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ORIGINAL ARTICLE

Early atherosclerosis development in female apolipoprotein E-deficient mice is associated with an increased vascular oxidative stress

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ABSTRACT

Objective: The mechanisms of atherosclerosis development in the apolipoprotein E-deficient (apoE$^{-/-}$) mice have been clearly described. In this genetically modified strain, oxidative stress contributes with atherosclerotic lesion progression. However, there are controversial criteria on the vulnerability of female apoE$^{-/-}$ mouse to oxidative stress and atherosclerosis during the fertile period. Thus, the aim of the present work was to examine the implication of vascular oxidative stress during early atherosclerosis development in young apoE$^{-/-}$ female mice.
Methods: Fifteen 4-week-old and 24-week-old female apoE$^{-/-}$ mice were fed with a high fat high cholesterol and compared with age-matched wild-type C57BL/6J female mice fed with standard diet. Aortic histopathology, serum lipid profile and redox biomarkers were performed for evaluation.
Results: Hematoxylin/eosin staining demonstrated the presence of early atherosclerotic lesions in the apoE$^{-/-}$ mice, which progressed with age. Dihydroethidium staining of aortic sections revealed a significant increase of superoxide anion generation in apoE$^{-/-}$ compared with the wild type mice. The increment of this reactive oxygen species was associated, at least, with the overexpression of Nox2 mRNA levels. In addition, the 8-week-old apoE$^{-/-}$ mice had a significant increment of lipid and protein damage, as well as a disruption of superoxide dismutase and catalase activity, together with a deprivation of reduced glutathione and an impairment of nitric oxide availability.
Conclusion: The present study shows that early atherosclerotic lesion formation in young apoE$^{-/-}$ female mice is associated with an increment of Nox2-mediated reactive oxygen species generation with the subsequent redox disruption, suggesting a key role of oxidative stress during atherogenesis in fertile apoE$^{-/-}$ female mice.

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INTRODUCTION

The influence of gender on atherosclerosis development is still controversial. A great number of studies have shown that during the reproductive years, women are less prone to developing atherosclerosis and cardiovascular diseases than males, but men and post-menopausal women at comparable ages are at an equal risk for developing atherosclerosis [1]. The antioxidant properties of female hormones have been considered the main mechanism by which hormones exert their protection against cardiovascular pathologies [2]. In contrast, menopause, characterized by the loss of estrogen-dependent effects, may be a key determinant for sex-specific differences with negative repercussion, also in the magnitude of oxidative stress [2-4].

The role of oxidative stress in atherosclerosis have been well documented by the observation that increased markers of lipid and protein oxidation predict the progression of coronary heart diseases [5]. The redox disruption predisposes vessels to long-term atherosclerotic lesions, triggering inflammation, immune cell activation, platelet activation, thrombus formation, and disturbed hemodynamic flow [6].

There are evidences suggesting that atherosclerotic lesions in apolipoprotein E-deficient mice (apoE$^{-/-}$) are greater in females than in males [7]. Other studies showed that apoE$^{-/-}$ mice developed plaques earlier in the females than in males [8, 9]. Nonetheless, the reasons for this apparent discrepancy remain unclear. Thus, in the present study we tested the hypothesis that vascular oxidative stress is a key early step that precedes the development and progression of atherosclerotic lesions in the female apoE$^{-/-}$ mice.

MATERIALS AND METHODS

Animals and Experimental Design

Fifteen 4-week-old and 24-week-old female apoE$^{-/-}$ mice were obtained from CENPALAB (Mayabeque, Cuba). As controls, age-matched wild-type C57BL/6J female mice were used in the study (referred to as C57). ApoE$^{-/-}$ mice were fed with a high fat high cholesterol (HFHC) diet containing 10% wt/wt pork fat and 1% wt/wt cholesterol (Sigma, USA), meanwhile the wild-type C57 were fed a standard chow diet. The mice were housed under controlled temperature (22-23°C) and humidity (60%), exposed to a 12 h light-dark cycle. All experimental procedures were performed in accordance with the guidelines for the care and handling of laboratory animals as recommended by the National Institute of Health (NIH, USA). The experimental protocol was approved by the Pharmacy and Food Sciences College Institutional Animal Ethical Committee.

At weeks 8 and 44, the animals were anesthetized with ketamine hydrochloride (5 mg/kg, i.m.), euthanized with an overdose of sodium pentobarbital (90 mg/kg, i.v.), and the vascular system was perfused with NaCl 0.9% solution at 4°C. Then, the aortic tree was removed and processed.

Histopathology

Aortas were fixed in 10% formaldehyde solution during 24 h and samples from the aortic arch were embedded in paraffin. Five-micrometer tissue sections were stained with hematoxylin/eosin under standard procedures and imaged using an optic microscope (Olympus BX51; Yokohama, Japan).

Serum Lipid Assay

Serum total cholesterol (TC), very low-density lipoproteins (VLDL)/LDL cholesterol (LDLc) and high-density lipoprotein cholesterol (HDLc) were determined using commercial enzymatic kits (MBL International Corporation, Woburn, MA, USA).

Determination of Redox Biomarkers

All redox biomarkers were determined in aortas homogenates by spectrophotometric methods using a Pharmacia 1000 Spectrophotometer (Pharmacia LKB, Uppsala, Sweden). Total protein content was determined using the Bradford´s method with bovine serum albumin as standard [10]. Superoxide dismutase (SOD) activity was determined by using RANSOD kit (Randox Labs, Crumlin, UK). Catalase (CAT) activity was determined by following the decomposition of hydrogen peroxide (H_2O_2) at 240 nm at 10 s intervals for 1 min [11]. After precipitation of thiol proteins, the reduced glutathione and oxidized glutathione (GSH/GSSG) ratio was determined using a commercial kit (Kit No. 371757, Merck Millipore, Germany). Total protein carbonyls (CG), a marker of oxidative damage, were determined as previously described [13]. Concentration of malondialdehyde (MDA) was determined using a commercial kit obtained from Calbiochem (La Jolla, CA, USA). Finally, nitrates/nitrites (NO_3^-/NO_2^-) level, as a surrogate marker of nitric oxide (NO•), were determined converting nitrates to nitrites using the enzyme nitrate reductase (Boehringer Mannheim Italia, Milan, Italy). Then, Griess reagent (1% sulphanilamide, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 0.25% phosphoric acid) was added and the absorbance of the reaction mixture was measured at 540 nm [14].

Detection of *In Situ* Superoxide Anion Generation

Fresh cross-sections from the aortic arch were rinsed in cold PBS and superoxide anion (O_2•$^-$) generation was determined by the addition of dihydroethidium (DHE, 5 μM, Molecular Probes) for 30 min at 37°C as previously described [15]. DHE fluorescence in aortic cross-sections was determined with a confocal microscope (Olympus CKX41SF). Quantification of fluorescence was carried out on five animals per group, using ten sections of 10 μm.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

The fresh aortic tissue was homogenized at 20 Hz for 2 min in a Tissue Lyser II (Qiagen, Chatsworth, CA, USA). Total RNA from homogenates was isolated with an RNeasyPlus Micro Kit (Qiagen), according to the manufacturer's instructions. First-strand cDNA was prepared from total RNA by Quantitect Reverse Transcription Kit (Qiagen). Genomic DNA was removed as described by the manufacturer. To assess genomic DNA contamination, controls without reverse transcriptase were included. Oligonucleotide primers were designed based on the cDNA sequences reported in the GenBank database. Primers sets were the following:

-NOX2, sense: 5'- GCACCTGCAGCCTGCCTGAATT-3'

antisense: 5'-TTGTGTGGATGGCGGTGTGCA-3'

-β-actin, sense: 5'-CCCAAGGCCAACCGCGAGAAGAT-3'

antisense: 5'-GTCCCGGCCAGCCAGGTCCAG-3'

The measurement of mRNA was carried out using the LightCycler system (Bio Rad Laboratories, Philadelphia, PA, USA) and quantified by the ΔΔCT method; meanwhile, the statistical analysis (only for this technique) was performed using the REST software [16]. The mRNA levels were expressed relative to the endogenous control β-actin.

Statistical Analysis

Experimental data were expressed as mean ± standard error of mean (SEM). Statistical analysis was performed using the software SPSS version 18.0. For multiple comparisons, one-way ANOVA was used followed by Turkey post-hoc test. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Hypercholesterolemia in Female ApoE$^{-/-}$ mice

Using a commercial enzymatic kit, we detected that serum lipids in young and aged wild type mice remained at basal levels. In contrast, a significant increment ($P < 0.05$) of TC and VLDL/LDL in 8-week-old apoE$^{-/-}$ mice was observed, being this increment more dramatic in aged apoE$^{-/-}$ mice. Furthermore, there was a significant reduction ($P < 0.05$) of HDL levels in apoE$^{-/-}$ mice compared with C57 mice (Table 1).

Early Atherosclerotic Lesions Development in Female ApoE$^{-/-}$ Mice

The hematoxylin/eosin staining showed a normal morphology of the aortic wall in C57 mice. On the contrary, early atherosclerotic lesions were observed in 8-week-old apoE$^{-/-}$ female mice. The lesions were characterized by an intimal thickening and extracellular space enlargement. In aged apoE$^{-/-}$ mice, advanced lesions were developed. These results showed that atherosclerosis affects young apoE$^{-/-}$ females mice, which develop advanced atherosclerotic lesions with age.

Table 1. Serum lipid profile of C57BL6/J and apoE$^{-/-}$ female mice

Experimental groups	TC (mmol/l)	LDL/VLDL (mmol/l)	HDL (mmol/l)	TG (mmol/l)
8-week-old C57	14.36 ± 2.27^{a}	9.29 ± 0.78^{a}	8.21 ± 0.34^{a}	1.1 ± 0.09^{a}
44-week-old C57	12.51 ± 2.93^{a}	10.25 ± 1.02^{a}	8.85 ± 0.29^{a}	1.14 ± 0.07^{a}
8-week-old apoE$^{-/-}$	24.5 ± 1.34^{b}	16.43 ± 1.65^{b}	5.01 ± 0.38^{b}	1.49 ± 0.1^{b}
44-week-old apoE$^{-/-}$	36.29 ± 2.42^{c}	22.54 ± 0.93^{c}	4.79 ± 0.43^{b}	2.26 ± 0.11^{c}

Values are means ± SEM. **TC**, total cholesterol; **TG**, triglycerides; **HDLc**, high-density lipoprotein cholesterol; **LDL**, low-density lipoprotein cholesterol; **VLDL**, very low-density lipoproteins. Different letters within the same set denote statistical differences (P < 0.05).

Aortic Oxidative Stress in Early Atherosclerosis Lesions

The spectrophotometric assessment of oxidative stress biomarkers revealed a significant increment (P < 0.05) of lipid and protein oxidative damage in the aortic tissue of young apoE$^{-/-}$ mice, which incremented significantly (P < 0.05) in aged apoE$^{-/-}$ mice compared with C57. The activity of antioxidant enzymes SOD and CAT, as well as the GSH/GSSG ratio were significantly lower (P < 0.05) in 8- and 44-week-old apoE$^{-/-}$ mice than in wild type mice. On the other hand, the aortic levels of NO• in young apoE$^{-/-}$ mice diminished; meanwhile incremented in aged apoE$^{-/-}$ mice (P < 0.05) compared with the wild type animals. These findings suggest that aortic redox impairment represents an early event during atherosclerosis development in female apoE$^{-/-}$ mice.

Superoxide Anion Generation is Increased in the Aortic Tissue of Female ApoE$^{-/-}$ Mice

The aortic superoxide generation was determined in situ by DHE staining, which reacts with superoxide to form a fluorescent product, 2-hydroxyethidium [17]. Quantitative fluorescence evaluation of DHE-stained aortic sections revealed a significant increment (P < 0.05) of superoxide levels in the aortas of female apoE$^{-/-}$ mice compared with controls, even in the aortic tissue of 8-week-old apoE$^{-/-}$ mice.

Nox2 is Overexpressed in the Aortic Tissue of Female ApoE$^{-/-}$ Mice

To investigate the contribution of Nox2 to atherosclerosis development in 8- and 44-week-old apoE$^{-/-}$ we performed a RT-qPCR assay. In accordance with the increment of O_2•$^-$ generation, NOX2 mRNA levels were significantly increased (P < 0.05) in the aortic tissue of both, young and aged apoE$^{-/-}$ mice in comparison with C57 wild type mice.

DISCUSSION

Experimental and clinical data have demonstrated the pathogenic role of oxidative stress throughout atherosclerosis development, from endothelial dysfunction to the rupture of advanced atherosclerotic plaques [18-20]. However, the influence of gender and the protective effect of estrogens against oxidative stress and atherogenesis in females remains under controversial

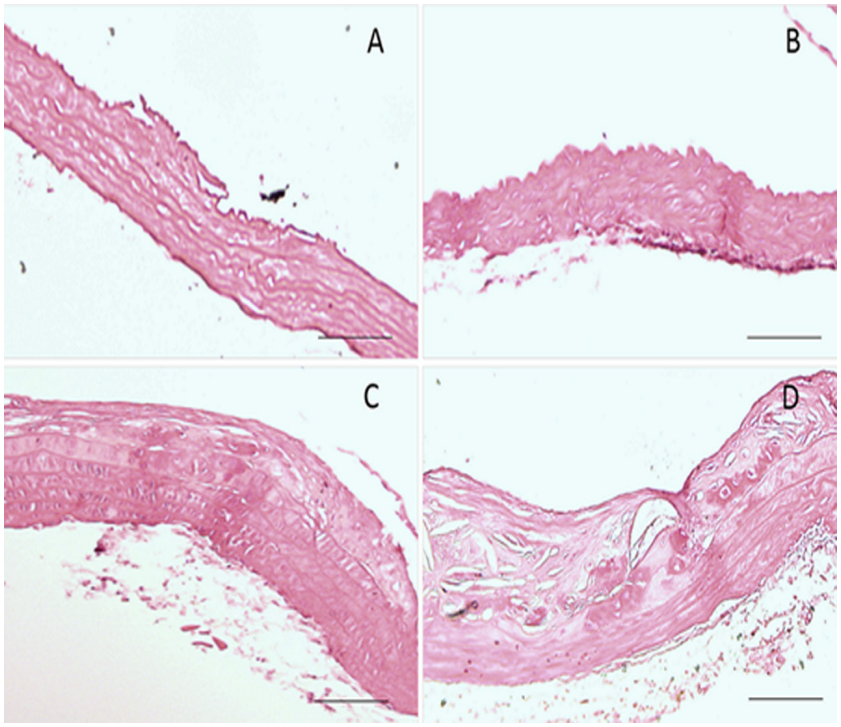

Figure 1. Histopathological analysis of aortic cross-sections.
Eosin-hematoxylin shows a normal morphology of the arterial wall in C57BL/6J female mice (**A**, **B**; n = 5). In contrast, an intimal thickening is observed in the aortas from 8- and 44-week-old apoE$^{-/-}$ female mice (**C**, **D**; represented by arrows). The lesions are characterized by a distortion in the vascular tissue architecture and extracellular spaces enlargement. In 44-week-old apoE$^{-/-}$ mice, these lesions are typical of advanced stages of atherosclerosis, showing high amount of cholesterol crystals. (20x magnification; scale bar = 50 µm)

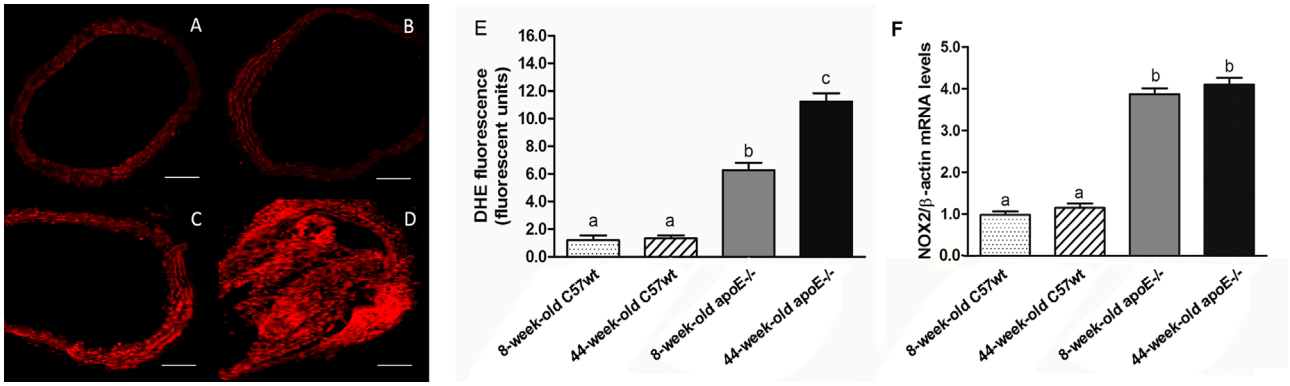

Figure 2. Detection of aortic superoxide anion generation and Nox2 gene expression in aortic cross-sections.
In situ dihydroethidium (DHE) staining of aortas and quantitative assessment of relative fluorescence levels shows a significant increment of superoxide generation in apoE$^{-/-}$ female mice (**C**, **D**) compared with C57 wild type mice (**A**, **B**). In panel **E**, the bars are the mean ± SEM of fluorescence units (n = 5). Besides, the RT-qPCR analysis shows an increased Nox2 expression in apoE$^{-/-}$ (**F**). In this panel, the bars are the mean ± SEM of relative Nox2/β-actin mRNA expression levels. Different letters denote statistical differences between groups (REST software; in all cases P < 0.05).

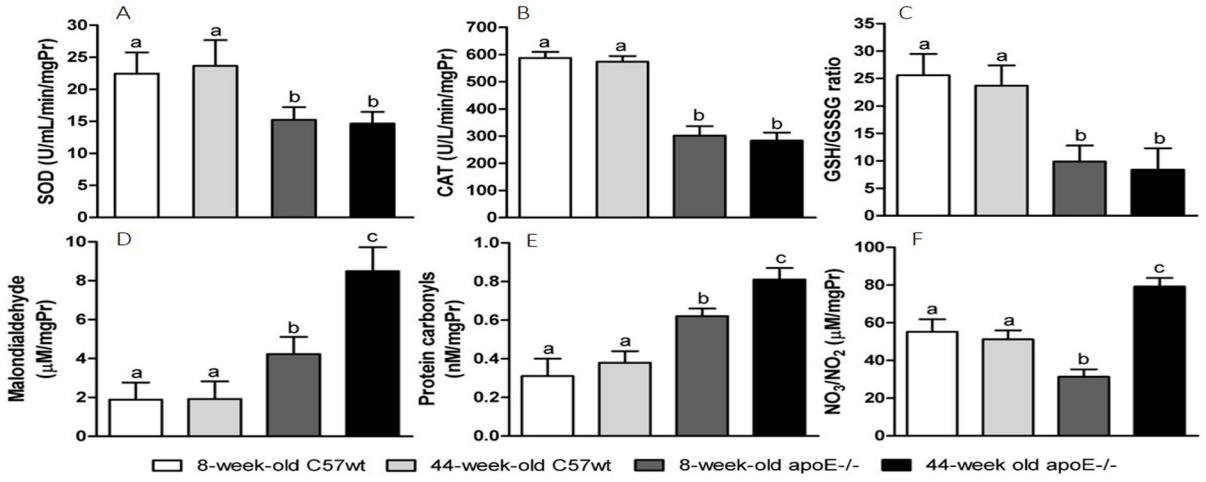

Figure 3. Behavior of aortic oxidative stress biomarkers and nitric oxide levels.
The spectrophotometric analysis shows an incremented susceptibility to lipid and protein oxidation (**D**, **E**; n = 5), together with a reduction of enzymatic and non-enzymatic antioxidant defenses in apoE$^{-/-}$ female mice compared with the C57 (**A-C**; n = 5). In addition, a disruption of nitric oxide availability was noted in the young and aged apoE$^{-/-}$ mice (**F**; n = 5). The values were expressed relative to protein concentration. Different letters denote statistical differences between groups (ANOVA, Turkey post-hoc test; in all cases P < 0.05).

discussion. The use of mice models of atherosclerosis show that during the fertile period, female mice are less prone to developing atherosclerotic lesions than males [21]. In contrast, other experimental evidences suggest that atherosclerotic lesions in apoE$^{-/-}$ mice (C57 genetic background) are greater in females than in males at comparable ages [9]. These contradictory observations have been associated with sex-dependent variations observed in the apoE$^{-/-}$ mouse, including the cellular immune response [8] and the content of apoA in HDL particles [22]. However, the contribution of vascular oxidative stress to the early atherosclerotic lesions development in female apoE$^{-/-}$ mice has been less explored.

In the present study, we show that 8-week-old apoE$^{-/-}$ mice developed atherosclerotic lesions, which progressed in aged animals. The development and progression of atherosclerotic lesions in young and aged apoE$^{-/-}$ mice was accompanied by a hyperlipidemic state. In the apoE$^{-/-}$ mouse, fed a chow diet, there is a shift in plasma lipoprotein profile, from HDL to predominantly VLDL, being the total cholesterol in the atherogenic lipoprotein fractions. When animals are supplemented with a hypercholesterolemic diet, the increment of proatherogenic lipoproteins fraction is even more dramatic [23]. In agreement with this report, a significant increase of LDL/VLDL fraction, as well as a diminishment of HDL levels was observed in 8- and 44-week-old apoE$^{-/-}$ mice.

The link between oxidative stress and atherosclerosis development has been established in the apoE$^{-/-}$ mice model [24]. The apoE deficiency-induced hypercholesterolemia promotes the overproduction of reactive oxygen species (ROS) contributing with atherogenesis [25]. However, the contribution of oxidative stress to the early atherosclerotic lesion formation in the females of apoE$^{-/-}$ mouse remain less studied. In the present work, using the DHE staining, we observed an increase of $O_2^{\bullet -}$ generation in the aortic cross-sections from the 8-week-old apoE$^{-/-}$ female mice compared with age-matched C57 animals. This result suggests a key role of superoxide generation in the early atherosclerotic

lesion development in apoE$^{-/-}$ female mice. Moreover, the fluorescence of DHE staining was more intense in the aortas of the 44-week-old apoE$^{-/-}$ mice. Thus, our finding identify the $O_2^{\bullet-}$ overproduction as a key event that contributes with the chronicity of oxidative stress and atherosclerosis progression in this animal model.

In the context of atherosclerosis, ROS overproduction and oxidative stress is largely determined by NADPH oxidases [26, 27]. The superoxide generation by NADPH oxidase family, has been implicated in endothelial dysfunction [28], LDL oxidation, activation of pro-inflammatory signaling pathways [29] and plaque instability and rupture [30]. The contributions of the specific vascular Nox subunits to atherogenesis are incompletely understood. However, it is well known that the development of human atherosclerotic lesions is associated with increased Nox2 expression [31]. Besides, in apoE$^{-/-}$ mice, deficiency of Nox2 results in ~50% less aortic lesion area [32]. Here, we demonstrated that Nox2 mRNA is overexpressed in the aortic tissue of 8-week-old apoE$^{-/-}$ mice. Furthermore, the progression of atherosclerotic lesion in 44-week-old apoE$^{-/-}$ mice was tightly associated with an increment of Nox2 expression and the subsequent superoxide generation. A limitation of our work was the impossibility to differentiate the relative contribution of vascular cell and inflammatory cell Nox2 to atherogenesis in this animal model. However, the present work provides clear evidences that Nox2, in general, play a key role in the pathogenesis of early atherosclerosis in female apoE$^{-/-}$ mice.

In response to $O_2^{\bullet-}$ overproduction, the antioxidant enzymes SOD and CAT were inactivated [33], as well as other antioxidant defenses such as GSH [28]. The GSH/GSSG ratio within cells is often used as an indicator of the global redox status [34]. In general, antioxidant mechanisms deprivation promoted the establishment of a prooxidant environment in which essential biomolecules were damaged. The increment of lipid and protein oxidation biomarkers, observed in the aortic tissue of 8-week-old apoE female mice, contributed with advanced atherosclerotic lesions formation in the aged apoE$^{-/-}$ mice.

At initial stages of atherosclerosis, $O_2^{\bullet-}$ radicals react rapidly with NO• leading to the formation of peroxynitrite ($ONOO^-$) and the loss of NO• bioavailability. Under this condition, eNOS switches from a NO• generating-coupled state to an uncoupled state in which oxygen is reduced to form $O_2^{\bullet-}$ [35], aggravating the vascular oxidative stress. As the disease progresses, the inducible isoform of NOS (iNOS) is overexpressed by pro-inflammatory macrophages in response to NO• deprivation and endothelial damage [36], resulting in a larger release of NO• with longer half-life. In turn, the excess of NO• might react with $O_2^{\bullet-}$ to form $ONOO^-$, contributing to endothelial dysfunction and atherosclerosis development [37]. This biphasic behavior of NO• was observed in our study, suggesting a central role of NO• metabolism disruption during atherosclerosis development and progression in the females of apoE$^{-/-}$ transgenic mouse.

In summary, our results show that vascular oxidative stress is an early event during atherogenesis in female apoE$^{-/-}$ mice. The increased oxidative stress is likely to be associated, at least, with the increment of Nox2-mediated ROS generation. In future studies, the behavior of redox signaling pathways should be addressed. Our results suggest that in presence of risk factors, such as hyperlipidemia, females are susceptible to atherosclerosis development even during the fertile period.

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REFERENCES

1. Perez-Lopez FR, Larrad-Mur L, Kallen A, Chedraui P, Taylor HS. Gender differences in cardiovascular disease: hormonal and biochemical influences. Reprod Sci 2010; 17:511-31.
2. Bellanti F, Matteo M, Rollo T, De Rosario F, Greco P, Vendemiale G, Serviddio G. Sex hormones modulate circulating antioxidant enzymes: impact of estrogen therapy. Redox Biol 2013; 1:340-6.
3. Miller AA, De Silva TM, Jackman KA, Sobey CG. Effect of gender and sex hormones on vascular oxidative stress. Clin Exp Pharmacol Physiol 2007; 34:1037-43.
4. Vasalle C, Sciarrino R, Bianchi S, Battaglia D, Mercuri A, Maffei S. Sex-related differences in association of oxidative stress status with coronary artery disease. Fertil Steril 2012; 97:414-9.
5. Lopes-Virella MF, Hunt KJ, Baker NL, Virella G, Moritz T. The levels of MDA-LDL in circulating immune complexes predict myocardial infarction in the VADT study. Atherosclerosis 2012; 224:526-31.
6. Leopold JA, Loscalzo J. Oxidative risk for atherothrombotic cardiovascular disease. Free Radic Biol Med 2009; 47:1673-706.
7. Meyrelles SS, Peotta VA, Pereira TMC, Vasquez EC. Endothelial dysfunction in the apolipoprotein E-deficient mouse: insights into the influence of diet, gender and aging. Lipids Health Dis 2011; 10:211-23.
8. Caligiuri G, Nicoletti A, Zhou X, Tornberg I, Hansson GK. Effects of sex and age on atherosclerosis and autoimmunity in apoE-deficient mice. Atherosclerosis 1999; 145:301-8.
9. Maeda N, Johnson L, Kim S, Hagaman J, Friedman M, Reddick R. Anatomical differences and atherosclerosis in apolipoprotein E-deficient mice with 129/SvEv and C57BL/6 genetic backgrounds. Atherosclerosis 2007; 195:75-82.
10. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976; 72:248-54.
11. Boehringer Mannheim. Biochemica information. A revised biochemical reference source. Enzymes for routine. Berlin, Germany, 1987.
12. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and non-protein sulfhydril groups in tissue with Ellman´s reagent. Anal Biochem 1968; 25:192-205.
13. Levine RL, Wehr N, Williams JA, Stadtman ER, Shacter E. Determination of carbonyl groups in oxidized proteins. Methods Mol Biol 2000; 99:15-24.
14. Granger DL, Taintor RR, Boockvar KS, Hibbs JB Jr. Determination of nitrate and nitrite in biological samples using bacterial nitrate reductase coupled with the Griess reaction. Methods companion. Methods Enzymol 1996; 7:78-83.
15. Miller FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. Circ Res 1998; 82:1298-305.
16. Pfafll MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucl Acids Res 2002; 30:e36.
17. Zhao H, Kalivendi S, Zhang H, Joseph J, Nithipatikom K, Vasquez-Vivar J, Kalyanaraman B. Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracelular fluorescence detection of superoxide. Free Radic Biol Med 2003; 34:1359-68.
18. Landmesser U, Hornig B, Drexler H. Endothelial function: a critical determinant in atherosclerosis? Circulation 2004; 109:II27-33.
19. Singh U, Jialal I. Oxidative stress and atherosclerosis. Pathophysiology 2006; 13:129-42.
20. Popolo A, Autore G, Pinto A, Marzocco S. Oxidative stress in patients with cardiovascular disease and chronic renal failure. Free Radic Res 2013; 47:346-56.
21. Bourassa PA, Milos PM, Gaynor BJ, Breslow JL, Aiello RJ. Estrogen reduces atherosclerotic lesion development in apolipoprotein E-deficient mice. Proc Natl Acad Sci USA 1996, 93:10022-7.
22. Surra JC, Guillen N, Arbones-Mainar JM, Barranquero C, Navarro MA, Arnal C, Orman I, Segovia JC, Osada J. Sex as a profound modifier of atherosclerotic lesion development in apolipoprotein E-deficient mice with different genetic backgrounds. J Atheroscler Thromb 2010, 17:712-21.
23. Plump AS, Smith JD, Hayek T, Aalto-Setälä K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell 1992; 71:343-53.
24. Letters JM, Witting PK, Christison JK, Eriksson AW, Pettersson K, Stocker R. Time-dependent changes to lipids and antioxidants in plasma and aortas of apolipoprotein E knockout mice. J Lipid Res 1999; 40:1104-12.
25. Prasad K, Kalra J. Oxygen free radicals and hypercholesterolemic atherosclerosis. Am Heart J 1993; 125:958-71.
26. Pignatelli P, Carnevale R, Cangemi R, Loffredo L, Sanguigni V, Stefanutti C, Basili S, Violi F. Atorvastatin inhibits gp91phox circulating levels in patients with hypercholesterolemia. Arterioscler Thromb Vasc Biol 2010; 30:360-7.
27. Violi F, Pignatelli P, Pignata C, Plebani A, Rossi P, Sanguigni V, Carnevale R, Soresina A, Finocchi A, Cirillo E, Catasca E, Angelico F, Loffredo L. Reduced atherosclerotic burden in subjects with genetically determined low oxidative stress. Arterioscler Thromb Vasc Biol 2013; 33:406-12.
28. Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. Arterioscler Thromb Vasc Biol 2005; 25:29-38.
29. Li H, Horke S, Forstermann U. Vascular oxidative stress, nitric oxide and atherosclerosis. Atherosclerosis 2014; 237:208-19.
30. Heistad DD. Oxidative Stress and Vascular Disease: 2005 Duff lecture. Arterioscler Thromb Vasc Biol 2006; 26:689-95.
31. Guzik TJ, Sadowski J, Guzik B, Jopek A, Kapelak B, Przybylowski P, Wierzbicki K, Korbut R, Harrison DG, Channon KM. Coronary artery superoxide production and Nox isoform expression in human coronary artery disease. Arterioscler Thromb Vasc Biol 2006; 26:333-9.
32. Judkins CP, Diep H, Broughton BR, Mast AE, Hooker EU, Miller AA, Selemidis S, Dusting GJ, Sobey CG, Drummond GR. Direct evidence of a role for Nox2 in superoxide production, reduced nitric oxide bioavailability, and early atherosclerotic plaque formation in ApoE−/− mice. Am J Physiol Heart Circ Physiol 2010; 298:H24-32.
33. Zhao R, Shen GX. Functional modulation of antioxidant enzymes in vascular endothelial cells by glycated LDL. Atherosclerosis 2005; 179:277-84.
34. Carelli S, Ceriotti A, Cabibbo A, Fassina G, Ruvo M, Sitia R. Cysteine and glutathione secretion in response to protein disulfide bond formation in the ER. Science 1997; 277:1681-4.
35. Miyoshi T, Li Y, Shih DM, Wang X, Laubach VE, Matsumoto AH, Helm GA, Lusis AJ, Shi W. Deficiency of inducible no synthase reduces advanced but not early atherosclerosis in apolipoprotein e-deficient mice. Life Sci 2006; 79:525-31.
36. Loscalzo J, Welch G. Nitric oxide and its role in the cardiovascular system. Prog Cardiovasc Dis 1995; 38:87-104.
37. Napoli C, de Nigris F, Williams-Ignarro S, Pignalosa O, Sica V, Ignarro LJ. Nitric oxide and atherosclerosis: An update. Nitric Oxide 2006; 15:265-79.

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Conflict of Interest: None declared