outcomes with HO-1 expression suggests that the effect may be mediated by activity of this enzyme. However, definitive proof of HO-1 dependence requires further investigation. (*Curr Pharm Des.* 2013;19(39):6896-905.)

3.7. The cellular autophagy markers Beclin-1 and LC3B-II are increased during reperfusion in fibrillated mouse hearts.

Autophagy is an intracellular bulk degradation process for elimination of damaged macromolecules and organelles. In the past decades, the scientific community has gained increasingly detailed understanding of the role of autophagy in myocardial homeostasis, although still many controversies remain. In the ischemic myocardium, autophagy appears to be beneficial for survival, whereas upon reperfusion the process may induce cell death. However, the overall effect of autophagy seems to depend on the duration and intensity of stress, as along with the extent of autophagy within myocardial tissue. Reperfusion of an ischemic heart maybe harmful, but it is an essential process for myocardial survival. One of the major adverse consequences of reperfusion is the occurrence of ventricular fibrillation (VF). In this study, we investigated the possible connection between autophagy and VF. Isolated mouse hearts were subjected to ischemia/reperfusion (I/R) and divided into two groups based on the development of VF at the beginning of reperfusion. Western blot analysis was conducted for autophagy-associated proteins LC3B, ATG-5, ATG-7, ATG-12, Bcl-2 and Beclin-1 proteins. Significantly higher level of Beclin-1 and LC3B-II/LC3B-I ratio (both definitive autophagy biomarkers) was observed in the fibrillated myocardium, versus tissue from the nonfibrillated hearts. Interestingly, although Bcl-2 is a major regulator of Beclin-1, level of this protein was not significantly altered in tissue from fibrillated, versus non-fibrillated hearts. Moreover, Atg7 expression showed a trend, albeit nonsignificant, towards elevation in fibrillated versus non-fibrillated hearts. Results of the present investigation demonstrate a possible link between VF and autophagy. Studies by authors of this report to evaluate potential etiologic relationships between the two processes are ongoing. (*Curr Pharm Des.* 2013;19(39):6912-8.)

3.8. Adverse impact of diet-induced hypercholesterolemia on cardiovascular tissue homeostasis in a rabbit model: time-dependent changes in cardiac parameters.

In this study we investigated whether diet-related hypercholesterolemia increases oxidative stress-related burden to cardiovascular tissue, resulting in progressively increased mortality, along with deterioration of electrophysiological and enzymatic function in rabbit myocardium. New Zealand white rabbits were divided into four groups, defined as follows: GROUP I, cholesterol-free rabbit chow for 12 weeks; GROUP II, cholesterol-free chow, 40 weeks; GROUP III, chow supplemented with 2% cholesterol, 12 weeks; GROUP IV, chow supplemented with 2% cholesterol, 40 weeks. At the 12 and 40-week time points, animals in each of the aforementioned cohorts were subjected to echocardiographic measurements, followed by sacrifice. Significant deterioration in major outcome variables measured in the present study were observed only in animals maintained for 40 weeks on 2% cholesterol-supplemented chow, with much lesser adverse effects noted in animals fed high cholesterol diets for only 12 weeks. It was observed that rabbits receiving high cholesterol diets for 40 weeks exhibited significantly increased mortality, worsened ejection fraction and general deterioration of cardiac functions, along with increased atherosclerotic plaque formation and infarct size. Additionally, myocardium of GROUP IV animals was observed to contain lower levels of heme oxygenase-1 (HO-1) and cytochrome c oxidase III (COX III) protein relative to the controls. (*Int J Mol Sci*. 2013 Sep 17;14(9):19086-108.)

3.9. Atherogenesis and iron: from epidemiology to cellular level

Iron accumulates in human atherosclerotic lesions but whether it is a cause or simply a downstream consequence of the atheroma formation is still an open question. According to the so called "iron hypothesis", iron is believed to be detrimental for the cardiovascular system, thus promoting atherosclerosis development and progression. Iron, in its catalytically active form can participate in the generation of reactive oxygen species and induce lipid-peroxidation, triggering endothelial activation, smooth muscle cell proliferation and macrophage activation; all of these processes are considered to be pro-atherogenic. On the other hand, the observation that hemochromatotic patients, affected by life-long iron overload, do not show any increased incidence of atherosclerosis is perceived as the most convincing evidence against the "iron hypothesis".

Epidemiological data and animal models have provided conflicting data regarding the role of iron in atherogenesis, emerging the need for more careful studies in which issues like the source and the compartmentalization of iron would be addressed. This review article summarizes what we have learnt about iron and atherosclerosis from the epidemiological studies, animal models and cellular systems. (This review is under revision at *Frontiers in Pharmacology*)

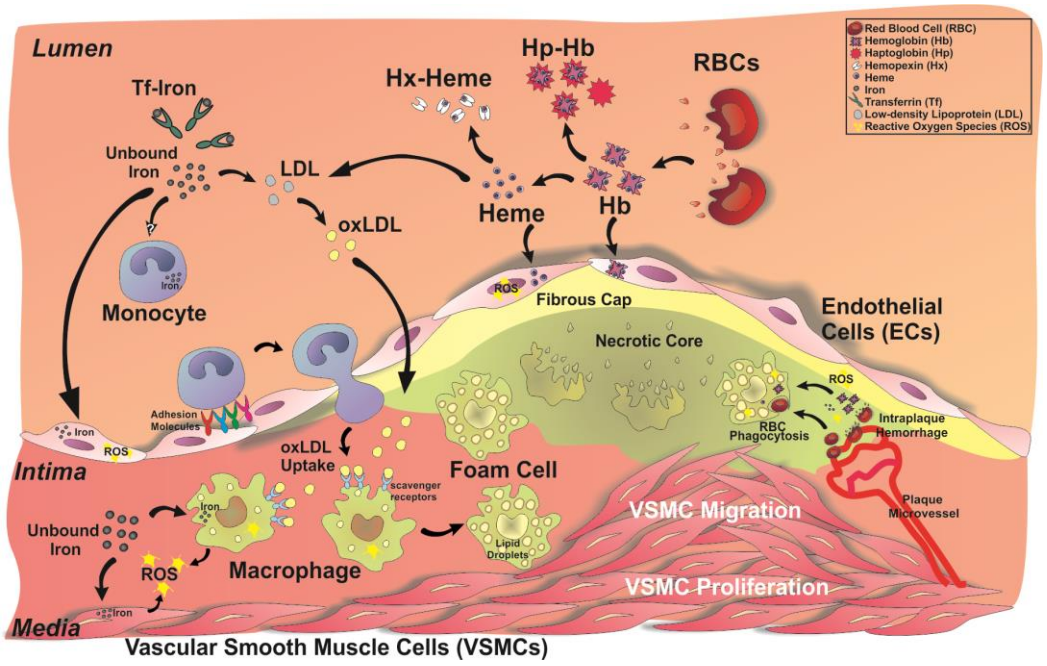


Figure 1.

How iron can access the lesions: a role for iron in atherosclerosis.

Iron can accumulate in the plaque as inorganic iron or hemoglobin-contained iron. Inorganic iron mainly derives from circulating iron, in the form of transferrin (Tf)-bound iron and non-transferrin-bound iron (NTBI). NTBI is generated upon increased iron absorption associated with pathological conditions, such as anemia and hemochromatosis. Circulating NTBI is thought to be accessible to many cell types in the context of an atherosclerotic plaque: endothelial cells, monocytes/macrophages and vascular smooth muscle cells (VSMCs). Hemoglobin- and heme-derived iron can access the plaque upon conditions of intravascular hemolysis and intraplaque hemorrhage, affecting endothelial cells and macrophages. Hemoglobin (Hb), heme and iron promote endothelial activation, by enhancing adhesion molecule expression. As a consequence, monocytes recruitment is expected to be increased. Circulating iron and Hb oxidize LDLs, thus enhancing subendothelial LDL retention and macrophage progression to foam cells. Iron also affects VSMC proliferation and migration into the lesion, favouring plaque progression.

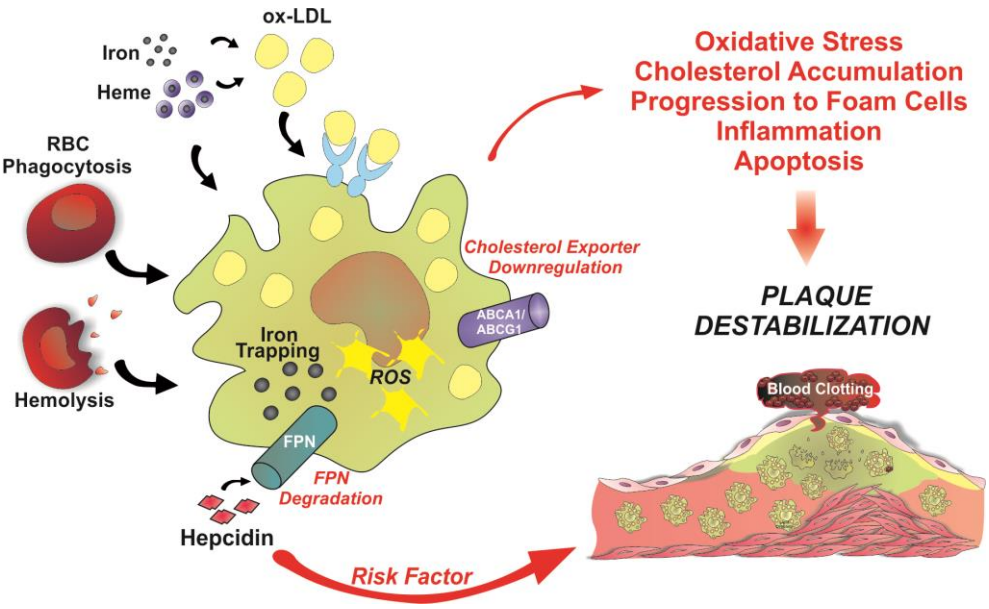


Figure 2.

Schematic overview of the refined iron hypothesis: a role for macrophage-retained iron in atherosclerosis.

Iron can be accumulated in the macrophage as inorganic iron and Hb-iron, upon erytrophagocytosis or hemolysis. Once stored into the cell, it can be recycled back via FPN-mediated export. According to the refined iron hypothesis, high hepcidin levels are considered a risk factor for plaque progression and destabilization. Hepcidin is known to bind FPN, thus promoting its degradation and blocking iron export. This is expected to increase intracellular ROS levels and to decrease cholesterol efflux. As a result, oxidative status alteration and LDL accumulation occur, promoting foam cell formation, inflammation and eventually plaque instability.

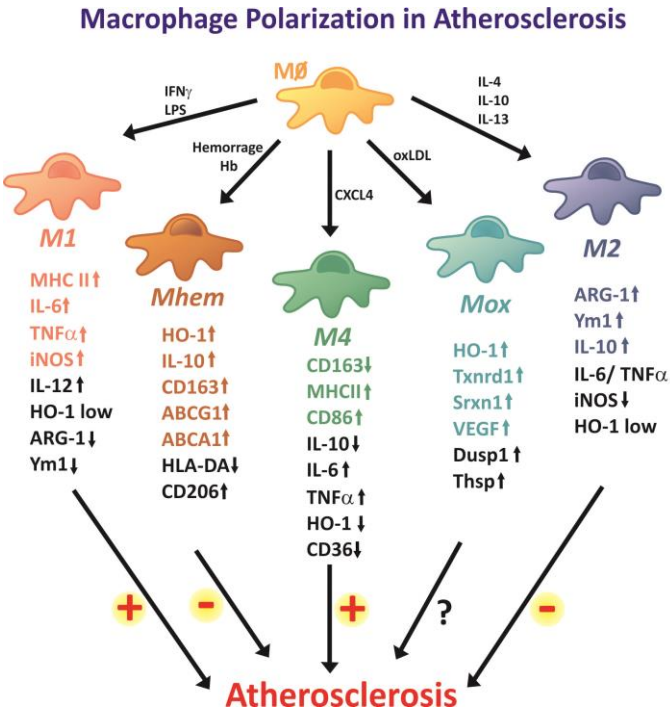


Figure 3.

Macrophage polarization in atherosclerosis.

In the atherosclerotic plaque macrophages could polarize towards different phenotypes, here depicted. M1 macrophages have strong pro-inflammatory properties, thus being potentially involved in lesion progression. M2 macrophages are considered anti-inflammatory and involved in tissue repair and remodeling. The M2 phenotype is reported as anti-atherogenic. Mhem macrophages originate upon intraplaque hemorrhage and are endowed with high Hb handling ability. These anti-atherogenic macrophages express high levels of the heme-degrading enzyme HO-1 and the Hp-Hb scavenger receptor CD163. M4 macrophages generate in response to the chemo-attractant CXCL4, thus showing pro-inflammatory and pro-atherogenic effects. These macrophages express low levels of CD163 and high levels of MHCII and CD86. M1 and M4 macrophages promote, while M2 and Mhem macrophages counteract foam cell formation, thus having opposite effect on atherosclerosis progression.