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Development of atherosclerosis in the diabetic BALB/c mice Prevention with Vitamin E administration

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Abstract

The aim of the present study was to determine in the BALB/c mice, a model of development of atherosclerosis when both hyperglycemia and hypercholesterolemia are present, whether the atherogenic effects of these parameters could be decreased with the administration of Vitamin E.

BALB/c mice were made diabetic and divided in three groups: one fed the standard rodent chow diet (D); the other two fed an atherogenic diet (D + A); one of them supplemented with Vitamin E (D + A + E). Two groups of non diabetic animals were also performed, one fed the standard diet (C) and the other the atherogenic diet (C + A).

After 16 weeks of treatment all the control animals survived, in contrast, a mortality rate of 12, 70 and 37% was observed, respectively, in the D, D + A and D + A + E groups. Neither fatty deposits nor macrophages were observed in the arterial wall of the animals fed the standard diet (D and C animals). In contrast, this finding was observed in 25% of the C + A, 71% of the D + A and 33% of the D + A + E.

In conclusion, diabetic mice fed an atherogenic diet showed in the aorta a higher number of fatty deposits and macrophages than the control animals. These effects were partially reversed with the administration of Vitamin E, supporting in this model the role of oxidative stress in the development of atherosclerosis.
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Keywords: Diabetes; Atherosclerosis; Vitamin E; Cardiovascular diseases

1. Introduction

Cardiovascular diseases secondary to atherosclerosis are a common cause of mortality and morbidity among subjects with type 1 diabetes mellitus (DM) [1–4]. In this population, there is a three- to five-fold higher rate of coronary heart diseases, leading to the development of the complications associated with atherosclerosis at a younger age than in the non diabetic population [1–4]. In fact, there are some reports showing, in subjects with type 1 DM, a mortality rate secondary to cardiovascular disease as high as 40% at 50 years of age [1,2].

Elevated levels of glucose are related to most of the complications secondary to diabetes [3,5]. The role of glucose in these complications is not completely understood and probably has a multifactorial origin. Although, an increase in oxidative stress seems to play a relevant role, since some of these complications including congenital malformations, neuropathy and retinopathy, can be prevented or ameliorated with the administration of antioxidants [6–10]. Therefore, the damage mediated by glucose could be secondary to the generation of free radicals. In fact, we and others have demonstrated that the oxidation of the LDL lipids, a key step in the development

Abbreviations: C, control mice fed the standard diet; C + A, control mice fed the atherogenic diet; D, diabetic mice fed the standard diet; D + A, diabetic mice fed the atherogenic diet; D + A + E, diabetic mice fed the atherogenic diet supplemented with Vitamin E; DM, diabetes mellitus; LDL, low-density lipoprotein; MDA, malondialdehyde; STZ, streptozotocin

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of atherosclerosis [11–13], is accelerated in vitro by the presence of glucose [14–16], linking glucose with LDL oxidation.

It has been reported that the BALB/c mouse, which normally exhibits resistance to high fat diet-induced atherosclerosis, develops fatty streak lesions when hyperglycemia is also present [17]. This makes the BALB/c mouse an excellent model to study the mechanism involved in the effects of glucose on the development of the atherosclerotic plaques. Thus, by using BALB/c mice made diabetic by the administration of streptozotocin and fed a high fat diet, in the present study we wanted to determine whether the administration of Vitamin E, a powerful free radical scavenger, is able to prevent the development of atherosclerosis in these animals.

2. Materials and methods

2.1. Animals

Five weeks old female BALB/cByJ (BALB/c) mice were obtained from IFFA CREDO Laboratory (France) and fed a pelleted rodent chow diet (RMN diet, Harlan Ibérica SA, Barcelona, Spain) for 2–3 weeks until they were adapted to our facilities and reached adult weight. The animals were maintained in a temperature-controlled room (25 °C), with a 12 h light:12 h dark cycle and free access to food and water. Initial body weights for BALB/c mice were 17 ± 1 g (mean ± S.E.M.). The experimental protocol was approved by the Animal Research Committee of the University San Pablo CEU, Madrid, Spain.

2.2. Diets and experimental design

Two different diets were used in this study: (a) an "atherogenic" diet (ICN Ibérica Laboratory, Barcelona, Spain) known to elicit fatty streak lesions in strain C57BL/6 [18], containing 15% fat (primarily cocoa butter), 1.25% cholesterol and 0.5% sodium cholate and (b) a standard rodent chow diet (RMN diet, Harlan Ibérica SA, Barcelona, Spain) which contained 2.2% fat, 15% protein and 5.2% crude fiber.

Mice were made diabetic by the daily intraperitoneal administration of streptozotocin (STZ), (Sigma–Aldrich, Madrid, Spain) (40 mg/kg of body weight), previously diluted in sterile citrate buffer (0.05 M sodium citrate, pH 4.5), during 5 consecutive days. Diabetes was diagnosed by measuring urine glucose with paper test strips (Ketodiabur test, Boehringer Manheim, Barcelona, Spain), and thereafter this test was repeated on a weekly base along the study. During the first 5 weeks, all the animals were fed the standard rodent chow diet and after that, the diabetic animals were divided into three different groups, one group including 17 animals received the standard rodent chow diet (D), and the remaining two groups, one with 30 animals received the atherogenic diet (D + A), and the other with 14 was supplemented with Vitamin E (D + A + E), administered by oral gavage (100 mg of ± α-tocopherol acetate/day, Sigma–Aldrich, Madrid, Spain). Vitamin E was previously dissolved in a volume of 50 μL of the vehicle (sunflower oil). The animals from the D + A group also received the same amount of the vehicle. In parallel, two groups of control animals were used, receiving the same volume of citrate buffer during 5 days as the diabetic animals; one of them including 10 animals was fed the standard rodent chow diet (C) and the other with 14 animals, the atherogenic diet (C + A). Seven weeks after the initiation of the study, a new dose of STZ was injected to those diabetic animals which at that time did not show any sign of glucosuria. Thirty-two weeks after the beginning of the study, the surviving mice were anesthetized with a mixture of ketamine 5% (Merial, Barcelona, Spain) and Xilocaine 2% (Bayer, Barcelona, Spain) given intraperitoneally. Blood was collected from cardiac puncture into tubes containing heparin. After laparotomy, the liver was immediately extracted, placed into liquid nitrogen and stored at −80 °C until analysis.

2.2.1. Processing and analysis of the samples

An aliquot of plasma was deproteinized with $Ba(OH)_2$ and $ZnSO_4$ [19] and supernatants were used for glucose (Boehringer, Mannheim, Germany) and β-hydroxybutyrate determination [20]. Aliquots of plasma were also used for triglycerides (Triglycerides Enzymatic trinder method; Menarini Diagnostics, Florence, Italy) and cholesterol determination (Cholesterol H.F., Enzymatic trinder method, Menarini Diagnostics, Florence, Italy). Malondialdehyde (MDA) and Vitamin E concentrations were determined in plasma and liver, following methods previously described [21–23]. The total lipid content of the liver was quantified following the Bligh–Dyer method [24].

2.2.2. Morphological examination of atherosclerotic lesions

The upper section of the aorta (from aortic valve to right carotid artery) was removed from animals, cleaned of peripheral fat under a dissecting microscope, and then embedded in OCT medium (Leica, Nussloch, Germany) and frozen in liquid nitrogen. Serial cryostat (Leica, Nussloch, Germany) cut sections (10 μm thick) kept at −80 °C were done for histochemistry and immunohistochemistry. Twenty alternate sections from each animal were evaluated for fatty streak lesions after staining with oil red O and counterstaining with haematoxylin. The number of lesions in each cross-section was counted and areas were estimated using an Image Analysis System (Analysis® 3.0, Soft Imaging System GmbH, Germany) [18].

2.2.3. Immunohistochemistry

The sections next to those analyzed with Oil Red O staining, were used for immunohistochemical detection of macrophages [25,17]. Macrophages were identified using a primary rat monoclonal antibody to the mouse and human Mac-1 (Anti-Mac-1 CD11 B/CD18, Boehringer Manheim,

Table 1
Animals weight, levels of glucose, β-hydroxybutyrate, cholesterol and triglycerides in plasma, in the different experimental groups

Experimental groups	Animals studied (*n*)	Animals weight (g)	Glucose (mg/dL)	β-Hydroxybutyrate (μmol/L)	Cholesterol (mg/dL)	Triglycerides (mg/dL)
D	15	19.95 ± 0.71***♦♦♦	539 ± 61***	438 ± 75	84 ± 3*♦♦♦	116 ± 15
D+A	9	15.10 ± 0.45***	469 ± 97**	374 ± 25	731 ± 189**	82 ± 22*
D+A+E	9	15.35 ± 0.67	528 ± 87**	439 ± 105	680 ± 156	86 ± 12
C	10	25.11 ± 0.61	251 ± 12	226 ± 30	71 ± 4♦♦♦	131 ± 16♦♦♦
C+A	14	25.94 ± 0.32	235 ± 10	337 ± 61	175 ± 7	35 ± 2

Data are presented as mean ± S.E.M. (*) Diabetic mice vs. control mice under the same diet. (♦) Mice fed the standard rodent chow diet vs. the atherogenic diet. (*) $p<0.05$; (**) $p<0.01$; (***, ♦♦♦) $p<0.001$.
D: diabetic mice fed the standard rodent chow diet; D + A: diabetic mice fed the atherogenic diet without any supplementation; D + A + E: supplemented with 100 mg/d Vitamin E; C: control mice fed the standard rodent chow diet; C + A: control mice fed the atherogenic diet.

Barcelona, Spain) and as secondary antibody the biotin-labeled rabbit anti-rat immunoglobulin G (Vectastain ABC kit Elite, Vector, CA, USA) was used. Briefly, sections were fixed in cold acetone for 10 min. Endogenous peroxidase activity was blocked by incubating slides in a solution of 3% hydrogen peroxide for 5 min and after this period of time, with rabbit serum in order to avoid cross-reactions and non specific staining. The slides were incubated in humidified chambers with the primary antibody (previously diluted 1:250 in phosphate buffer saline PBS) overnight at 4 °C, followed by 30 min incubation at room temperature with the second antibody (diluted 1:200 in PBS). In order to amplify the signal obtained, the ABC reagent (Avidin–biotin–peroxidase, Vectastain ABC Kit Elite, Vector, CA, USA) was added to the slides, with 30 min incubation at room temperature. Sections were visualized by chromogenic detection with AEC (AEC substrate kit for peroxidase, Vector, CA, USA). After treatment, sections were washed with PBS and counterstained with haematoxylin. Control slides were incubated without the primary or secondary antibody.

2.2.4. *Statistical analysis*

Data are expressed by the mean ± S.E.M. The significance of the difference between the means of the different groups for parameters with normal distribution was obtained with the analysis of variance and the Tuckey test for multiple comparisons. Non parametric methods (Mann–Whitney *U*-test and Kruskal–Wallis test) were used for the analysis of parameters that did not follow a normal distribution. To study the differences in the survival rate the Chi-square test was used. For the statistical analysis the Systat program was used (Systac Inc., Evanston, USA).

3. Experimental results

3.1. *Animals weight*

As expected the diabetic mice (D, D + A and D + A + E) had lower body weight than control animals (Table 1). A further decrease in body weight was observed in the diabetic animals fed the atherogenic diet (D + A, D + A + E) when compared with the D animals fed the standard diet (Table 1). In contrast, no differences were observed in body weight in control animals, independently of the diet used (Table 1).

3.2. *Metabolic parameters*

Plasma glucose and β-hydroxybutyrate were measured as an index of the metabolic condition of the animals. As shown in Table 1, plasma concentration of glucose was higher in the diabetic mice (D, D + A and D + A + E) than in the control groups (C, C + A). A trend toward higher levels of plasma β-hydroxybutyrate was observed in the diabetic mice when

Table 2
Vitamin E and MDA concentration in plasma and liver and total hepatic lipids

Experimental groups	Plasma		Liver		
	Vitamin E (μg Vitamin E/mg lipid)	MDA (μmol/L)	Total Lipids (mg lipids/g tissue)	Vitamin E (nmol Vitamin E/mg lipid)	MDA (nmol/g tissue)
D	1.035 ± 0.061	6.47 ± 0.383	64.64 ± 5.13♦♦♦	0.481 ± 0.045	4.824 ± 0.172
D+A	1.110 ± 0.185	6.697 ± 0.531***	195.48 ± 5.09***	0.521 ± 0.076	5.446 ± 0.323***
D+A+E	2.530 ± 0.510•	4.300 ± 0.379••	172.11 ± 7.63	5.128 ± 1.699•••	3.505 ± 0.143•••
C	1.196 ± 0.064	5.404 ± 0.453	70.22 ± 1.14♦♦♦	0.489 ± 0.019	4.746 ± 0.243♦♦♦
C+A	1.052 ± 0.048	4.141 ± 0.251	134.38 ± 7.98	0.249 ± 0.014	3.572 ± 0.094

The number of animals included in each group is shown in Table 1. Data are presented as mean ± S.E.M. (*) Diabetic mice vs. control mice under the same diet. (♦) Mice fed the standard rodent chow diet vs. the atherogenic diet. (•) Diabetic mice from the D + A + E group vs. diabetic mice from the D + A group. (•) $p<0.05$; (••) $p<0.01$; (***,•••, ♦♦♦) $p<0.001$.
D: diabetic mice fed the standard rodent chow diet; D + A: diabetic mice fed the atherogenic diet without any supplementation; D + A + E: supplemented with 100 mg/d Vitamin E; C: control mice fed the standard rodent chow diet; C + A: control mice fed the atherogenic diet.

compared to control animals, although the differences were not statistically significant (Table 1). The administration of either the atherogenic diet or Vitamin E had no effect on plasma levels of glucose or β-hydroxybutyrate in the diabetic animals.

The concentration of plasma cholesterol was significantly higher in the animals treated with the atherogenic diet than in those receiving the standard diet. This effect was common to both control and diabetic animals, although in the diabetic mice receiving the atherogenic diet, the plasma cholesterol levels reached values significantly higher than in control animals fed the same diet (Table 1). The administration of an atherogenic diet to diabetic mice (D + A) did not induce any change in triglycerides levels compared to the animals fed the standard diet (D). However, in control animals, the atherogenic diet led to a decrease in this parameter (Table 1). The administration of Vitamin E to the diabetic mice receiving the atherogenic diet did not have any effect in the plasma levels of both cholesterol and triglycerides. As expected, the administration of Vitamin E to the diabetic mice fed the atherogenic diet led to an increase in both liver and plasma Vitamin E content when compared with the concentration observed in the D and D + A animals (Table 2). No differences were observed in both plasma and liver MDA concentration between the diabetic and control animals, when they were fed the standard diet (Table 2). In contrast, higher levels of this parameter were found in plasma and liver of the D + A mice compared with C + A animals (Table 2). With the administration of Vitamin E, a marked decrease was observed in both plasma and liver MDA content (Table 2). The administration of an atherogenic diet to diabetic and control animals led to an increase in the liver content of lipids (Table 2).

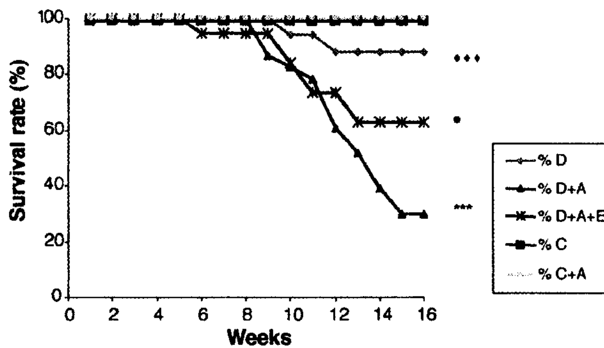

Fig. 1. Survival rate in the different experimental groups. Initially 17, 30 and 14 animals were included, respectively, in the D, D + A, D + A + E group. Of them 15 animals survived in the D group and 9 in the D + A; D + A + E groups. In contrast, in the C and C + A groups, initially we included 10 and 14 animals; all of them survived until the end of the study. D: diabetic mice fed the standard rodent chow diet; D + A: diabetic mice fed the atherogenic diet without any supplementation and D + A + E: supplemented with 100 mg/d Vitamin E; C: control mice fed the standard rodent chow diet; C + A: control mice fed the atherogenic diet. (*) Diabetic mice vs. control mice under the same diet. (♦) Mice fed the standard rodent chow diet vs. the atherogenic diet. (•) Diabetic mice from the D + A + E group vs. diabetic mice from the D + A group. (•) $p < 0.05$; (***, ♦♦♦) $p < 0.001$.

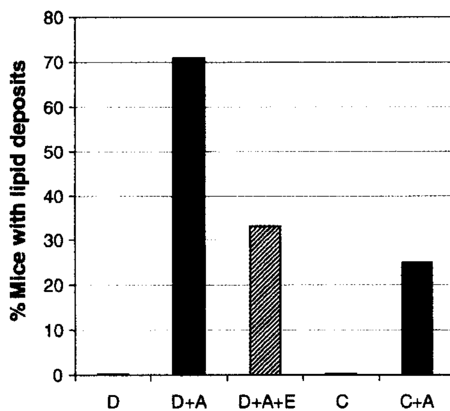

Fig. 2. Percentage of animals where lipid deposits were observed. In this figure, we shown the percentage of animals containing lipid deposits in the sections of the arterial wall studied. D: diabetic mice fed the standard rodent chow diet; D + A: diabetic mice fed the atherogenic diet without any supplementation; D + A + E: supplemented with 100 mg/d Vitamin E; C: control mice fed the standard rodent chow diet; C + A: control mice fed the atherogenic diet.

3.3. Mortality

The atherogenic diet did not increase the mortality rate in the control mice. This result was in clear contrast with the diabetic animals where a marked increase in the mortality rate was observed in the animals treated with the atherogenic diet (Fig. 1). The high mortality observed in the D + A group

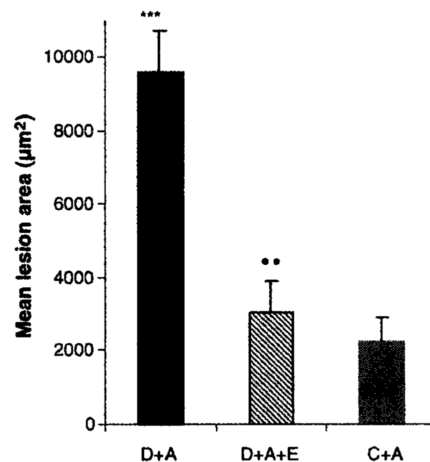

Fig. 3. Mean area of the aortic lipid deposits in the different experimental groups. In the present figure, the analysis was performed only in animals from the following groups: D + A: diabetic mice fed the atherogenic diet without any supplementation; D + A + E: supplemented with 100 mg/d Vitamin E; C + A: control mice fed the atherogenic diet. In the remaining two groups no lipid deposits were observed (D: diabetic mice fed the standard rodent chow diet; C: control mice fed the standard rodent chow diet). (*) Diabetic mice vs. control mice under the same diet. (•) Diabetic mice from the D + A + E group vs. diabetic mice from the D + A group. (••) $p < 0.01$; (***) $p < 0.001$.

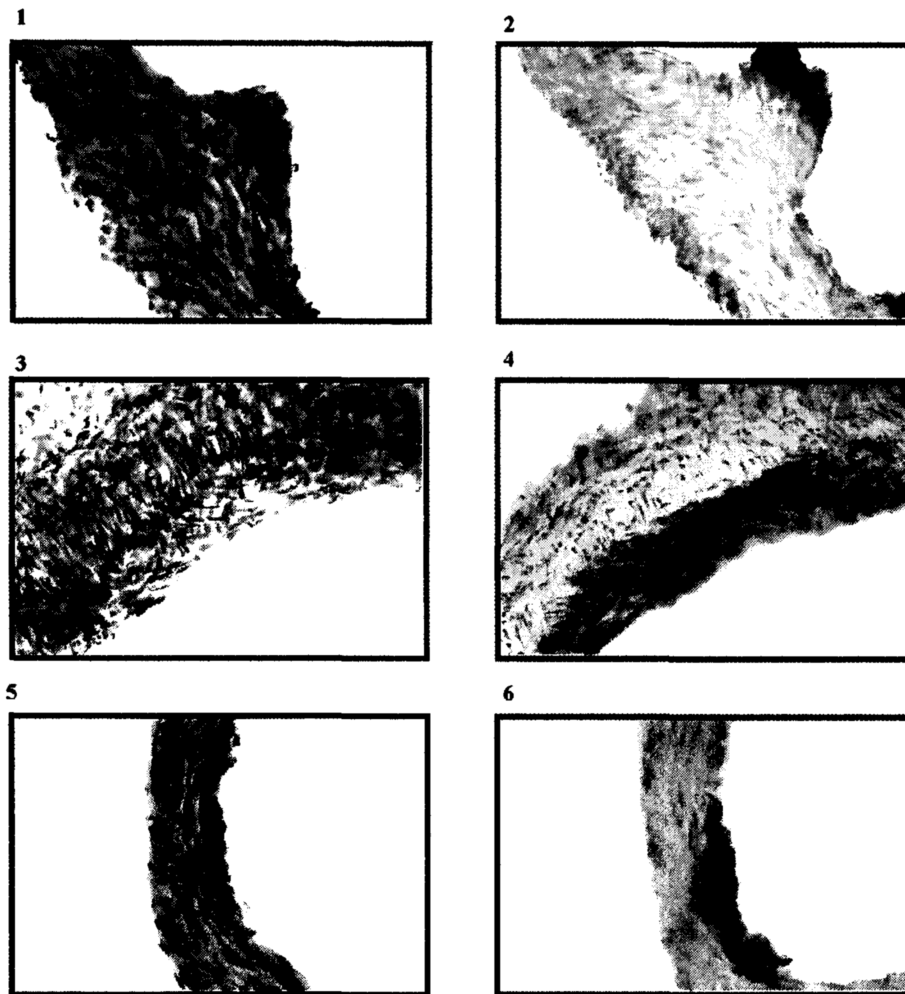

Fig. 4. Photomicrographs of adjacent sections of aortas obtained from mice fed the atherogenic diet. Sections 1, 3 and 5 were stained with haematoxylin and oil red and sections 2, 4 and 6 immunostained with anti-Mac-1 antibody (100×).

during the 16 weeks of the study was diminished with the administration of Vitamin E, (D + A + E) (Fig. 1), although in this group, the mortality was still higher than in the D group receiving the standard diet.

3.4. *Fatty deposits and macrophages accumulation in the arterial wall*

In the animals treated with the standard diet, neither fatty deposits nor macrophages were observed in the arterial wall, both in C and D animals. With the administration of the atherogenic diet in the C + A group, fatty deposits and macrophages were observed in 25% of the animals studied (Fig. 2), with a mean lesion area containing lipid deposits of 2235 μm^2 (Fig. 3). In contrast, 71% of the D + A mice showed fatty deposits (Fig. 2) and accumulation of macrophages, with a mean lesion area containing lipid deposits of 9597 μm^2 (Fig. 3). The administration of Vitamin E the diabetic mice receiving the atherogenic diet, decreased the number of animals with fatty deposits to 33%, with a mean lesion area containing lipid deposits and macrophages of 3053 μm^2.

As shown in Fig. 4, the adjacent sections of the aorta containing lipid deposits showed macrophages accumulation, suggesting a close relationship between both processes. Although the methods used do not allow us to precise whether the deposits were intra or extracellular.

4. Discussion

Present results show in an experimental mouse model of atherosclerosis secondary to both hypercholesterolemia and hyperglycemia, that the administration of Vitamin E, a powerful lipid soluble antioxidant, decreases in the arterial wall both the fatty deposits and the accumulation of macrophages, and substantially decreases mortality, linking oxidative stress to atherosclerosis development.

Although the mechanism involved in the development of atherosclerosis in subjects with diabetes is not completely understood; in other conditions, there is evidence that lipid per-

oxidation plays a central role in this process [11–13,26,27]. Compounds derived from the LDL lipid oxidation, either directly or indirectly are able to stimulate the synthesis of inflammatory factors, induce the accumulation of cholesterol in macrophages from wall and are toxic to endothelial cells as well as for other cells of the arterial wall [11–13,26,27]. These processes are involved, among others, in the development of atherosclerosis [11–13,26,27]. Because of their double bonds, lipids are the biological compounds most susceptible to the damage induced by the reaction with free radicals [28]; therefore, in states with high plasma lipid levels, there is more substrate for the reaction with free radicals. In fact, in different conditions, hyperlipidemia has been associated with increased plasma levels of lipid peroxides and higher LDL susceptibility to oxidation, condition linked to atherosclerosis development [29,30]. The presence of a high glucose concentration could further accelerate and increase the generation of lipid peroxides. In fact, we and others have shown in vitro that a high glucose concentration in LDL submitted to oxidation by copper is able to accelerate and increase the generation of lipid peroxidation products [14–16]. Therefore, in the animal model used here, the combination of high glucose and high cholesterol levels could accelerate lipid peroxidation and lead to both lipid deposition and macrophages accumulation in the arterial wall, as our results show. The fact that the administration of Vitamin E, a powerful lipid soluble antioxidant, was able to decrease the extension of the fatty deposits and macrophages infiltration in the arterial wall further emphasize the role of the oxidative stress in the development of atherosclerosis. In the present model, the beneficial effect of Vitamin E is not secondary to a decrease in plasma glucose or cholesterol, since their respective plasma concentrations remain unchanged in the diabetic mice supplemented with Vitamin E.

In clinical practice, despite of intensive treatment [3,5], it is not always easy to normalize blood glucose, and even this intensive treatment is not free of complications. Thus, any means of preventing the development of cardiovascular diseases secondary to diabetes by other mechanisms than the normalization of glucose levels, as in the present experimental conditions Vitamin E does, could help to decrease the development of atherosclerosis, the leading complication of mortality and morbidity among subjects with diabetes.

In the present experimental conditions, the treatment with an atherogenic diet to mice made diabetic with the administration of streptozotocin led to a much higher plasma levels of cholesterol than in control animals. Similar results have been shown by other authors in this experimental model [17] and could be secondary to the decrease of lipoproteins clearance that occurs in subjects with diabetes [31]. In contrast, this diet decreased the plasma levels of triglycerides, both in D and C animals. This alteration also has been found by other authors using the same model [17,32], and could be secondary to the fact that long chain fatty acids inhibits the synthesis of fatty acids and increases the synthesis of ketone bodies leading to the secretion of VLDL with a low content in triglycerides [33]. A fatty diet can also increase lipoprotein lipase activity [34], which could decrease the plasma levels of triglycerides.

Another relevant finding from the present study is the high mortality rate observed in the diabetic mice submitted to the atherogenic diet. Although we do not know the factors involved in this high mortality, oxidative stress might play a relevant role as Vitamin E administration was able to decrease the mortality. As a consequence of the administration of the atherogenic diet, a marked increase in the liver lipid storage was observed both in C + A and D + A animals, being this effect more pronounced in diabetic animals. Increased lipid deposits in liver can lead to the development of steatohepatitis and cirrhosis, phenomenon associated with increased oxidative stress. In humans, the administration of Vitamin E to subjects with fatty liver and steatohepatitis decreases the liver cell mortality [35]. In fact, the D + A + E group despite of a high liver lipid content showed a lower MDA content than the D + A group suggesting a decrease in oxidative stress. It is attractive to speculate that the lower mortality observed in this group, could be partially due to decreased oxidative stress, secondary to the higher Vitamin E content.

In summary, our results show that in the mouse model of atherosclerosis associated to hyperglycemia and hypercholesterolemia, the administration of Vitamin E decreases in the arterial wall, both the fatty deposits and the macrophages accumulation as well as the mortality rate. Overall, present results allow us to suggest that subjects with diabetes might need a higher intake of Vitamin E than current recommendations.

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