High Levels of Lipid Peroxidation Induced by Lipofundin Administration Correlate with Atherosclerotic Lesions in Rabbits.

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Summary

Lipid peroxidation induces disturbance of cellular membrane organization, functional loss and modification of proteins and DNA bases, and it has been implicated in the pathogenesis of various diseases such as atherosclerosis. Under oxidative stress conditions, the oxidized low-density lipoproteins are unregulated uptake by macrophages with the subsequent foam cell formation and atherogenic development. The aim of the present work was to investigate the impact of Lipofundin 20% on lipids peroxidative processes and its role in atherogenic process in New Zealand rabbits. Malondialdehyde, total hydroperoxides, lipid peroxidation susceptibility and total antioxidant status were determined using spectrophotometric techniques. The eosin/hematoxylin staining was used in order to examine the atherosclerotic lesions formation. The administration of Lipofundin 20% (2 mL/kg) enhanced the plasma lipid profile and consequently the malondialdehyde and hydroperoxides levels. Also was observed a decreased total antioxidant status and a high susceptibility to lipid peroxidation in the treated animals. The results showed that high levels of lipid peroxidation products correlated with atherosclerotic lesions formation in this animal model of atherosclerosis.

Key words: Lipofundin 20%, lipid peroxidation, atherosclerosis.

Introduction

Elevation of plasma concentrations of low-density lipoprotein (LDL) is a key event in the development of atherosclerosis, a leading cause of death in the western world [1]. A well documented and consistent hypothesis for atherogenesis postulates that this disorder is triggered by oxidative modifications of LDL caused by reactive oxygen species (ROS) from vascular wall cells [2].

The cellular macromolecules, in particular lipids, proteins and DNA, are natural targets of ROS oxidation. These oxidants are capable of initiating lipid peroxidation (LPO) by abstraction of an allylic proton from a polyunsaturated fatty acid. This process, by multiple stages leading to the formation of lipid hydroperoxides, is a known contributor to the development of atherosclerosis [3]. Also, various small molecular weight aldehydes such as malonaldehyde and 4-hydroxy-2-nonenal are formed during LPO as secondary or decomposition products [4]. These end products of LPO are cytotoxic for the vascular wall cells and immunogenic. The elevated levels of these reactive molecules lead to oxidative stress, inflammatory response, and immune system activation that contribute to atherosclerosis development [5].

Artificial fat emulsions are widely used in parenteral nutrition. The soya oil-based fat emulsions represent a major part of energy and are also a necessary source of essential fatty acids in mentioned therapy [6]. Lipofundin 10% constitutes a usually indicated fat emulsion as a source of calories for patients requiring parenteral nutrition, but investigations by Jellinek et al. and Noa et al. demonstrated that Lipofundin 20% induces atherosclerotic lesions formation in rabbits [7,8]. The impact of Lipofundin 20% administration on the systemic LPO products formation had not been studied. In the present work we evaluated some of these indicators in New Zealand white rabbits bearing Lipofundin-induced lesions.

Materials and methods

Animals

Twenty New Zealand white male rabbits, weighing 2.0-2.5 kg and 12 weeks old, were obtained from CENPALAB (Bejucal, Havana, Cuba). Rabbits were housed under conventional conditions exposed to light-dark cycle of 12 h with free access to water and food. All procedures were performed in accordance with the guidelines stipulated by the Institutional Animal Care Committee and in accordance with the European Union Guidelines for animal experimentation.

Lipofundin

Lipofundin MCT/LCT 20% (Braun Melsungen AG, Melsungen, Germany) is a lipid emulsion containing soya oil 100 g, medium-chain triglycerides 100 g, glycerol 25 g, egg lecithin 12 g, α-tocopherol 170 ± 40 mg, and sodium oleate/water for injection in sufficient quantity to 1000 ml.

Induction of atherosclerotic lesions

Two groups of 10 rabbits were used in the study. The first group received an intravenous injection of physiologic solution of NaCl 0.9% (control group), and the second one received a slow intravenous injection of 2 ml/kg of Lipofundin MCT/LCT 20 %, as an infusion during 1-2 min. This procedure was repeated daily during a period of 8 days [7,8].

Serum samples collection

Blood samples (3 ml) were taken on day 0 (before Lipofundin administration) and on day 9 (at the end of the study), for biochemical analyses. Blood was withdrawn from the rabbit's marginal ear vein. These samples were immediately centrifuged at 2500 g, at 4 ºC for 10 min. The serum was collected and aliquots were stored at -70 ºC until analysis.

Histopathology

For histopathological studies, five-micrometer tissue sections were cut, air-dried on glass slides, deparaffinized and rehydrated. Then, tissue sections were stained with hematoxylin and eosin under standard procedures.

Serum lipid assay

Serum total cholesterol, triglycerides, c-LDL and c-HDL were determined using commercial enzymatic kits (Randox, Crumlin, U.K.).

Lipid peroxidation products determination

The serum levels of malondialdehyde (MDA) and total hydroperoxides (TH) were determined by spectrophotometric methods using a Pharmacia 1000 Spectrophotometer (Pharmacia LKB, Uppsala, Sweden). MDA levels were determined using the LPO-586 kit obtained from Calbiochem (La Jolla, CA, USA). In the assay, the production of a stable chromophore after 40 min of incubation at 45 ºC was measured at 586 nm. For standards, freshly prepared solutions of malondialdehyde bis [dimethyl acetal] (Sigma St Louis, MO, USA) were employed and assayed under identical conditions [9,10]. Quantification TH was measured by Bioxytech H_2O_2-560 kit (Oxis International Inc., Portland, OR, USA). The assay is based on the oxidation of Fe^{2+} to Fe^{3+} by hydroperoxides under acidic conditions. Ferric ions bind with the indicator xylenol orange (3,3`-bis(N,N-di(carboxymethyl)-aminomethyl)-o-cresolsulfone-phtalein, sodium salt) to form a stable colored complex, which can be measured at 560 nm.

Susceptibility to lipid peroxidation assay

In order to determine susceptibility to lipid peroxidation, serum samples were incubated with a solution of copper sulphate (final concentration 2 mM) at 37 ºC for 24 h. So the PP was calculated by subtracting the MDA levels before the induction of lipid peroxidation from that obtained at 24h [11].

Total antioxidant status determination

The total antioxidant status was determined using a Randox TAS kit Cat No. 2332. In the assay ABTS (2,2´-Azino-di-[3-ethylbenzthiazoline sulphonate]) is incubated with a peroxidase (metmyoglobin) and H_2O_2 to produce the radical cation $ABTS^{\bullet+}$. This has a relatively stable blue-green color, which is measured at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which is proportional to their concentrations.

Statistical analysis

The homogeneity variance test (Bartlett-Box) was applied. In addition, differences between groups were determined by student's t-test (two-tailed). Data were expressed as the mean ± standard deviation. The level of statistical significance employed was at least $p<0.05$, for all determinations.

Results

Histopathology

In the control animals, a normal morphology of different layers of the aortic tree was observed. In this group, microscopic analyses of hematoxylin and eosin-stained aortic sections did not show an intimal thickening (Figure 1A and B). In contrast, aortic sections from the animals who received intravenously 2 ml/kg of Lipofundin 20% during 8 days showed thickening of the intima with apparent lipid accumulation and distortion of tissue architecture (Figure 1C and D).

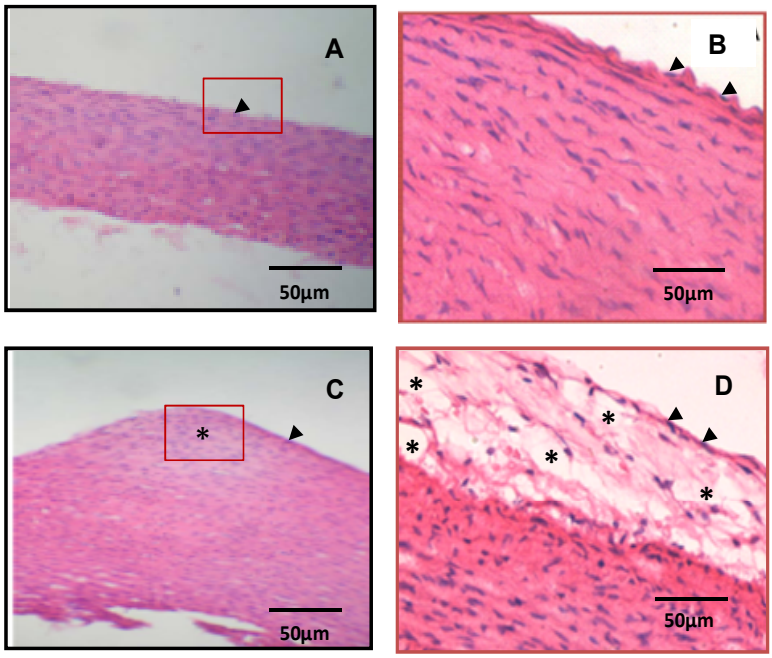

Figure 1. Histopathological analysis of rabbit´s aortic tree. Eosin/hematoxylin staining reveals a normal morphology of aortas in control animals (A, B). In the group treated with Lipofundin 20%, the aortas showed an intimal thickening, characterized by empty spaces, which suggest lipid accumulation (asterisks) and a cellular architecture distortion (C, D). Magnification 10x (A, C) and 40x (B, D). Arrow: endothelial cells. Scale bar, 50 µm.

Serum lipid profile

Serum total cholesterol, triglycerides, c-LDL and c-HDL levels showed a significant increase ($p<0.05$) in those animals who were treated during 8 days with the lipid-rich emulsion Lipofundin 20%, while no significant changes in serum lipids occurred in the control rabbits throughout the study (Figure 2).

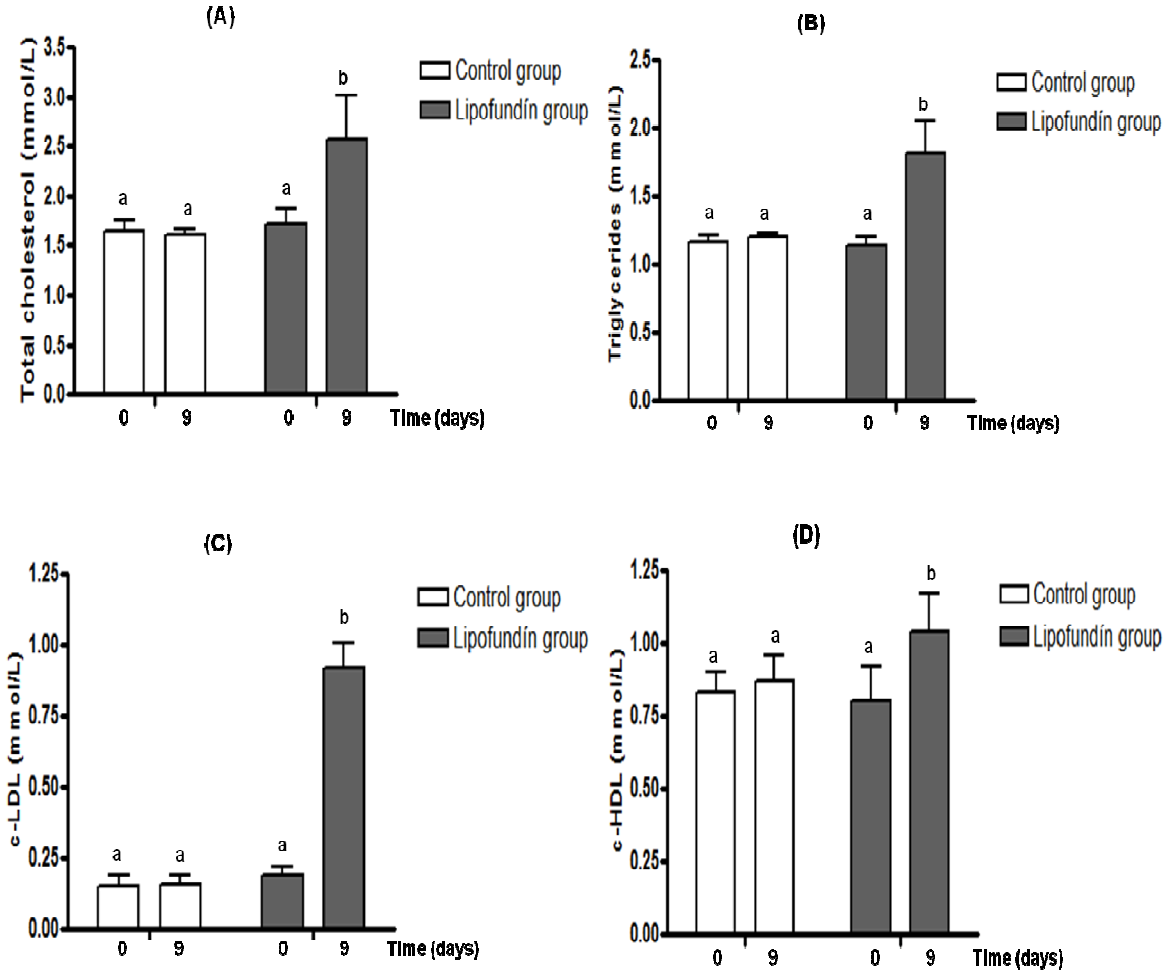

Figure 2. Effects of Lipofundin 20% administration on serum lipid profile. The figures showed a significant increase of all lipid biomarkers in the treated animals after eight days of Lipofundin 20% administration. Different letters represent statistical differences ($P<0.05$).

Lipid peroxidation biomarkers and total antioxidant status

In **Table 1** are represented the biomarkers of LPO-induced damages and the behaviour of total antioxidants present in the analyzed samples. The results showed that biomolecules damages biomarkers were significantly ($P<0.05$) modified after 8 days of Lipofundin 20% administration compared to non treated group. At the end of the experimental period the MDA levels, one of the end-products of LPO, showed an increase in Lipofundin group while TH concentration decreased.

The peroxidation potential was determined in order to know the total reactive antioxidant power in the samples. The susceptibility to lipid peroxidation was higher in those animals who received Lipofundin 20%. After 8 days of treatment, in these animals a significant increase ($P<0.05$) of peroxidation potential was observed compared to the one calculated in controls.

On the other hand, in the rabbits treated with the lipid emulsion the total antioxidant status was significantly lower ($P<0.05$) than in control group, which is in correspondence with the high levels of LPO products and the elevated susceptibility to oxidative modifications in lipids, observed in the animals with Lipofundin 20% treatment.

Table 1. The results showed the behaviour of determined biomarkers in both group at the end of the experiment. MDA: malondialdehyde, PP: peroxidation potential, TAS: total antioxidant status, TH: total hydroperoxides. Different letters represent statistical differences ($P<0.05$).

Biomarkers	Control group (N=10)	Lipofundin group (N=10)
MDA (µmol/L)	2.64 ± 0.22 [a]	6.34 ± 0.81 [b]
TH (µmol/L)	39.07 ± 5.57 [a]	27.12 ± 8.69 [b]
PP (µmol/L of MDA)	4,16 ± 0.12 [a]	8.85 ± 0.86 [b]
TAS (µmol/L)	2.78 ± 0.93 [a]	1.02 ± 0.67 [b]

Discussion

Serum lipid profile

The role of cholesterol and triglycerides on atherogenic process has been well documented in the scientific literature [12]. There is a causal relationship between the elevated plasma lipids and the development of atherosclerotic lesions [13]. Hyperlipidemia is a major cause of atherosclerosis and atherosclerosis-associated conditions [14].

Artificial fat emulsions are widely used in parenteral nutrition. The soya oil-based fat emulsions represent a major part of energy and are also a necessary source of essential fatty acids in mentioned therapy [15]. Lipofundin 10% constitutes a usually indicated fat emulsion as a source of calories for patients requiring parenteral nutrition.

In the present study, an elevated serum level of triglycerides, total cholesterol, c-LDL and c-HDL was observed in the animals treated with Lipofundin 20% respect to control rabbits. These results could be associated with the high content of triglycerides in Lipofundin 20%. Plasma cholesterol and triglycerides values provide information concerning the lipoprotein particles that are increased. Total cholesterol values include both unesterified cholesterol and cholesteryl

esters. The elevation of plasma cholesterol usually indicates that LDL is increased and high levels of plasma triglycerides points to an elevation in chylomicrons, very low-density lipoproteins (VLDL), and/or remnants [16].

Although, cholesterol is not present in Lipofundin composition, its serum increased level after 8 days of the experiment may be associated with the exogenous triglycerides administration. In the liver, high levels of triglycerides can induce the ApoB100 synthesis and ultimately the ensemble of VLDL [17]. The synthesized VLDL fraction may contains endogenous triglycerides from the liver and lipid particles of the infused fat emulsion. There are mutual exchanges of lipids and apolipoproteins between VLDL, LDL and HDL on the one hand and the infused triglyceride/phospholipid particles on the other. Transfer proteins in the plasma also take part in such exchanges [18]. A human study demonstrated that Lipofundin 10% caused an increase by 60% of total serum cholesterol [19]. The observed behaviour in the rabbit´s lipid profile after 8 days of Lipofundin 20% administration, a fat emulsion that contains no cholesterol, may be explained in this way.

Lipid peroxidation biomarkers

LPO was first studied in relation to the deterioration of foods in 1930s, when the study on the chemistry of free radical reactions made remarkable advancements [20]. With increasing evidence showing the involvement of free radicals in biology [21], LPO has received renewed attention from wider viewpoints in the fields of chemistry, biochemistry, biology, nutrition, and medicine. Later studies revealed that, like proteins, carbohydrates and nucleic acids, lipids are targets of various ROS, and oxidized to give a diverse array of products. The mechanisms, dynamics, and products of LPO in vitro have been studied extensively and are now fairly well understood and documented [22], but the physiological levels and biological effects of LPO and its products have been partially elucidated. It has been shown that LPO induces disturbance of fine structures, alteration of integrity, fluidity, and permeability, and functional loss of biomembranes, modifies LDL to proatherogenic and proinflammatory forms, and generates potentially toxic products [23].

Although several criteria are without doubt required to adequately describe a biomarker, the entire basis of the biomarker phenomenon is the measurement of a compound that directly reflects certain biological events related to the pathogenesis of a disease or condition. In case of MDA, LPO is the biological event and atherogenesis is the pathology it is related to. Thus, the rationale of MDA as a biomarker relies both on that it is (solely) derived from lipid peroxides, that changes in MDA concentrations reflect changes in LPO level and that LPO is in fact somehow predictive of atherosclerotic events.

In the present study we noted an increasing MDA levels in those animals treated with Lipofundin 20%, and consequently atherosclerotic lesions could be observed. This result is in accordance with the criteria that the end-product of LPO MDA is strongly associated with the development of atherosclerosis [24]. The Lipofundin-induced high serum lipids levels, especially in those atherogenic such as cholesterol and LDL permit to explain, in part, the fact that in administered animals LPO products are higher than in controls. The higher plasma MDA level, which is also a major epitope of oxidized LDL, suggests that Lipofundin 20% induces an increase in LPO closely associated with an elevation of atherogenic LDL particles and related ApoB100-containing lipoproteins. Also, the significant increase of the PP in sera from rabbits bearing atherosclerotic lesions produced by Lipofundin 20% administration compared with those from

control group reinforces the role of LPO in the loss of redox systemic status in the former animals which were under atherogenic stimuli caused by Lipofundin 20% treatment.

Lipid hydroperoxides and hydrogen peroxide (H_2O_2) generated by LPO play a central role in atherosclerosis, mainly during endothelial dysfunction. Transition metals (iron or cupper) may produce H_2O_2 decomposition and cause the generation of the highly toxic and reactive hydroxyl radical ($^{\bullet}OH$), which reacts with cellular components [25]. The lower TH concentration detected in rabbits treated with Lipofundin 20% in comparison to the value detected in the control group at the end of study may be a consequence of free transition metals induced-H_2O_2 decomposition. The reduction of TH suggests that in the animals treated with Lipofundin 20% was established an endothelial metabolic disruption. This process may affect the delicate balance between vasodilators and vasoconstrictors factors, which promotes an atherogenic development in the treated rabbits.

On the other hand, TAS is an experimental technique in which the bioavailability of antioxidant substances is measured. The experimental results reflected a reduced antioxidant capacity in the animals of the Lipofundin group. To date, evidence suggests that the variation in tissue responses to oxidative stress is related to the metabolic capacity of the tissue as well as its antioxidant content. In the literature is well established that atherosclerosis development is closely related with an increase of oxidative stress but also with a decrease of antioxidant mechanisms [26].

Histopathology

The histopathological analyses of the aortic sections of those animals who received Lipofundín showed an intimal thickening and a distortion of cellular architecture. This emulsion, rich in triglycerides and other lipids cause oxidative stress and consequently may cause oxidative modifications of these lipids and serum LDL. The accumulation and oxidation of LDL within the arterial wall initiates a complex series of biochemical and inflammatory/immunomodulatory reactions involving multiple cell types ultimately leading to the development of atherosclerotic process [27]. In the present study, the Lipofundin-induced lesion was characterized by intimal thickening and is known that early atherosclerotic lesions are classified as either intimal xanthomas or intimal thickening. Intimal xanthomas or fatty streaks are accumulations of foam cells without a necrotic core while intimal thickening is characterized by the accumulation of smooth muscle cells, immune competent cells, and oxidized lipids [27]. The histopathological analyses reinforce the criteria that Lipofundin 20% administration induces atherosclerotic lesion formation.

Conclusions

In summary, the present study demonstrates that Lipofundin 20% induces a high susceptibility to LPO which correlates with atherosclerotic lesions formation. Also we show additional evidences that reinforce the active role of oxidized lipids in atherosclerosis development in this animal model.

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