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Atheroprotective action of a modified organoselenium compound: *in vitro* evidence

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ABSTRACT

Oxidation of low-density lipoprotein (LDL) has been strongly suggested to play a significant role in the pathogenesis of atherosclerosis. Thus, reducing LDL oxidation is a potential approach to decrease the risk of the atherosclerosis. Organoselenium compounds have demonstrated promising atheroprotective properties in experimental models. Herein, we tested the *in vitro* atheroprotective capability of a modified organoselenium compound, Compound HBD, in protecting isolated LDL from oxidation as well as foam cells formation. Moreover, the glutathione peroxidase (GPx)-like activity of Compound HBD was analyzed in order to explore the mechanisms related to the above-mentioned protective effects. The Compound HBD in a concentration-dependent manner reduced the Cu²⁺-induced formation of conjugated dienes. The protein portion from LDL were also protected from Cu²⁺-induced oxidation. Furthermore, the Compound HBD efficiently decreased the foam cell formation in J774 macrophage cells exposed to oxidized LDL. We found that the atheroprotective effects of this compound can be, at least in part, related to its GPx-like activity. Our findings demonstrated an impressive effect of Compound HBD against LDL-induced toxicity, a further *in vivo* study to investigate in more detail the antioxidant and antiatherogenic effects of this compound could be considered.

Key words: atherosclerosis, Compound HBD, foam cell, GPx-like activity, LDL oxidation, organoselenium compounds.

INTRODUCTION

Atherosclerosis, classically defined as a progressive inflammatory disease, remains the most common cause of morbidity and mortality in industrialized countries. This pathophysiological condition

affects the vascular wall and leads to coronary artery diseases and cerebrovascular accidents (stroke) (Go et al. 2014). Hypercholesterolemia, especially high concentration of serum low density lipoproteins (LDL) has been considered one of the major risk factors for atherosclerosis development and progression (Ross 1999, Barter 2005).

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LDL, the main blood cholesterol carrier, becomes atherogenic after undergoing oxidative modifications (Brown et al. 1981, Itabe et al. 2011, Ganini and Mason 2014). The hypothesis that oxidative modification of LDL contributes to the progression of atherosclerosis is supported by an impressive body of *in vitro* findings and by persuasive results in animal models of atherosclerosis (Bird et al. 1998, Berrougui et al. 2006, Pirillo et al. 2013). All major vascular cell types are capable of oxidizing LDL, and several lines of evidence have demonstrated the *in vivo* occurrence of oxidized lipoproteins in atherosclerotic lesions (Steinberg and Witztum 2010, Yoshida and Kisugi 2010).

Oxidized LDL is intensively taken up by macrophages through scavenger receptors that subsequently promote foam cell formation which compose fatty streaks (hallmark of early atherogenesis) followed by the development of fibrous and atheromatous plaques (Steinberg 1997, 2002, Miller et al. 2010). Furthermore, oxidized LDL has been shown to enhance atherogenesis by other mechanisms, such as cytotoxicity towards endothelial cells and macrophages and stimulation of thrombotic and inflammatory events (Witztum 1993).

One possible method to prevent atherosclerotic diseases would be the administration of antioxidant substances thereby making LDL less sensitive to this oxidative process. In fact, it has been evidenced that the antioxidant capability of LDL can be increased by dietary antioxidant supplementation, i.e. LDL can incorporate endogenous and exogenous antioxidants in its supramolecular structure, decreasing its susceptibility to be oxidized. In fact, many endogenous and exogenous compounds have been reported to display beneficial effects against LDL oxidation (Noguchi et al. 2000, Chu and Liu 2005, Barcelos et al. 2011). However, the strategies with antioxidants supplementation had generated both “positive” and “no response” effects in decreasing atherogenesis (Rahman et al. 2014).

Reports have shown that selenium-containing organic molecules are generally more potent antioxidants than “classical” antioxidants, and this fact serves as an impetus for an increased interest in the rational design of synthetic organoselenium compounds (Mugesh et al. 2001, Nogueira et al. 2004, Nogueira and Rocha 2011). In the last years our laboratory have been studying the *in vitro* and *in vivo* antioxidant and anti-inflammatory properties of a simple diorganoselenium compound, diphenyl diselenide (PhSe)₂ (a prototype of this class of compounds) in models of atherosclerosis (de Bem et al. 2008, Hort et al. 2011, 2014, Stralioetto et al. 2013b, Mancini et al. 2014). Notably, this compound inhibited LDL oxidation induced by copper ions (Cu^{2+}) (de Bem et al. 2008) as well as potentially reducing the formation of atherosclerotic lesions in hypercholesterolemic low density lipoprotein receptor knockout ($\text{LDLR}^{-/-}$) mice (Hort et al. 2011). Importantly, chemical modifications in this organoselenium compound could confer higher pharmacological efficiency and less toxicological effects (Nogueira and Rocha 2011). In this regard, we recently demonstrated the powerful effect of the disubstituted diaryl diselenides, p-methoxydiphenyl diselenide and p-chloro-diphenyl diselenide against LDL-induced toxicity (Stralioetto et al. 2013a).

Considering that, the purpose of the present study was to investigate the potential beneficial effects of a modified organoselenium compound, Compound HBD (Fig. 1a), i.e. 2-((1-(2-(2-(2-(1-(2-hydroxybenzylideneamino) ethyl) phenyl) diselanyl) phenyl) ethylimino) methyl) phenol, in protecting *in vitro* isolated LDL from oxidation, as well as foam cells formation, which are the main elements involved in the early steps of atherogenesis. Moreover, the GPx-like activity of Compound HBD was also evaluated in an attempt to delve into molecular mechanisms related to the aforementioned protective effects.

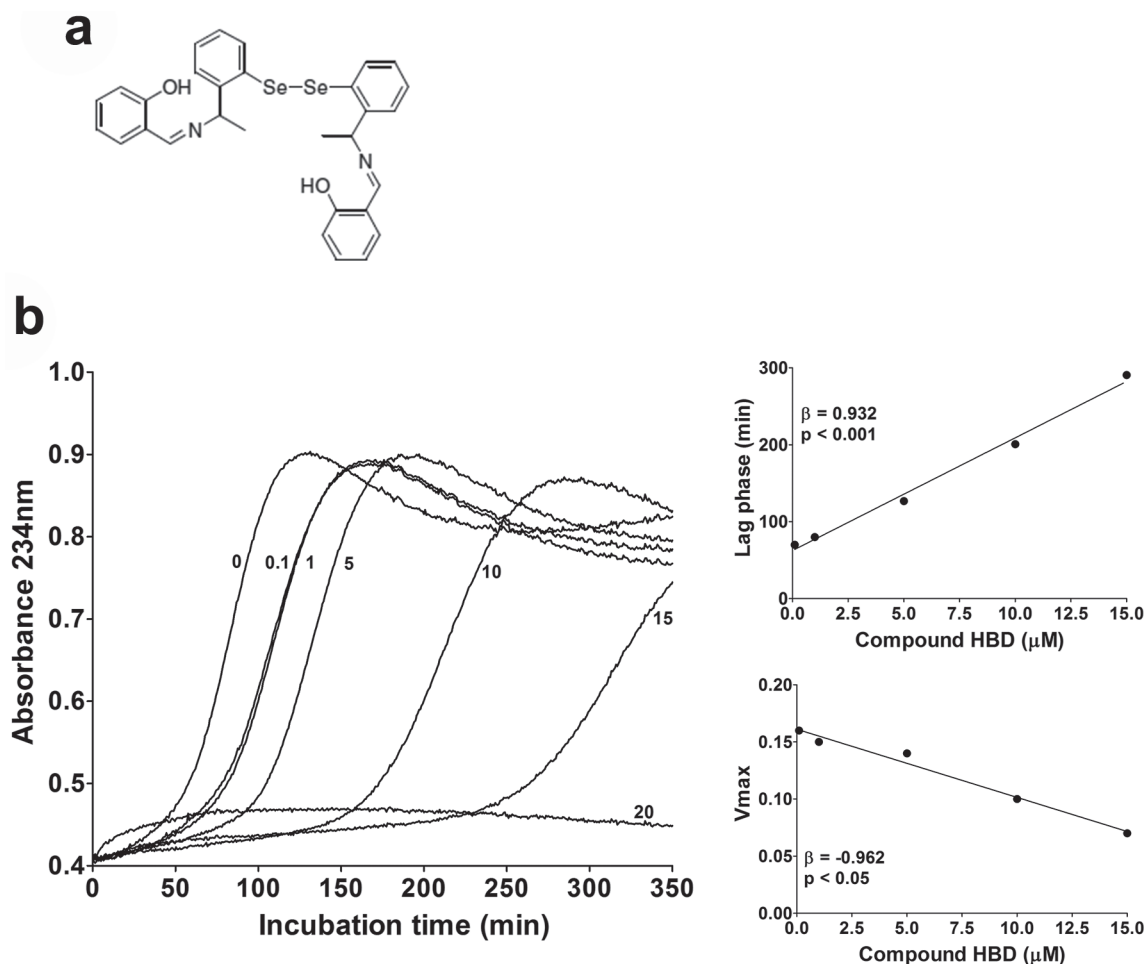


Figure 1 - Effect of Compound HBD on Cu²⁺-induced lipid peroxidation in human LDL. (a) Chemical structure of Compound HBD, i.e. 2-((1-(2-(2-(2-(1-(2-hydroxybenzylideneamino) ethyl) phenyl) diselanyl) phenyl) ethylimino) methyl) phenol. (b) At different time points (indicated in the abscissa axis), conjugated dienes (CD) were analyzed. LDL samples (50 μg protein/mL) were incubated in the presence of 5 μM CuSO₄ in the absence or presence (0.1-20 μM) of Compound HBD. CD are expressed as absorbance at 234nm. The inset shows the effect of Compound HBD on the lag phase and oxidation rate (V_{max}) of CD formation. Experiments were repeated at least three times, showing similar results. Linear regression of lag phase and V_{max} was used in order to verify the concentration dependent effect.

MATERIALS AND METHODS

COMPOUND HBD

Compound HBD, 2-((1-(2-(2-(2-(1-(2-hydroxybenzylideneamino) ethyl) phenyl) diselanyl) phenyl) ethylimino) methyl) phenol (Fig. 1a), was synthesized according to literature methods (Braga et al. 2005, Liu et al. 2005) with little modifications. Analysis of the ¹H NMR and ¹³C NMR spectra showed that the compound obtained (with 99.9%

purity) presented analytical and spectroscopic data in full agreement with their assigned structure.

LDL ISOLATION AND OXIDATION

LDL from human plasma was isolated by discontinuous density-gradient ultracentrifugation, as previously indicated by de Bem et al. (2008). The protein concentration in LDL preparation was measured using the method of Lowry et al. (1951). To prepare oxidized LDL (oxLDL), isolated LDL

(1 mg protein/ml) was oxidized with 10 μM CuSO_4 at 37 °C for 16 h. The reaction was terminated by adding 1.5 mM EDTA and the samples dialyzed against 148 mM phosphate buffer for 24 h at 4 °C. The experimental protocol was approved by the institutional ethics committee of the Universidade Federal de Santa Catarina (no 943/11).

LDL OXIDATION ASSAY

Incubation of LDL with Cu^{2+} initiates lipid peroxidation and causes extensive oxidation of the LDL lipids (Ziouzenkova et al. 1998). LDL oxidation was monitored by following the conjugated dienes (CD) formation. LDL samples (50 μg protein/mL) were preincubated at 37 °C in a medium containing 10 mM potassium phosphate buffer, pH 7.4 and different concentrations of Compound HBD (0 to 20 μM). After 10 min, CuSO_4 5 μM was added to the reaction medium and the reaction was monitored for 6 h for evaluating CD production. The oxidation was continuously monitored by measuring the increase in absorbance at 234 nm due to CD formation as previously described (Esterbauer et al. 1989). In addition, the value of the lag phase was determined as the intercept of the tangent of the slope of the absorbance curve in propagation phase with the time axis, and was expressed in min. The oxidation rate (V_{max}) was obtained from the slope of the absorbance curve during the propagation phase (Giese and Esterbauer 1994).

MEASUREMENT OF LDL-TRP FLUORESCENCE

The time course of tryptophan (Trp) fluorescence emission intensity is used to monitor Cu^{2+} induced apoB-100 LDL oxidation. The fluorescence spectra of native LDL display a single band centered at approximately 332 nm, which is assigned to the Trp residues in apoB-100 (Giessauf et al. 1995). Loss of Trp fluorescence is a marker for oxidations at the protein core of LDL (Reyftmann et al. 1990, Giessauf et al. 1995). The kinetics of LDL oxidation was followed by measuring the decrease of Trp-

fluorescence, corresponding to the decomposition of this amino acid, after the addition of 3.3 μM CuSO_4 , in absence or presence of different Compound HBD (0 to 15 μM) concentrations. Trp fluorescence was measured at different time points (0 to 360 min) (excitation at 282 nm and emission at 331 nm). Then the parameter “half-time” ($t_{\text{max}/2}$) was used to characterize the fluorescence changes in quantitative terms for practical purposes. It is defined as the time needed to observe a reduction in fluorescence of 50% of the difference between initial and residual fluorescence intensity (Jerlich et al. 2000).

CELL CULTURE AND FOAM CELL FORMATION ASSAY

Murine macrophage cell line J774A was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were maintained with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2 mM glutamine, 10 mM HEPES, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% fetal bovine serum (FBS) in a 5% CO_2 humidified atmosphere at 37 °C. For the foam cells formation, J774 cells were plated in 12-well plate at equal density (2×10^5 cells per well) in DMEM medium supplemented with 10% FBS for 24 h. After that, the medium was replaced by DMEM medium without FBS and the cells were incubated with Compound HBD 1 μM or vehicle in a 5% CO_2 humidified atmosphere at 37 °C. After 24 h, oxLDL (100 $\mu\text{g}/\text{mL}$) was added to the medium for additional 3 h. Foam cell formation assay was performed with the Oil-Red O staining method (Koopman et al. 2001). Briefly, after oxLDL incubation, cells were fixed with 4% paraformaldehyde, washed in phosphate buffer, then stained by 0.3% Oil-Red O for 10 min. Hematoxylin was used as counterstaining. Images of cells were acquired using a light microscopy (Olympus, BX-41). Six images were captured from each group, and the total pixels intensity was determined using NIH ImageJ 1.36b imaging

software (National Institutes of Health, Bethesda, MD), and lipid content was expressed as arbitrary units (a.u.).

GLUTATHIONE PEROXIDASE (GPX) LIKE ACTIVITY

The GPx-like activity of Compound HBD was measured according to a method previously described by Wilson et al. (1989). Compound HBD at different concentrations (1 to 30 μ M) was incubated at 37 °C in a medium containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM reduced glutathione (GSH), 1 mM azide, 0.2 U of GR and 0.25 mM NADPH. The reaction was initiated by addition of 0.2 mM of H_2O_2 . The activity was followed by the decrease of NADPH absorption at 340 nm.

STATISTICAL ANALYSIS

Values are presented as mean \pm SEM. The statistical analyses were performed by using one-way analysis of variance (ANOVA) followed by the post hoc Duncan's multiple range test. Linear regression analysis was also used to test concentration-dependent effects. A value of $p < 0.05$ was considered to be significant. All tests were performed using the Statistica software package (StatSoft Inc., Tulsa, OK, USA).

RESULTS

COMPOUND HBD EFFECTS ON Cu^{2+} - INDUCED LDL OXIDATION

LDL was subjected to oxidation with 5 μ M Cu^{2+} . The Cu^{2+} -ions exerted peroxidative modification of LDL polyunsaturated fatty acids and led to a molecular rearrangement, thus forming CD. The kinetic profile of the LDL oxidation was characterized by an initial *lag time* followed by a propagation period, where the rate of CD formation was maximal, and then by a decomposition phase. Notably, Compound HBD inhibited Cu^{2+} -induced generation of CD in a concentration-

dependent manner (Fig. 1b). Compound HBD in a concentration-dependent manner prolongs the *lag period* ($\beta = 0.932$, and $p < 0.001$), and decreases the oxidation rate $-V_{max}$ ($\beta = -0.962$, and $p < 0.05$), evidenced by changes in the propagation phase slope. It is noteworthy that Compound HBD presented maximal efficacy at 20 μ M.

COMPOUND HBD EFFECTS ON LDL-PROTEIN OXIDATION FLUORESCENCE KINETICS

Probably, oxidative modification of the lipid part of the LDL particle is followed by a modification of apoB-100 (Esterbauer et al. 1987). Fig. 2 shows that protein fraction of LDL are also oxidized as function of time in the presence of Cu^{2+} (3.3 μ M), resulting in a decrease in the kinetic of tryptophan fluorescence. When LDL was incubated with Compound HBD, the LDL-protein oxidation was prevented in a concentration-dependent manner ($\beta = 0.856$, and $p < 0.05$), evidenced by 50% inhibition of fluorescence tryptophan ($T_{max/2}$), reaching more than 50% of inhibition in the higher concentrations (10 and 15 μ M).

EFFECTS OF COMPOUND HBD IN THE oxLDL MEDIATED FOAM CELL FORMATION

The oxLDL uptake by macrophages and consequent foam cell formation was induced in macrophages exposed to oxLDL. The pretreatment with Compound HBD (1 μ M) significantly reduced the oxLDL uptake by macrophages, a critical step involved in the atherogenic process (Fig. 3a and b; $p < 0.01$). It is important mentioning that we preliminarily performed a MTT assay to analyze the cell viability after Compound HBD incubation. We exposed the macrophages cells to different concentrations of Compound HBD during 24 hours to attest that 1 μ M is a safe concentration (data not show).

GPX-LIKE ACTIVITY

The next step was to investigate the mechanisms by which Compound HBD was able to prevent

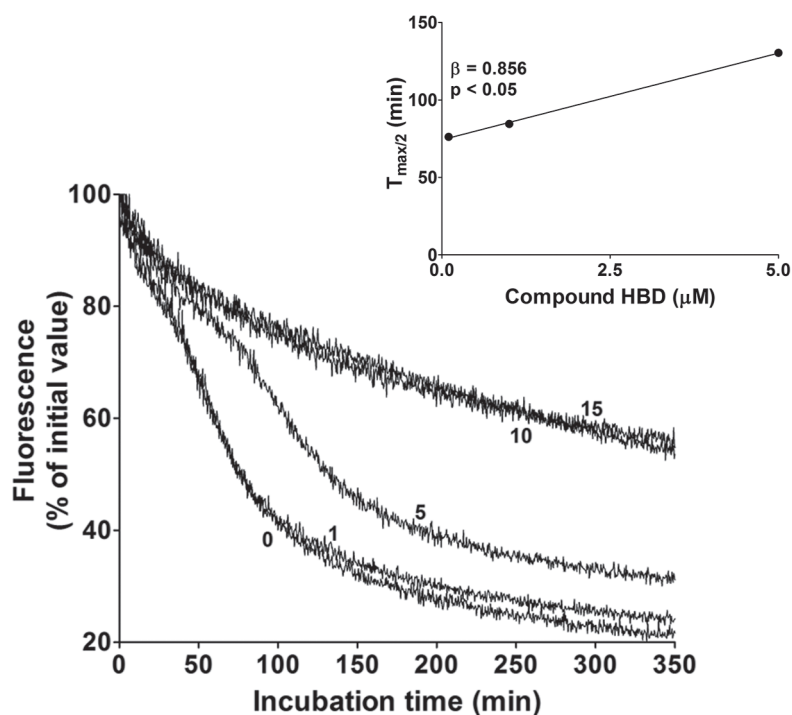


Figure 2 - Effect of Compound HBD on Cu^{+2} -induced loss of tryptophan fluorescence in human LDL. LDL samples ($50 \mu\text{g}$ protein/mL) were incubated at 37°C in the presence of $3.3 \mu\text{M}$ CuSO_4 in the absence or presence ($1\text{--}15 \mu\text{M}$) of Compound HBD. Tryptophan fluorescence (excitation at 282 nm and emission at 331 nm) was measured at different time points ($0\text{--}300 \text{ min}$). Inset shows the 50% inhibition ($t_{\text{max}/2}$) of loss of tryptophan fluorescence. Data are expressed as percentage of the initial value of emission intensity measured before Cu^{+2} addition. Experiments were repeated at least three times, showing similar results. Linear regression of loss of tryptophan fluorescence was used in order to verify the concentration-dependent effect.

LDL oxidation. The GPx is critically involved in cell protection against oxidative stress by reducing of numerous reactive species, at the expenses of GSH. It is well documented the GPx-like activity of organoselenium compounds, such as ebselen and $(\text{PhSe})_2$ (Wilson et al. 1989). Therefore, using a GR-coupled assay, we investigated the GPx-like activity of Compound HBD. Notably, the Compound HBD displayed a concentration-dependent GPx-like activity in an *in vitro* system (Fig. 4; $\beta = 0.98$, and $p < 0.001$).

DISCUSSION

Atherosclerosis is a multifactorial complication leading to cardiovascular diseases and stroke.

Elevated levels of total cholesterol and LDL-cholesterol stand among the major risk factors of atherosclerosis. Specifically, the oxidized form of LDL is supposed to set the ground of this pathophysiology (Brown and Goldstein 1984, Ross 1999, Rahman et al. 2014). The antioxidants that can inhibit these oxidative processes might be useful in preventing atherosclerosis-related pathological conditions (Steinberg and Witztum 2010). Epidemiological studies show that there is a positive correlation between the consumption of antioxidants and a decrease of the incidence of coronary artery disease (Pandey and Rizvi 2009). In this regard, it has been suggested that organoselenium compounds have various

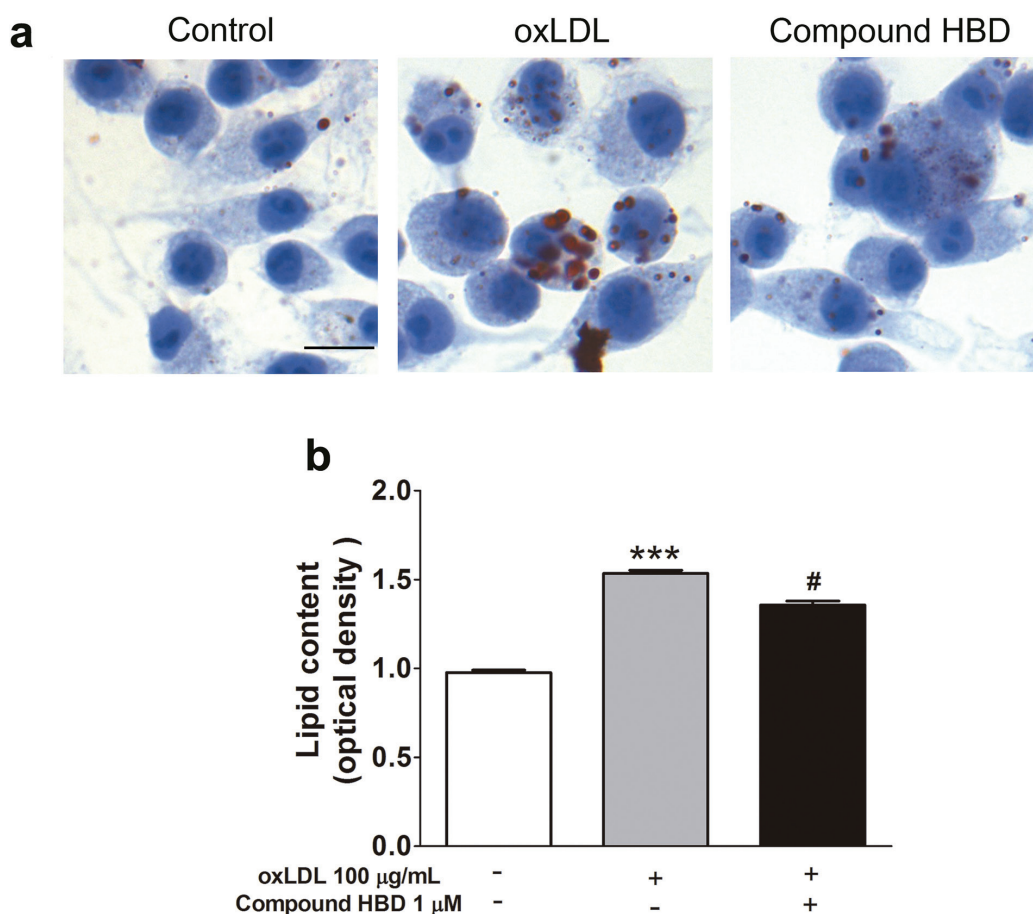


Figure 3 - Effect of Compound HBD on foam cell formation. (a) J774 macrophage cells were preincubated for 24 h with 1 µM of Compound HBD or vehicle, then, oxidized LDL (oxLDL) 100 µg/mL was added to the medium for 3 h. The cells were stained with Oil Red O and hematoxilin and observed under light microscope. Representative images were from three independent experiments. Scale bar, 10 µm. (b) Quantification of foam cells lipid content (optical density). The data presented were means ± SEM. * $p < 0.001$ compared with control cells and # $p < 0.05$ compared with oxLDL-treated cells (one-way ANOVA).

pharmacological activities, such as antioxidant, anti-inflammatory, and cardioprotective activities (Nogueira and Rocha 2011). For instance, our previous studies demonstrated that (PhSe)₂, the most simple organoselenium compound, presented atheroprotective properties in different *in vivo* and *in vitro* models (de Bem et al. 2008, 2009, Hort et al. 2011, 2014, Stralioetto et al. 2013b).

In this work, Compound HBD was chosen as a potential beneficial molecule against oxidation of human isolated LDL based on their chemical similarity with (PhSe)₂. We hypothesized that the chemical modifications present in the Compound

HBD could provide higher pharmacological efficiency and less toxicological effects. Previously, Hassan et al. (2009) demonstrated *in vitro* antioxidant effects of Compound HBD on Fe²⁺-induced lipid peroxidation in rat's tissue homogenates and phospholipids extract, not only at physiological pH but also under acidic conditions, pointing this compound as a possible pharmacological agent in ischemic/reperfusion injury (Hassan et al. 2009). Our data shows that the Compound HBD presented beneficial effects against oxidation induced by Cu²⁺ in isolated LDL and prevented foam cell formation.

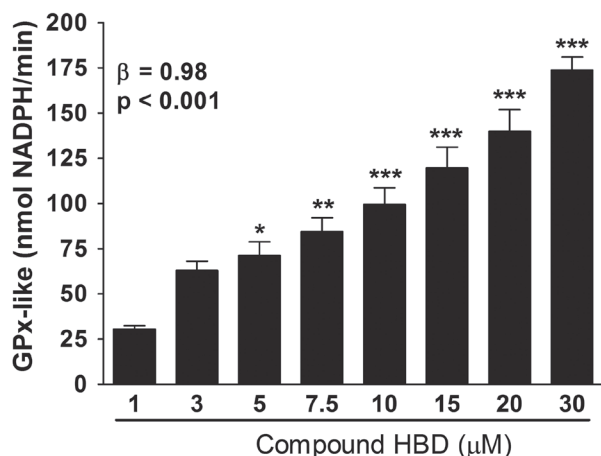


Figure 4 - The GPx-like activity of Compound HBD (1-30 μM). Linear regression of GPx-like activity was used in order to verify the concentration-dependent effect. Results are represented as mean ± SEM from at three independent experiments (one-way ANOVA).

It has been suggested that among all *in vitro* antioxidant capacity assays, measuring the LDL antioxidant activity is the most physiopathologically important and the most informative approach for screening antioxidant activity in compounds in order to prevent atherosclerosis (Katsube et al. 2004). LDL has multiple reduction sites with different affinities for Cu^{2+} . A common method for measuring the inhibition of LDL oxidation *in vitro* is by determining the formation of CD in response to peroxidative modification of the polyunsaturated fatty acids (PUFAs) present in the LDL molecule mediated by Cu^{2+} (Ziouzenkova et al. 1998). This approach was based on previous research findings that LDL oxidation at cell-free system by redox-active metal ions (copper and iron) is physiological and biochemically similar to that of cellular systems (Gunathilake and Rupasinghe 2014). Epidemiologic studies indicate the higher level of copper and iron ions in the arterial walls of the atherosclerotic individuals and these redox-active metal ions have been implicated in playing a vital role in oxidizing the native LDL molecule both *in vivo* and *in vitro* (Rahman et al. 2014). In the present study, the Compound HBD prevented

Cu^{2+} -induced isolated LDL peroxidation in a concentration-dependent manner by decreasing the formation of CD, thus extending the lag phase and lowering the oxidation rate (V_{max}). It is noticeable that, the Compound HBD presented higher potential in inhibiting LDL oxidation when compared to $(\text{PhSe})_2$ using the same *in vitro* model (de Bem et al. 2008). This evidence suggest that other organoselenium compounds, besides $(\text{PhSe})_2$ and ebselen (the prototypes of these class) deserve to be evaluated as antiatherogenic agents.

Considering that during oxidation process the apoB-100 present in LDL is also modified, another significant result of our study was the ability of Compound HBD to prevent Cu^{2+} -induced loss of Trp fluorescence in human isolated LDL. In this regard, it has been reported that the fluorescence spectrum of native LDL displays a single band centered at approximately 332 nm, which is assigned to the Trp residues in apoB-100, and the loss of Trp fluorescence is a marker for oxidations at the protein core of LDL (Reyftmann et al. 1990). The protective effect of Compound HBD against Cu^{2+} -induced loss of Trp fluorescence indicates that, besides its beneficial effect against the oxidation of lipid moieties of LDL, this organoselenium compound also prevents the oxidation of protein part of human LDL, pointing to an additional mechanism that could contribute to inhibition of the atherogenic process.

It is worth mentioning that other well-established antioxidants also protect LDL from the oxidation induced by different insults in *in vitro* as well as *in vivo* assays. For instance, several studies using vitamin C e E demonstrated remarkable cardiovascular protective role of these molecules in prevent the LDL oxidation (Sabharwal and May 2008, Ghaffari et al. 2011, Shariat et al. 2013, Nadeem et al. 2012). The report of Nadeem et al. (2012) demonstrated that vitamin E derivatives, namely the tocopherols, become incorporated into LDL protecting this lipoprotein against oxidation.

Specifically, preincubation with α - and γ -tocopherol (final concentration range, 0–5 μ M) prevent the LDL oxidation induced by copper (II) chloride solution (CuCl₂). Moreover, previously Galli et al. (2004) observed that vitamin E metabolites, carboxyethyl-6-hydroxychromans (concentration range 0.015–5 μ M), exert concentration-dependent inhibition of the Cu²⁺-induced lipid oxidation of plasma. On the other hand, some molecules approved by US Food and Drug Administration for the use in the cardiovascular disease also present antioxidant properties. One interesting example is Rosuvastatin, a novel 3-Hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase inhibitor widely used in the treatment of hypercholesterolemia, reducing the risk of myocardial infarction and stroke (MacDonald 2010). Rosuvastatin is able to deal with reactive species generated in the vascular environment and shows *in vitro* antioxidant capacity protecting against tissue lipid peroxidation induced by Fenton's reaction (Gómez-García et al. 2007, Ajith et al. 2008). In addition, a recent clinical study reported that rosuvastatin treatment reduce significantly plasma levels of oxLDL in hypercholesterolemic subjects (Homma et al. 2015).

Macrophage cholesterol accumulation is positively correlated with atherogenesis (Steinberg et al. 1989, Moore and Tabas 2011, Steinberg 2013). The oxidative modification of LDL resulted in diminished affinity for LDL receptors and increased affinity for macrophage scavenger receptors. The active uptake of the oxidized LDL by macrophages leads to their transition to foam cells, initiating plaque formation (Lv et al. 2014). This process is largely mediated and supported by the metabolism of endothelial and smooth muscle cells in response to oxidized LDL, concomitant with the release of proinflammatory cytokines from emerging foam cells (Nambiar et al. 2014). In this study, we have shown that pretreatment of J774 macrophages with Compound HBD significantly decreased

oxidized LDL uptake and, consequently, foam cell formation. Consistent with this observation, we have reported that (PhSe)₂ (Stralio et al. 2013b) as well as its derivatives (Stralio et al. 2013a) potentially inhibited the foam cell formation induced by oxidized LDL. Indeed, the effect of Compound HBD in prevent foam cell formation could be through extracellular and intracellular mechanisms, and it is important to establish this process in next studies. For instance, Bartolini et al. (2015a) showed that a microencapsulated formulation of PhSeZnCl (M-PhSeZnCl) was uptake through an endocytosis like mechanism in the MCF-7 cells (Bartolini et al. 2015a).

With the intention of understanding the mechanism by which the Compound HBD reduces the LDL oxidation, we further investigated the GPx-like activity of this compound. Notably, the Compound HBD displayed concentration-dependent GPx-like activity, as already demonstrated for other organoselenium compounds that efficiently prevent LDL oxidation (de Bem et al. 2008, Stralio et al. 2013a). The antioxidant activity of organoselenium compounds have been attributed to its GPx-like activity (Sies 1993, 1994). In this sense, two pioneer studies showed that ebselen is efficient in reducing oxidative modification of LDL through its hydroperoxide-reducing activity (Noguchi et al. 1994, Lass et al. 1996). The decade of 1990s was also characterized by an enormous development of small synthetic organoselenium compounds that mimic GPx catalytic activity. One of them is (PhSe)₂, the simplest structure in this series, that reacts very efficiently with hydroperoxides and organic peroxides, mimicking the reaction cycle of the GPx enzyme in the presence of reduced thiols. Importantly, the apparent GPx-like activity of (PhSe)₂ has been reported to be superior than that of ebselen (Mugesh 2000, de Bem et al. 2013). One possible explanation for this more efficient activity of (PhSe)₂ is the higher contribution of Se equivalents in the reaction medium (Bartolini

et al. 2015a). Some new disubstituted diaryl-diselenides, such as Compound HBD and p-Cl-diphenyl diselenide (Straliotto et al. 2013a) display GPx-like activity comparable or even higher than that of $(\text{PhSe})_2$, while this enzymatic like effect was not evidenced for other compound of this series as p-metoxyl- diphenyl diselenide (Straliotto et al. 2013a). In line with this, Bartolini et al. (2015a) recently showed that phenylselenium zinc chloride (PhSeZnCl) presents higher apparent GPx-like activity than ebselen, but not superior than $(\text{PhSe})_2$ (Bartolini et al. 2015a). Mechanistically, this result indicated that the GPx-like activity of Compound HBD display an important role in preventing LDL oxidation, pointing it as possible pathway involved in the antiatherogenic effect of this class of compounds. In this regard, clinical studies have suggested an important antiatherogenic role for the antioxidant enzyme GPx (Blankenberg et al. 2003). In animal studies, the lack of functional GPx1 has been shown to accelerate diabetes-associated atherosclerosis via the upregulation of proinflammatory and profibrotic pathways in $\text{ApoE}^{-/-}$ mice (Lewis et al. 2007). Furthermore, reduced GPx1 expression has been associated with an increase in cell-mediated oxidation of LDL (Guo et al. 2001).

Considering our previous reports about antiatherogenic effect of the simple organoselenium compound $(\text{PhSe})_2$, we can not discard that other mechanisms can be involved in the antiatherogenic action of Compound HBD in *in vitro* and *in vivo* models (de Bem et al. 2013, Straliotto et al. 2013b). For instance, $(\text{PhSe})_2$ promotes Nrf-2 activation in endothelial cells, which increases the expression of antioxidant genes leading to an improvement in cellular redox environment and consequently protecting these cells against oxidative insults (de Bem et al. 2013, Hort et al. 2014). Furthermore, Bartolini et al. (2015b) demonstrated that a new class of diselenides derived from $(\text{PhSe})_2$ - particularly 2,2'-diselenyl dibenzoic acid - behave as mild thiol peroxidases leading to a moderate

generation of oxidative stress, which ultimately stimulated Nrf-2 nuclear translocation and then the transcription of the same Nrf-2 gene as well as of glutathione-S-transferase and other phase II genes. This resulted in a higher degree of protection against H_2O_2 cytotoxicity in cells (Bartolini et al. 2015b).

In conclusion, the Compound HBD, an organoselenium compound derived from $(\text{PhSe})_2$, presented significant atheroprotective effects: i) showed a high potential of inhibiting the lipid and protein oxidation induced by copper in isolated LDL, and ii) exerted a direct effect on macrophage cholesterol metabolism by reducing the cellular uptake of oxidized LDL and, consequently, foam cell formation. Probably, these effects are at least in part related to its GPx-like activity. Taken together, such data renders Compound HBD as a promising molecule for pharmacological studies with respect to the atherogenic process.

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RESUMO

A oxidação da lipoproteína de baixa densidade (LDL) apresenta um papel importante na patogênese da aterosclerose. Nesse sentido, reduzir a oxidação da LDL é uma abordagem importante para diminuir o risco de aterosclerose. Compostos orgânicos de selênio têm demonstrado propriedades ateroprotetoras promissoras em modelos experimentais. Neste estudo, nós testamos a capacidade ateroprotetora *in vitro* de um composto orgânico de selênio modificado, Composto HBD, em proteger a LDL isolada da oxidação, bem como a formação de células espumosas. Além disso, a atividade

mimética da enzima glutathione peroxidase (GPx) do composto HBD foi analisada com o intuito de explorar os mecanismos relacionados com os efeitos protetores apresentados pelo Composto HBD. O Composto HBD reduziu a formação de dienos conjugados induzida pelo Cu^{2+} em uma maneira dependente da concentração. A parte proteica da LDL também foi protegida da oxidação induzida pelo Cu^{2+} . Além disso, o composto HBD eficientemente diminuiu a formação de células espumosas em macrófagos J774 expostos à LDL oxidada. Nós observamos que os efeitos ateroprotetores deste composto podem ser, ao menos em parte, relacionados à sua atividade mimética da GPx. Nossos resultados demonstraram um efeito *in vitro* promissor do Composto HBD em reduzir a toxicidade induzida pela LDL. Nesse sentido, um outro estudo *in vivo* para investigar de maneira mais detalhada os efeitos antioxidantes e ateroprotetores deste composto pode ser considerado.

Palavras-chave: aterosclerose, Composto HBD, célula espumosa, atividade tipo glutathione peroxidase, oxidação da LDL, compostos orgânicos de selênio.

REFERENCES

- AJITH TA, RIJI T AND ANU V. 2008. *In vitro* anti-oxidant and DNA protective effects of the novel 3-hydroxy-3-methyl-glutaryl coenzyme A reductase inhibitor rosuvastatin. Clin Exp Pharmacol Physiol 35(5-6): 625-629.
- BARCELOS RP, PORTELLA RL, DA ROSA EJ, FONSECA AS, BRESOLIN L, CARRATU V, SOARES FA AND BARBOSA NV. 2011. Thiosemicarbazone derivate protects from AAPH and Cu^{2+} -induced LDL oxidation. Life Sci 89(1-2): 20-28.
- BARTER P. 2005. The inflammation: lipoprotein cycle. Atheroscler Suppl 6: 15-20.
- BARTOLINI D, COMMODI J, PIRODDI M, INCIPINI L, SANCINETO L, SANTI C AND GALLI F. 2015b. Glutathione S-transferase pi expression regulates the Nrf2-dependent response to hormetic diselenides. Free Radic Biol Med pii: S0891-5849(15)00307-X.
- BARTOLINI D ET AL. 2015a. Reaction kinetics and targeting to cellular glutathione S-transferase of the glutathione peroxidase mimetic PhSeZnCl and its D,L-poly lactide microparticle formulation. Free Radic Biol Med 78: 56-65.
- BERROUGUI H, ISABELLE M, CHERKI M AND KHALIL A. 2006. Marrubium vulgare extract inhibits human-LDL oxidation and enhances HDL-mediated cholesterol efflux in THP-1 macrophage. Life Sci 80(2): 105-112.
- BIRD DA, TANGIRALA RK, FRUEBIS J, STEINBERG D, WITZTUM JL AND PALINSKI W. 1998. Effect of probucol on LDL oxidation and atherosclerosis in LDL receptor-deficient mice. J Lipid Res 39(5): 1079-1090.
- BLANKENBERG S ET AL. 2003. Glutathione peroxidase 1 activity and cardiovascular events in patients with coronary artery disease. N Engl J Med 349: 1605-1613.
- BRAGA AL, PAIXÃO MW AND MARIN G. 2005. Seleno-imine: a new class of versatile, modular N, Se ligands for asymmetric palladium-catalyzed allylic alkylation. Synlett 11: 1675-1678.
- BROWN MS AND GOLDSTEIN JL. 1984. How LDL receptors influence cholesterol and atherosclerosis. Sci Am 251: 58-66.
- BROWN MS, KOVANEN PT AND GOLDSTEIN JL. 1981. Regulation of plasma cholesterol by lipoprotein receptors. Science 212: 628-635.
- CHU YF AND LIU RH. 2005. Cranberries inhibit LDL oxidation and induce LDL receptor expression in hepatocytes. Life Sci 77(15): 1892-1901.
- DE BEM AF, FARINA M, PORTELLA RL, NOGUEIRA CW, DINIS TC, LARANJINHA JA, ALMEIDA LM AND ROCHA JB. 2008. Diphenyl diselenide, a simple glutathione peroxidase mimetic, inhibits human LDL oxidation *in vitro*. Atherosclerosis 201: 92-100.
- DE BEM AF, FIUZA B, CALCERRADA P, BRITO PM, PELUFFO G, DINIS TC, TRUJILLO M, ROCHA JB, RADI R AND ALMEIDA LM. 2013. Protective effect of diphenyl diselenide against peroxynitrite-mediated endothelial cell death: a comparison with ebselen. Nitric Oxide 31: 20-30.
- DE BEM AF, PORTELLA RL, COLPO E, DUARTE MM, FREDIANE A, TAUBE PS, NOGUEIRA CW, FARINA M, DA SILVA EL AND ROCHA JB. 2009. Diphenyl diselenide decreases serum levels of total cholesterol and tissue oxidative stress in cholesterol-fed rabbits. Basic Clin Pharmacol Toxicol 105: 17-23.
- ESTERBAUER H, JURGENS G, QUEHENBERGER O AND KOLLER E. 1987. Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. J Lipid Res 28: 495-509.
- ESTERBAUER H, STRIEGL G, PUHL H AND ROTHENEDER M. 1989. Continuous monitoring of *in vitro* oxidation of human low density lipoprotein. Free Radic Res Commun 6: 67-75.
- GALLI F, PIRODDI M, LANNONE A, PAGLIARANI S, TOMASI A AND FLORIDI A. 2004. A comparison between the antioxidant and peroxynitrite-scavenging functions of the vitamin E metabolites alpha- and gamma-carboxyethyl-6-hydroxychromans. Int J Vitam Nutr Res 74(5): 362-373.
- GANINI D AND MASON RP. 2014. Absence of an effect of vitamin E on protein and lipid radical formation during lipoperoxidation of LDL by lipoxygenase. Free Radic Biol Med 76C: 61-68.
- GHAFFARI T, NOURI M, IRANNEJAD E AND RASHIDI M. 2011. Effect of vitamin E and selenium supplement on

- paraoxonase-1 activity, oxidized low density lipoprotein and antioxidant defense in diabetic rats. *Bioimpacts* 1(2): 121-128.
- GIESEG SP AND ESTERBAUER H. 1994. Low density lipoprotein is saturable by pro-oxidant copper. *FEBS Lett* 343: 188-194.
- GISSAUF A, STEINER E AND ESTERBAUER H. 1995. Early destruction of tryptophan residues of apolipoprotein B is a vitamin E-independent process during copper-mediated oxidation of LDL. *Biochim Biophys Acta* 1256: 221-232.
- GO AS ET AL. 2014. Executive summary: heart disease and stroke statistics--2014 update: a report from the American Heart Association. *Circulation* 129: 399-410.
- GÓMEZ-GARCÍA A, MARTÍNEZ TORRES G, ORTEGA-PIERRES LE, RODRÍGUEZ-AYALA E AND ALVAREZ-AGUILAR C. 2007. Rosuvastatin and metformin decrease inflammation and oxidative stress in patients with hypertension and dyslipidemia. *Rev Esp Cardiol* 60(12): 1242-1249.
- GUNATHILAKE KD AND RUPASINGHE HP. 2014. Inhibition of human low-density lipoprotein oxidation in vitro by ginger extracts. *J Med Food* 17: 424-431.
- GUO Z, VAN REMMEN H, YANG H, CHEN X, MELE J, VIJG J, EPSTEIN CJ, HO YS AND RICHARDSON A. 2001. Changes in expression of antioxidant enzymes affect cell-mediated LDL oxidation and oxidized LDL-induced apoptosis in mouse aortic cells. *Arterioscler Thromb Vasc Biol* 21: 1131-1138.
- HASSAN W, IBRAHIM M, DEOBALD AM, BRAGA AL, NOGUEIRA CW AND ROCHA JB. 2009. pH-dependent Fe (II) pathophysiology and protective effect of an organoselenium compound. *FEBS Lett* 583: 1011-1016.
- HOMMA K ET AL. 2015. Comparison of the effects of low-dose rosuvastatin on plasma levels of cholesterol and oxidized low-density lipoprotein in 3 ultracentrifugally separated low-density lipoprotein subfractions. *J Clin Lipidol* 9: 751-757.
- HORT MA, STRALIOTTO MR, DE OLIVEIRA J, AMOEDO ND, DA ROCHA JB, GALINA A, RIBEIRO-DO-VALLE RM AND DE BEM AF. 2014. Diphenyl diselenide protects endothelial cells against oxidized low density lipoprotein-induced injury: Involvement of mitochondrial function. *Biochimie* 105: 172-181.
- HORT MA, STRALIOTTO MR, NETTO PM, DA ROCHA JB, DE BEM AF AND RIBEIRO-DO-VALLE RM. 2011. Diphenyl diselenide effectively reduces atherosclerotic lesions in LDLr^{-/-} mice by attenuation of oxidative stress and inflammation. *J Cardiovasc Pharmacol* 58: 91-101.
- ITABE H, OBAMA T AND KATO R. 2011. The Dynamics of Oxidized LDL during Atherogenesis. 2011. *J Lipids* 2011: 418313.
- JERLICH A, FRITZ G, KHARRAZI H, HAMMEL M, TSCHABUSCHNIG S, GLATTER O AND SCHAUR RJ. 2000. Comparison of HOCl traps with myeloperoxidase inhibitors in prevention of low density lipoprotein oxidation. *Biochim Biophys Acta* 1481: 109-118.
- KATSUBE T, TABATA H, OHTA Y, YAMASAKI Y, ANUURAD E, SHIWAKU K AND YAMANE Y. 2004. Screening for antioxidant activity in edible plant products: comparison of low-density lipoprotein oxidation assay, DPPH radical scavenging assay, and Folin-Ciocalteu assay. *J Agric Food Chem* 52: 2391-2396.
- KOOPMAN R, SCHAART G AND HESSELINK MK. 2001. Optimisation of oil red O staining permits combination with immunofluorescence and automated quantification of lipids. *Histochem Cell Biol* 16: 63-68.
- LASS A, WITTING P, STOCKER R AND ESTERBAUER H. 1996. Inhibition of copper- and peroxy radical-induced LDL lipid oxidation by ebselen: antioxidant actions in addition to hydroperoxide-reducing activity. *Biochim Biophys Acta* 1303(2): 111-118.
- LEWIS P ET AL. 2007. Lack of the antioxidant enzyme glutathione peroxidase-1 accelerates atherosclerosis in diabetic apolipoprotein E-deficient mice. *Circulation* 115: 2178-2187.
- LIU D, DAI Q AND ZHANG X. 2005. A new class of readily available and conformationally rigid phosphino-oxazoline ligands for asymmetric catalysis. *Tetrahedron* 61: 6460-6471.
- LOWRY OH, ROSEBROUGH NJ, FARR AL AND RANDALL RJ. 1951. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265-275.
- LV YC ET AL. 2014. MicroRNA-19b promotes macrophage cholesterol accumulation and aortic atherosclerosis by targeting ATP-binding cassette transporter A1. *Atherosclerosis* 236: 215-226.
- MACDONALD GP. 2010. Cost-effectiveness of rosuvastatin for primary prevention of cardiovascular events according to Framingham Risk Score in patients with elevated C-reactive protein. *J Am Osteopath Assoc* 110(8): 427-436.
- MANCINI G, DE OLIVEIRA J, HORT MA, MOREIRA EL, RIBEIRO-DO-VALLE RM, ROCHA JB AND DE BEM AF. 2014. Diphenyl diselenide differently modulates cardiovascular redox responses in young adult and middle-aged low-density lipoprotein receptor knockout hypercholesterolemic mice. *J Pharm Pharmacol* 66: 387-397.
- MILLER YI, CHOI SH, FANG L AND TSIMIKAS S. 2010. Lipoprotein modification and macrophage uptake: role of pathologic cholesterol transport in atherogenesis. *Subcell Biochem* 51: 229-251.
- MOORE KJ AND TABAS I. 2011. Macrophages in the pathogenesis of atherosclerosis. *Cell* 145: 341-355.
- MUGESH G. 2000. Synthetic organoselenium compounds as antioxidants: glutathione peroxidase activity. *Chem Soc Rev* 29: 347-357.

- MUGESH G, DU MONT WW AND SIES H. 2001. Chemistry of biologically important synthetic organoselenium compounds. *Chem Rev* 101: 2125-2179.
- NADEEM N, WOODSIDE JV, KELLY S, ALLISTER R, YOUNG IA AND MCENENY J. 2012. The two faces of α - and γ -tocopherols: an *in vitro* and *ex vivo* investigation into VLDL, LDL and HDL oxidation. *J Nutr Biochem* 23: 845-851.
- NAMBIAR SS, SHETTY NP, BHATT P AND NEELWARNE B. 2014. Inhibition of LDL oxidation and oxidized LDL-induced foam cell formation in RAW 264.7 cells show anti-atherogenic properties of a foliar methanol extract of *Scoparia dulcis*. *Pharmacogn Mag* 10: S240-248.
- NOGUCHI N, GOTOH N AND NIKI E. 1994. Effects of ebselen and probucol on oxidative modifications of lipid and protein of low density lipoprotein induced by free radicals. *Biochim Biophys Acta* 1213(2): 176-182.
- NOGUCHI N, WATANABE A AND SHI H. 2000. Diverse functions of antioxidants. *Free Radic Res* 33: 809-817.
- NOGUEIRA CW AND ROCHA JB. 2011. Toxicology and pharmacology of selenium: emphasis on synthetic organoselenium compounds. *Arch Toxicol* 85: 1313-1359.
- NOGUEIRA CW, ZENI G AND ROCHA JB. 2004. Organoselenium and organotellurium compounds: toxicology and pharmacology. *Chem Rev* 104: 6255-6285.
- PANDEY KB AND RIZVI SI. 2009. Plant polyphenols as dietary antioxidants in human health and disease. *Oxid Med Cell Longev* 2: 270-278.
- PIRILLO A, NORATA GD AND CATAPANO AL. 2013. LOX-1, OxLDL, and atherosclerosis. *Mediators Inflamm* 2013: 152786.
- RAHMAN MA, ABDULLAH N AND AMINUDIN N. 2014. Inhibitory effect on *in vitro* LDL oxidation and HMG Co-A reductase activity of the liquid-liquid partitioned fractions of *Herichium erinaceus* (Bull.) Persoon (lion's mane mushroom). *Biomed Res Int* 2014: 828149.
- REYFTMANN JP, SANTUS R, MAZIERE JC, MORLIERE P, SALMON S, CANDIDE C, MAZIERE C AND HAIGLE J. 1990. Sensitivity of tryptophan and related compounds to oxidation induced by lipid autoperoxidation. Application to human serum low- and high-density lipoproteins. *Biochim Biophys Acta* 1042: 159-167.
- ROSS R. 1999. Atherosclerosis--an inflammatory disease. *N Engl J Med* 340: 115-126.
- SABHARWAL AK AND MAY JM. 2008. α -Lipoic acid and ascorbate prevent LDL oxidation and oxidant stress in endothelial cells. *Mol Cell Biochem* 309: 125-132.
- SHARIAT SZAS, MOSTAFAVI SA AND KHAKPOUR F. 2013. Antioxidant effects of vitamins C and E on the low-density lipoprotein oxidation mediated by myeloperoxidase. *Iran Biomed J* 17(1): 22-28.
- SIES H. 1993. Ebselen, a selenoorganic compound as glutathione peroxidase mimic. *Free Radic Biol Med* 14(3): 313-323.
- SIES H. 1994. Ebselen: a glutathione peroxidase mimic. *Methods Enzymol* 234: 476-482.
- STEINBERG D. 1997. Low density lipoprotein oxidation and its pathobiological significance. *J Biol Chem* 272: 20963-20966.
- STEINBERG D. 2002. Atherogenesis in perspective: hypercholesterolemia and inflammation as partners in crime. *Nat Med* 8: 1211-1217.
- STEINBERG D. 2013. In celebration of the 100th anniversary of the lipid hypothesis of atherosclerosis. *J Lipid Res* 54: 2946-2949.
- STEINBERG D, PARTHASARATHY S, CAREW TE, KHOO JC AND WITZTUM JL. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 320: 915-924.
- STEINBERG D AND WITZTUM JL. 2010. Oxidized low-density lipoprotein and atherosclerosis. *Arterioscler Thromb Vasc Biol* 30: 2311-2316.
- STRALIOTTO MR, DE OLIVEIRA J, MANCINI G, BAINY AC, LATINI A, DEOBALD AM, ROCHA JB AND DE BEM AF. 2013a. Disubstituted diaryl diselenides as potential atheroprotective compounds: Involvement of TrxR and GPx-like systems. *Eur J Pharm Sci* 48: 717-725.
- STRALIOTTO MR, HORT MA, FIUZA B, ROCHA JB, FARINA M, CHIABRANDO G AND DE BEM AF. 2013b. Diphenyl diselenide modulates oxLDL-induced cytotoxicity in macrophage by improving the redox signaling. *Biochimie* 95: 1544-1551.
- WILSON SR, ZUCKER PA, HUANG RRC AND SPECTOR A. 1989. Development of synthetic compounds with glutathione peroxidase activity. *J Am Chem Soc* 111: 5936-5939.
- WITZTUM JL. 1993. Murine models for study of lipoprotein metabolism and atherosclerosis. *J Clin Invest* 92: 536-537.
- YOSHIDA H AND KISUGI R. 2010. Mechanisms of LDL oxidation. *Clin Chim Acta* 411: 1875-1882.
- ZIOUZENKOVA O, SEVANIAN A, ABUJA PM, RAMOS P AND ESTERBAUER H. 1998. Copper can promote oxidation of LDL by markedly different mechanisms. *Free Radic Biol Med* 24: 607-623.