HISTOPATHOLOGICAL AND ULTRASTRUCTURAL CHARACTERIZATION OF LIPOFUNDIN-INDUCED ATHEROSCLEROTIC LESIONS IN RABBITS

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ABSTRACT
Atherosclerosis is a disease of the vascular wall that leads to myocardial infarction, stroke, ischemia, gangrene and aneurysm. The primary lesion of atherosclerosis is an elevated focal plaque within the intima, with a central lipid core (mainly cholesterol and cholesterol esters) and a fibrous layer that covers it. The aim of this study was the histopathological and ultrastructural characterization of the atherosclerotic lesions induced in rabbits by the administration of Lipofundin 20%, a rich-lipid emulsion. Nineteen New Zealand rabbits were distributed in two groups. Group A (8 animals) only received intravenous injections of PBS and Group B (11 animals) received 2 mL / kg weight of Lipofundin 20% for 8 days. On day 9 animals were sacrificed and the aortic archs were isolated for histological and ultrastructural examination. In Group A, non-histopathology alterations were observed in the artery intima. However, all aorta samples from rabbits of Group B (treated with Lipofundin 20%) showed atherosclerotic lesions characterized by intima thickening, media-to-intima migration of vascular smooth muscle cells and an increased presence of proteoglycan and collagen fibers. In some samples the injured area was thicker than media layer. Ultrastructural analysis presence of large amounts of foam cells with lipid droplets at the intracellular level and abundant extracellular lipid droplets. These lesions were classified as Type III/ IV according to the classification of the Committee on Vascular Lesions of the American Heart Association**.**

Keywords: Atherosclerosis, Foam cell, Histopathology, Lipofundín 20%.

INTRODUCTION

Atherosclerosis is a chronic inflammatory response of the vascular wall initiated by various events that can occur early in life. Multiple pathogenic mechanisms contribute to the formation and progression of the plaque, including endothelial dysfunction, adhesion and infiltration of monocytes, the proliferation of smooth muscle cells (SMCs), extracellular matrix deposition, accumulation of lipids and thrombosis [1]. Epidemiological studies indicate that genetic or acquired factors increase the risk of atherosclerosis, some of them such as age, sex, familial predisposition, are inevitable partners in our life, but others are potentially controllable, such as hyperlipidemia, obesity, smoking, arterial hypertension, diabetes mellitus and alcoholism [2].

In Western countries, atherosclerosis is responsible for about half or more of total mortality and for significant morbidity, which overwhelmingly outweigh those of any other process. Its distribution

is so broad that it has reached epidemic proportions in the economically developed populations [3].

Atherosclerosis mainly affects large or elastic arteries (e.g., aorta, carotid and iliac arteries) and medium-sized or muscular arteries (e.g., popliteal arteries) [4]. Myocardial infarction, stroke and aneurysms of the aorta, are the main consequences of this disease. Therefore, the epidemiological data of atherosclerosis are expressed primarily in terms of incidence or the number of deaths from ischemic heart disease [5].

In Cuba, as in all those countries where infections are not the primary cause of death, atherosclerosis occupies this central place and its consequences are responsible for more than 50% of deaths [6].

Essential lesions of atherosclerosis are intimal thickening and lipid accumulation mainly cholesterol and its esters, which produces the characteristic atheromatous plaques. Atherosclerotic plaques consist of three components: 1) cells, like (SMC), macrophages and other leukocytes, 2) the extracellular matrix of connective tissue that contains collagen, elastic fibers and proteoglycans, and 3) intracellular and extracellular deposits of lipid.

These three components are in varying proportions in the different plaques, resulting in a spectrum of lesions [7].

Atherosclerosis is begins very early, progressing slowly in a silent manner until approximately the fifth or sixth decade, when complications with clinical and pathological damage start. Early intimal alterations of the coronary arteries are detectable in the prenatal and infancy perios, and may be significantly associated with maternal smoking [8]. Studies in coronary arteries of pacients younger than one year the altereations ranged from focal areas with mild myointimal thickening to diffuse moderate thickening. In those lesions, SMC showed loss of polarity, infiltrating the subendothelium, mostly with rupture of the IEL and without neoangiogenasis. These lesions can be present very early in life and SMC seem to play an essential role [9].

Several animal models have been characterized and used since the last century for the study of atherosclerosis and the impact of different treatments in the prevention and progression. At present there are a variety of models developed in birds, primates and no primate mammals [10]. Diets rich in cholesterol and other fats have been widely used for induction of atherosclerosis, which accounts for the fact that not all animal species spontaneously develop the disease [11]. The use of transgenic variants of mice and rabbits has also been widely reported in the literature [12-14]. This experimental alternative allows spontaneous elevation of serum cholesterol, rapid progression of atherosclerotic lesions, and from the histopathological point of view there is a greater similarity to the pathophysiological and metabolic processes that occur in humans [13].

In 1982 was for the first time described a model for the induction of atherosclerosis in rats using Lipofundin MCT/LCT 20% [15]. Later, studies in Cuba reported that daily infusion of Lipofundin for 8 days induced aortic lesions characterized by intima thickening and foam cell formation in rats and rabbits [16, 17]. The administration of Lipofundin MCT/LCT20% has the advantage of produce atherosclerotic lesions only in 8 days, unlike the atherosclerosis animal models that use hypercholesterolemic diets where the lesions appear after months of administering these diets.

In this study we reproduced the Lipofundin atherosclerosis model in New Zealand rabbits and further characterize the histopathological and ultrastructural changes developed in the aortas of the animals.

MATERIALS AND METHODS

Lipofundin

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

Animals

In the study were used 19 male New Zealand white rabbits (NZW) (2.0-2.5 kg, 12 wk) obtained from the National Center for Laboratory Animal Production (CENPALAB, Bejucal, Havana, Cuba). Rabbits were maintained under conventional conditions (25°C, 60 ± 10% humidity), 12h day/night cycles with water and food *ad libitum.*

The animals were then randomized into two experimental groups. The study was conducted with the approval of the Ethics Committee of the Institute of Food and Drugs. All experimental procedures were performed in accordance with the Institutional Animal Care guidelines for use of experimental animals, and conforming to the Guide for the Care and Use of Laboratory Animals of the European Union.

Experimental groups and induction of atherosclerotic lesions

Group A (8 animals) received an intravenous injection daily for 8 days of 2 mL / kg body weight of phosphate buffered saline (PBS), pH 7.2. Group B (11 animals) received 2 mL / kg body weight of Lipofundin MCT / LCT 20% as an infusion over 1-2 minutes [15]. On day 9 of the experiment the rabbits were anesthetized with ketamine hydrochloride (35 mg/kg and 5 mg/kg xylazine HCl, IM) followed by intracoronary injection of KCl and then the animals were euthanized by an overdose of sodium pentobarbital.

Histopathology and morphometry

Tissue samples were obtained from the proximal end of the aortic arch, at the level of the aortic valves, because the preferential development of Lipofundin-induced atherosclerotic lesions in this segment. The samples were fixed in neutral solution of 10% buffered formaldehyde (pH 7.4) for 24 hours, and then they were performed in a tissue processor (Sakura, Model RH-12EP-2). The samples were paraffin-embedded and serially sectioned into 5-µm widths using a microtome (Leica, Model RM2135, Meyer Instruments, Houston, TX, USA).

Aortic paraffin-sections were subjected to routine hematoxylin-eosin staining to detect intimal thickening. In addition, Masson´s Trichrome and Alcian Blue special stainings were used for the detection of collagen fibers and glycosaminoglycans, respectively [18,19]. To differentiate the intima of the muscular layer was used the Verhoeff-Van Giesson staining specific for elastic laminae. The reagents used in the different staining techniques were of analytical grade and were obtained from Sigma-Aldrich (Sigma St Louis, United States). To visualize and capture images was used a light microscope

(Nikon Eclipse model 501) equipped with a DP20 camera.

Morphometrical analyses were performed to calculate intima-media ratio (IMR) in Verhoeff-Van Giesson stained sections, because this special staining is the one that best defines internal and external elastic laminae. The maximum thickness of the intima (MIT) was determined measuring the tissue between the internal elastic lamina (IEL) and the vascular endothelium, and the media width (MW) measuring the tissue between the IEL and external elastic lamina (EEL), using an analyzer Image (Image J version 1.38). The results obtained were used to estimate the (IMR) [18, 19] using the formula:

$$IMR = MIT / MW \quad (1)$$

Figure 1 shows the scheme used for morphometric measurements.

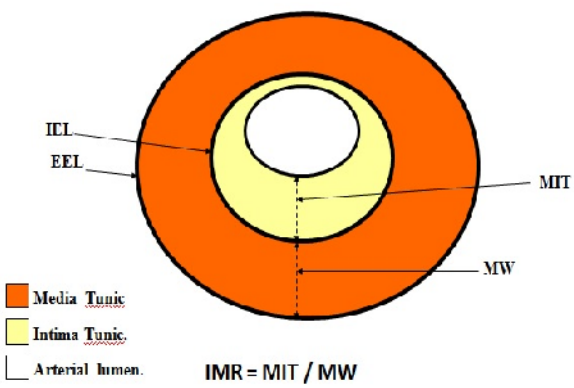

Fig. 1. Schematic representation of the method used to assess the severity of the damage in the arterial intima from morphometric methods. MIT: maximum intima thickness, MW: media width, IEL: internal elastic lamina, EEL: external elastic lamina.

Classification according to the Committee on Vascular Lesions of the Council of Atherosclerosis of the American Heart Association

Vascular lesions were classified according to the Committee on Vascular Lesions of the Council on Arteriosclerosis of the American Heart Association [7, 20-23]. In the qualitative evaluation were used the results obtained in the samples from the 11 Lipofundin-treated rabbits. For classification of the lesion type were taken into account the histopathological and ultrastructural results.

However, in 2000 Virmani *et. al.* have devised a simplified classification that relies on descriptive morphology, with minimal implication of the mechanisms involved. This classification scheme highlights specific morphological events that are appropriate targets for development of animal models or human diagnostic procedures, which will permit us to test hypotheses as to the final stages of the disease. The main limitation is that the lesions in current animal models rarely progress beyond the stage of atheroma (*ie*, a well-developed fibrous cap overlying a necrotic core) [24].

Ultrastructural analysis.

The transmission electron microscopy (TEM) study of the samples was performed as described previously [25]. Briefly, samples from rabbit aortic arch were fixed for 1h at 4°C in 3.2% glutaraldehyde (Agar Scientific, UK), 0.1 mol/L PBS (pH 7.4) and postfixed in 1% OsO4 (Agar Scientific, UK) for 1h at 4°C. After graded ethanol dehydration, samples were embedded in Spurr low-viscosity epoxy resin for 24h at 37°C. Ultrathin sections were cut into 400-500 Å thick slice with an LKB Ultramicrotome (Nova LKB), counterstained with uranyl acetate and lead citrate, and analyzed in a JEOL JEEM-2000EX. A total of 500 photomicrographs were analyzed.

Statistical analysis

Statistical analysis was performed using SPSS for Windows (version 11.5, SPSS Inc). The normal distribution of data was confirmed by Kolmogorov-

Smirnov test and homogeneity of variances by Bartlett's test. Differences between groups were determined by Student's t test (two-tailed). Data are expressed as mean ± standard deviation (SD). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Hematoxylin-eosin staining

In the aortic samples from the 8 animals in the Group A, the histological features of the arterial wall were within normal parameters. The intima was composed of the vascular endothelium and the underlying connective tissue (subendothelium) was almost indistinguishable by light microscopy. The tunica media or muscular was also normal, with a thickness typical of a large artery (elastic arteries) and with the smooth muscle cells arranged longitudinally to the surface. Figure 2A shows a photomicrograph from a representative rabbit of Group A.

However, in all samples from the aortas of the 11 rabbits from Group B showed lesions characterized by thickening of the intima with numerous empty extracellular spaces suggesting the presence of lipids that could be removed during the staining technique. An evident media-to-intima migration of SMCs was observed, since in some cases the SMCs were oriented vertically to the surface as a sign of migration to the area of the damage. More to the depth of media layer the cells were observed oriented longitudinally to the surface (Figure 2B). In some samples the tunica intima was thicker than the thickness of tunica media.

Alcian Blue staining

Alcian Blue staining revealed in the intima of the aortic arch of the eight animals in Group A a thin blue area immediately below the vascular endothelium showing the normal presence of glycosaminoglycans in the intima (Figure 3A). In contrast in the aortic samples of the eleven animals of group B this special staining revealed thickening of intima with increased quantities of GAGs (Figure 3B). In addition, a slight increase in blue positive staining in the tunica media was observed in comparison to that detected in samples from the control rabbits (Group A).

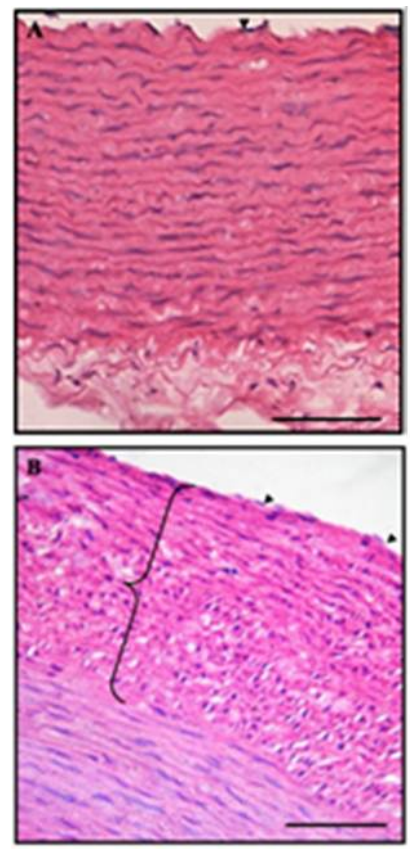

Fig. 2. Eosin/hematoxylin staining revealed a normal morphology of aortas in control animals (A). The bracket in B represents the thickness of the intima. Arrowheads indicate endothelium. Magnification 40X. Scale bars: 50 µm.

Masson's Trichrome staining

In the aortic arch of all rabbits in Group A was observed immediately below the vascular endothelium the presence of SMC, without any blue positive reaction, as a sign of absence or limited presence of collagen fibers in this area (Figure 4 A). However, in the aortic samples of the animals in Group B it was observed an increment of the blue reaction in the thickened areas of the intima, indicative of the high presence of collagen fibers in the lesions (Figure B).

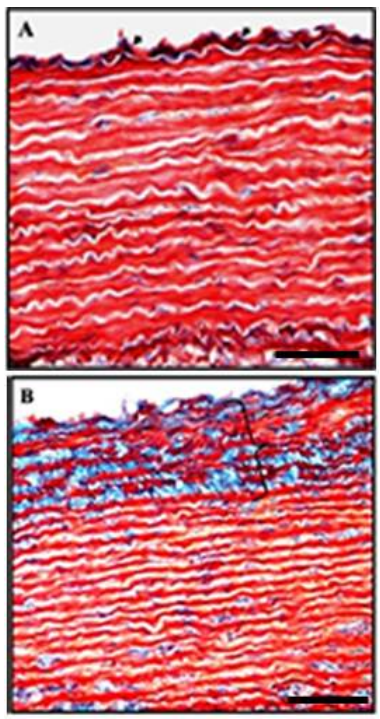

Fig 3. Alcian Blue staining A and B: photomicrographs of representative rabbits of control and Lipofundin treated groups, respectively. The bracket in B represents the thickness of the intima. Arrowheads indicate endothelium. Magnification 40X. Scale bars: 50 µm.

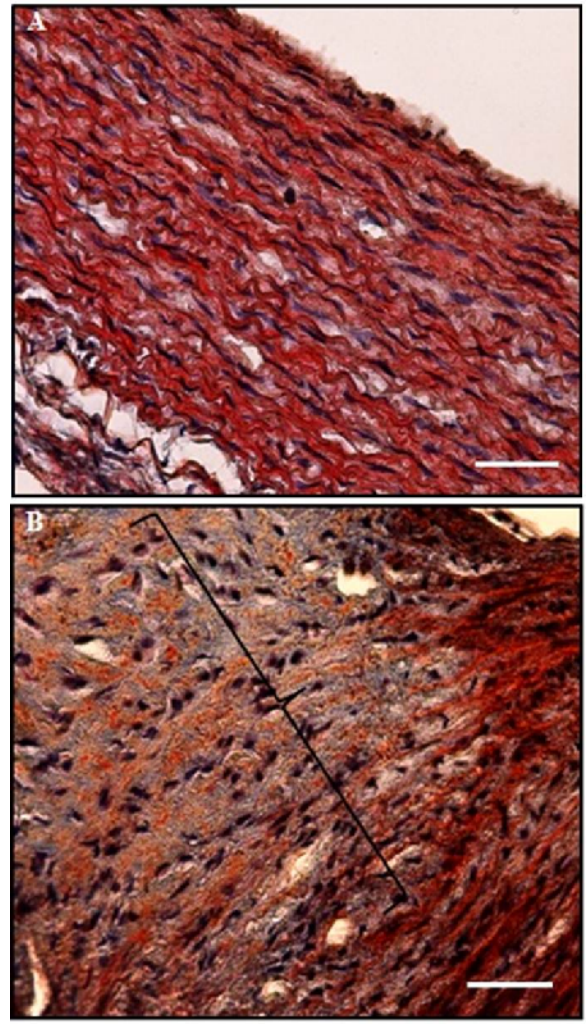

Fig. 4. Masson Trichrome staining A and B: photomicrographs of representative rabbits of control and Lipofundin treated groups, respectively. The bracket in B represents the thickness of the intima. Magnification 40X. Scale bars: 50 µm.

Verhoeff-van Giesson

Verhoeff-Van Giesson staining revealed abundant deposition of collagen fibers in tunica intima and media (red tincion) as well as disruption of elastic fibers (black tincion) in tunica intima of aortas from Lipofundin treated rabbits (Group B), whereas no such changes were observed in aortas from control group (Group A) (Figure 5).

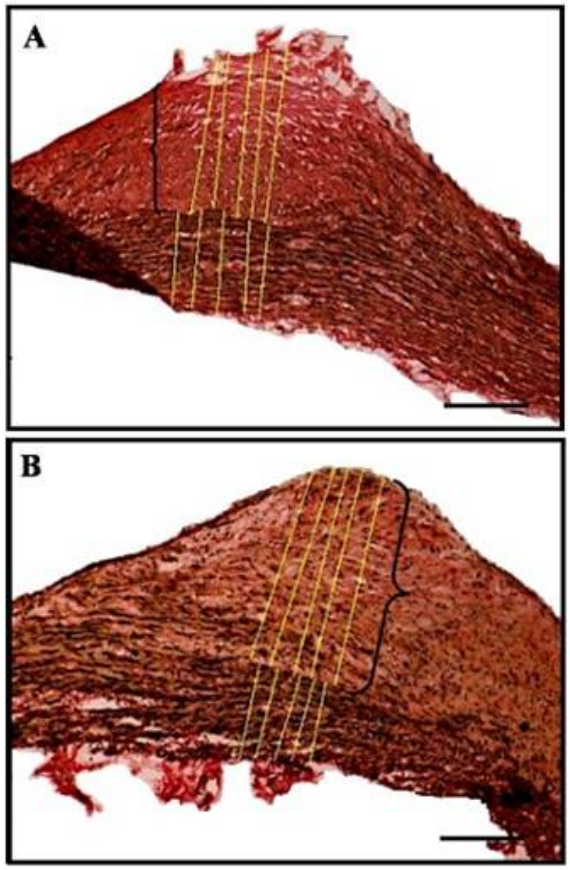

Fig. 5. Representative photomicrographs of Verhoeff-Van Gieson-stained aortic paraffin-embedded sections from two animals of Group B The yellow lines were the five measurements made at the intima and muscular tunicas to calculate the IMR. The brackets represent the thickness of the tunica intima. Magnification10X. Scale bars: 100 μm.

The administration of Lipofundin 20% in Group B produced a significant increase in IMR (1.75 ± 0.34) compared with control rabbits (Group A) (0.015 ± 0.004, $p < 0.001$).

Ultrastructural analysis

Transmission electron microscopy ultrastructural examination confirmed the results observed by light microscopy. In the samples from the 3 rabbits evaluated of Group A were not observed alterations in the aortic artery wall, characterized by a thin intima layer with SMCs immediately underlying the endothelium. No morphological changes were observed in endothelial cells (EC) and their junctions were well preserved Figure 6 A (box) and B.

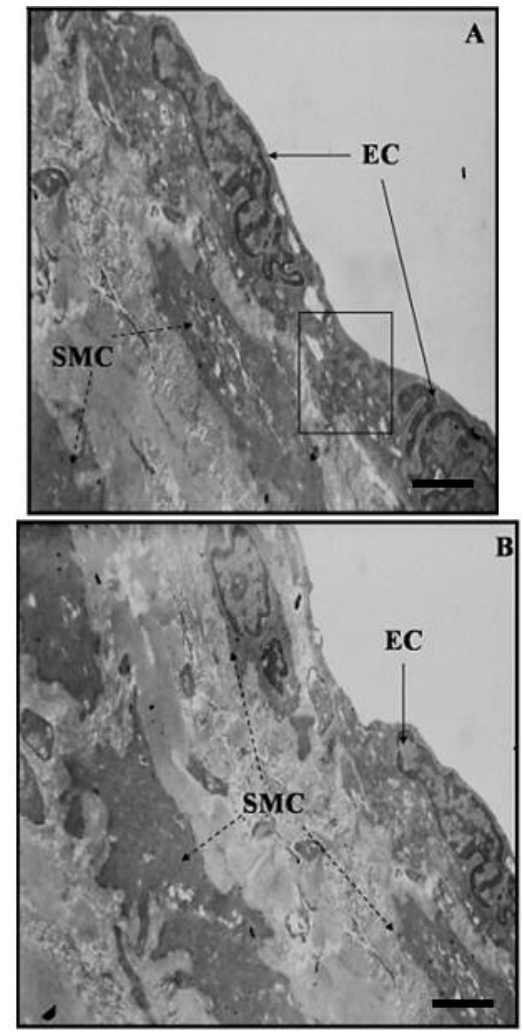

Fig. 6. Transmission electron microscopy photomicrographs of aortic arch from controls. Scale bars = 1 μm.

In contrast, in the samples from the 5 rabbits evaluated of Group B, Lipofundin caused thickening of the intima due to structural changes both at the cellular and extracellular matrix levels. In the extracellular matrix was observed accumulation of basement membrane-like material, high amounts of collagen fibers (in both transverse TCF and longitudinal LCF dispositions), and high number of

foam cells derived both from macrophages and SMCs (Figure 7 A and B). The presence of SMC-derived foam cells was demonstrated because the presence of remains of the basement membranes of these cells (Figure 7A arrowheads). Also, the presence of large number of myofibroblasts was detected (data not shown). In addition, abundant extracellular lipid droplets were observed in the intima, in greater amounts compared to those found at the intracellular level (Figure 8 A and B).

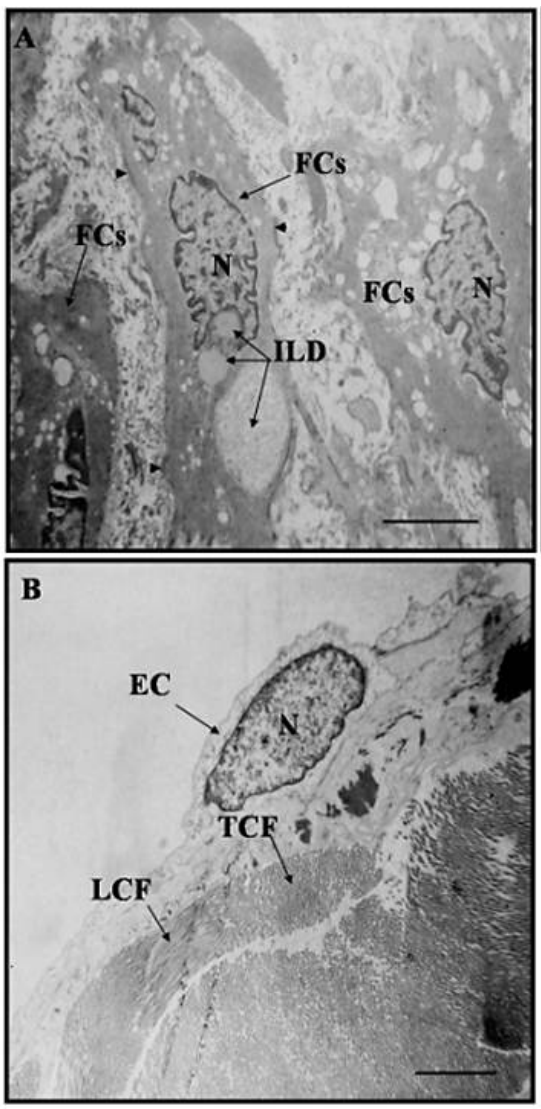

Fig. 7. Transmission electron microscopy photomicrographs of aortic arch from two animals of group B. Scale bars Bar = 1 μm.

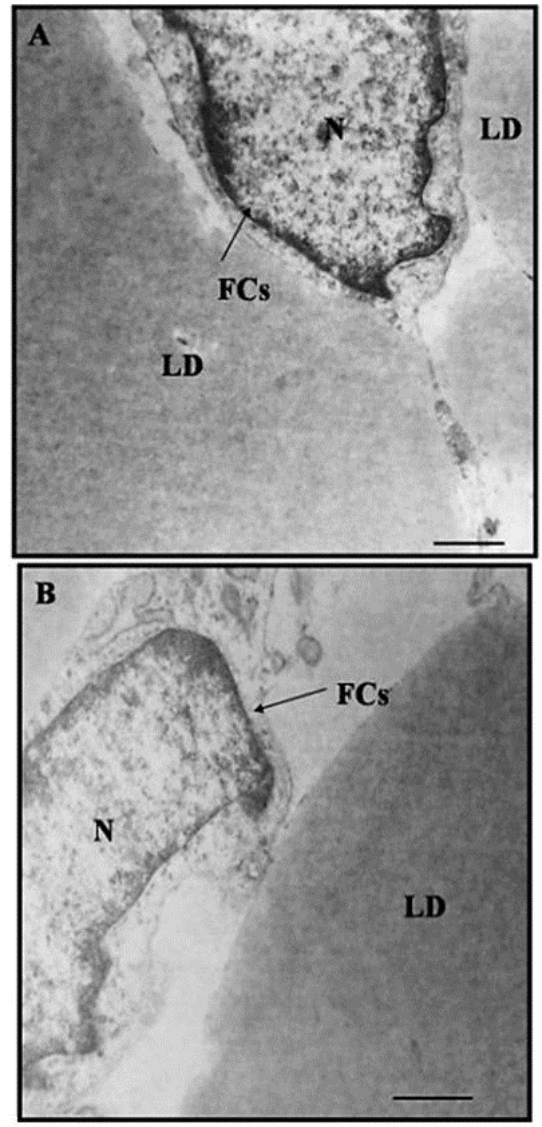

Fig. 8. Transmission electron microscopy photomicrographs of aortic arch from two animals of group B. A and B represent the area corresponding to the intima. Scale bars Bar = 500 nm.

For classification of the lesion type were taken into account the histopathological and ultrastructural results. Of the 11 rabbits, six manifested human-like type III (pre-atheroma) and five type IV atherosclerotic lesions classified as the first advanced lesion or atheroma. Lesions classified as type IV were those in which although there was not observed a well-defined lipid core, did have a clear formation of the fibrous plaque just below the endothelium.

DISCUSSION

In the present study we confirmed that the intravenous administration of Lipofundin 20% for only 8 days, induces the formation of atherosclerotic lesions at the level of the arterial intima of the aortas of New Zealand rabbits [16].

We use several histopathological special stainings allowed to demonstrate that the atherosclerotic lesions produced by the administration of Lipofundin 20% were characterized by an increased presence of GAGs, collagen fibers, foam cells and extracellular lipids.

Previous published reports have suggested that the calculation of IMR is a sensitive method for measure atherosclerotic lesion severity in humans [18, 19, 26, 27, 28].

The IMR of the arteries may vary from near to 0 to 1.0 or more in normal arteries from humans, and the thick segments represent physiological adaptations to changes in flow and wall tensions [21]. In addition, the IMR should be used to compare the severity of atherosclerosis in the same artery, but should not be used to compare atherosclerosis in 2 different arteries [19].

In our study, the mean of IMRs calculated in the aortic sections of all animals in Group B was above 1 (1.75 ± 0.34) and this value was significant higher than the one obtained in the aortic samples from the rabbits intravenously injected with PBS (Group A). It is noteworthy, that in the Lipofundin-atherosclerosis rabbit model used in the present study, the values of IMR obtained were higher compared with those obtained by Burns et al (0.412) and Hayashi et al (0.19) using experimental models were atherosclerotic lesions were induced in the femoral and aorta arteries of the rabbits by atherogenic diets [25 , 26].

It has been recently reported the use of IMR to show that the diffuse intimal thickening (DIT) was strongly expressed from an early age in arteries that are considered to be prone to atherosclerosis , so they concluded that the development of atherosclerosis depends at least partly on the degree of DIT [29].

Another study revealed the importance of the DIT and the fatty streakas a reservoir for lipid retention and identifies a family of extracellular matrix (ECM) molecules that may be involved in lipid retention, contributing to the early phases of lesion formation before the stage of the pathologic intimal thickening (PIT). This study was only performed in the coronary arteries, but they expect the same mechanism to occurs in other atherosclerosis prone arteries, such as abdominal aorta, because well developed DIT is present in their intima as well [30].

Previous reports [16, 17] demonstrated that atherosclerotic lesions were induced in animals by daily administration of Lipofundin. Now using TEM, we detected the presence of large amounts of foam cells of macrophage origin. Also, it was demonstrated SMC migration towards the intima with subsequent differentiation to foam cells and myofibroblasts. This characteristic of SMCs is called by several authors dedifferentiation process [31, 32]. The presence of myofibroblasts demonstrated the process of dedifferentiation of SMCs to synthetic phenotype. On the other hand, the fact that some of the foam cells presented remains of basement membranes demonstrated that the origin of these cells was from SMCs and not from monocytes. The ultraestructural study also showed that the aortic atherosclerotic

lesions produced by the infusion of Lipofundin were characterized by a high accumulation of extracellular lipid vacuoles.

The classification recommended by the Committee on Vascular Lesions of the Council of Atherosclerosis of the American Heart Association [7,20,21,22] includes a sequence of morphological and histological changes denoted with Roman numbers. This classification was originally developed to classify the human atherosclerotic lesions, but it have been also used to classify the ones produced in rabbit models of atherosclerosis, due to the morphological similarities of the lesions of both species [33, 34, 35, 36].

In our study we used for the first time the above classification because the characteristics of the atherosclerotic lesions produced by intravenous administration of Lipofundin 20% were also similar to human lesions.

Although it was not observed the lipid core, the lesions were characterized by the presence of huge quantities of collagen fibers immediately below the endothelium and ahead of the area rich in extracellular lipids, thus constituting the structure called fibrous plaque which it is formed ahead the lipid core. Therefore, we decided to classified these lesions as Type IV because the already evident formation of the fibrous envelope.

CONCLUSIONS

Intravenous administration of Lipofundin 20% for 8 days in New Zealand rabbits caused the formation of atherosclerotic lesions on the aortic arches classified as type III and type IV, with IMR values greater than 1.

REFERENCES

[1] Strong J.P. (1991) "The natural history of atherosclerosis in child-hood" *Ann. N.Y. Acad. Sci*. 623:9,

[2] Neaton J.D, Wentworth D. (1992) "Serun cholesterol, blood pressure, cigarette smoking, and death from coronary heart disease. Overall findings and differences by age for 316,099 white men" *Arch. Intern. Med.* 152:56.

[3] Global Atlas on Cardiovascular Disease Prevention and Control. Mendis S, Puska P, Norrving B editors. *World Health Organization, Geneva* 2011

[4] Gonzalez M., Martín-Bautista E., Carrero J.J., Fonollá J., Baró L, Bartolomé M.V., Gil-Loyzaga P., López-Huertas E. (2006) "One-month administration of hydroxytyrosol, a phenolic antioxidant present in olive oil, to hyperlipemic rabbits improves blood lipid profile, antioxidant status and reduces atherosclerosis development" *Atherosclerosis*.188:35-42.

[5] Glass C.K., Witztum J.L. (2001) "Atherosclerosis. the road ahead" *Cell*. 104:503-516.

[6] MINSAP. Anuario estadístico. Ministerio de Salud Pública. Ciudad de La Habana 2009. Disponible en: http://www.sld.cu/servicios/estadisticas/

[7] Stary HC (2000). "Natural History and Histological Clasification of atherosclerotic lesion" *Arterioscler Thromb Vasc Biol*. 20:1177-1178.

[8] Mileiu J, Ottaviani G, Lavezzi AM, Grana DR, Stella I, Matturri L. (2008) "Perinatal and infant early atherosclerotic coronary lesions" *Can J Cardiol;*24:137-41.

[9] Milei J, Grana DR, Navari C, Azzato F, , Guerri-Guttenberg RA, Ambrosio G. (2010) "Coronary intimal thickening in newborn babies and 1 – year-old infants". *Angiology*. 61:350-6.

[10] Russell JC, Proctor SD (2006). "Small animal models of cardiovascular disease: tools for the study of the roles of metabolic syndrome, dyslipidemia, and atherosclerosis" *Cardiovasc Pathol* 15:318-30.

[11] Narayanaswamy M., Wright K.C., Kandarpa K. (2000) "Animal models for atherosclerosis, restenosis, and endovascular graft research" *J Vasc Interv Radiol*. 11:5-17.

[12] Antischkow N (1914). "Uber die atherosclerose der aorta beim kaninchen and uber dering entstehungs bedin gungen". *B Pathol Anat Zur Al Pathol* 59:308-48.

[13] Paigen BB, Ishida BY, Verstuyft J (1990). "Atherosclerosis susceptibility differences among progenitors of recombinant inbred strains of mice". *Arteriosclerosis* 10:316-23.

[14] Nakashima Y, Plump AS, Raines EW (1994). "Apo E deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree". *Arterioscler Thromb* 14:133-40.

[15] Coleman R, Hayek T, Keidar S, Aviram M (2006). "A mouse model for human atherosclerosis: long-term histopathological study of lesion development in the aortic arch of apolipoprotein E-deficient (ApoE-/-) mice". *Acta Histochem* 108:415-24.

[16] Jellinek H, Harsing J, Siztesi SZ (1982). "A new model for atherosclerosis. And electom microscopy stady of the lesion induced by i.v administred fat". *Aterosclerosis* 43:7-18

[17] Noa M y Mas R (1992). "Ateromisol y lesión ateroscleróticas en conejos inducida por Lipofundín". *Progreso en Ciencias Médicas Venezuela* 6:14-19.

[18] Noa M, Mas R, De la Rosa MC y Magraner J (1995). "Efect of Policopsanol on Lipofundin-induced atherosclerotic lesion in rat". *Journal of Farmacy and Farmacology* 47(4):289-91.

[19] Ruengsakulrach P, Sinclair R, Komeda M, Raman J,Gordon I, Buxton B (1999). "Comparative histopathology of Radial artery versus internal thoracic artery and risk factors for development of intimal hyperplasia and atherosclerosis". *Circulation* 100:II-139-II-144.

[20] Ström A, Franzén A, Wängnerud Ch, Knutsson A, Heinegard D, Hultgardh-Nilsson A (2004). "Altered vascular remodeling in osteopontin-deficient atherosclerotic mice". *J Vasc Res* 41:314-322.

[21] Stary HC, Blankenhorn DH, Chandler AB, Glavov S, Insull W Jr, Richardson M, Rosenfeld ME, Schaffer A, Schwartz CJ, Wagner WD, Wissler RW (1992). "A definition of the intima of human arteries and of its atherosclerosis-prone regions". *Arterioscler Thromb*. 12:120-134.

[22] Stary HC, Chandler AB, Glavov S, Guyton JR, Insull W Jr, Rosenfeld ME, Schaffer A, Schwartz

CJ, Wagner WD, Wissler RW (1994). "A definition of initial, fatty streak, and intermediate lesion of atherosclerosis". *Arterioscler Thromb.* 14:840-856.

[23] Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glavov S, Insull W Jr, Rosenfeld ME, Schwartz CJ, Wagner WD, Wissler RW (1995). "A definition of advanced types of atherosclerotic lesion and a histological clasification of atherosclerosis". *Arterioscler Thromb Vasc Biol* 15:1512-1531.

[24] Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM.(2000) "Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions" *Arterioscler Thromb Vasc Biol.* 20:1262-1275.

[25] Spurr, A.R. (1969). "A low-viscosity epoxy resin embedding medium for electron microscopy". *Journal of Ultrastuctural Research.* 26, 31-43.

[26] Bustos C, Hernández-Presa MA, Ortego M, Tuñón J, Ortega L, Pérez F, Díaz C, Hernández G, Egido J (1998). "HMG-CoA reductase inhibition by atorvastatin reduces neointimal inflammation in a rabbit model o atherosclerosis". *J. Am. Coll. Cardiol.* 32:2057-2064.

[27] Kobayashi H, Kitamura S, Kawachi K, Morita R, Konishi Y, Tsutsumi M. (1993) "A pathohistological and biochemical study of arteriosclerosis in the internal thoracic artery, a vessel commonly used as a graft in coronary artery bypass surgery". *Surg Today*. 23:697–703.

[28] Kaufer E, Factor SM, Frame R, Brodman RF.(1997) "Pathology of the radial and internal thoracic arteries used as coronary artery bypass grafts". *Ann Thorac Surg.* 63:1118 –1122.

[29] Nakashima Y, Kinukawa ChN, Sueishi K.(2002) "Distributions of diffuse intimal thickening in human arteries: preferential expression in atherosclerosis-prone arteries from an early age". *Virchows Arch* 441:279–288.

[30] Nakashima Y, Fujii H, Sumiyoshi S, Wight TN, Sueishi K.(2007) "Early human atherosclerosis. Accumulation of lipid and proteoglycans in intimal thickenings followed by macrophage infiltration" *Arterioscler Thromb Vasc Biol.* 27:1159-1165.

[31] Hayashi T, Arockia J, Fukatsu A, Matsui-Hirai H, Osawa M, Miyazaki A, Tsunekawa T, Kano-Hayashi H, Iguchi A, Sumi D, Ignarro LJ (2004). "A new HMG-CoA reductase inhibitor, pitavastatin remarkably retards the progression of high cholesterol induced atherosclerosis in rabbits". *Atherosclerosis* 176:255-263.

[32] Fernández-Brito JE, Bielokrinitzki V, Morgalo R, Candas A, Dujarric R, Candas M (1981). "Diseño experimental de la investigación. Estudio de la aterosclerosis coronaria, aórtica y cerebral". *Rev Cubana Hig Epidemiol* 19:137-49.

[33] Daley SJ, Klemp KF, Guyton JR, Rogers KA (1994). "Cholesterol-fed and casein-fed models of atherosclerosis. Part 2: Differing morphological severity of atherogenesis despite matched plasma cholesterol levels". *Arterioscler Thromb Vasc Biol.* 14:105-141.

[34] Mehrad H, Mokhtari-Dizaji M, Ghanaati H, Shahbazfar A, Mohsenifar A(2011). "Developing a rabbit model of neointimal stenosis and

atherosclerotic fibrous plaque rupture". *J Teh Univ Heart Ctr* 6:117-125.

[35] Castro M, Pacheco MMR, Machado MRF (2009). "Morphology of aortic arch in rabbits with aterosclerosis treated with resveratrol". *Intern J Appl res Vet Med* 7:190-195.

[36] Ishino S, Mukai T, Kuge Y, Kume N, Ogawa M, Takai N, Kamihashi J, Shiomi M, Minami M, Kita T, Saji H(2008). "Targeting of lectinlike oxidized low-density lipoprotein receptor 1 (LOX-1) with 99mTc-labeled anti–LOX-1 antibody: potential agent for imaging of vulnerable plaque". *J Nucl Med* 49:1677–1685.