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Identification of the atherosclerosis phenotype *in vivo* by vascular duplex ultrasonography in ApoE-deficient dogs

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ABSTRACT

Background and aims: This study evaluated the atherosclerosis phenotype by vascular duplex ultrasonography (VDU) in ApoE-deficient dogs.
Methods: A total of 108 beagle dogs were examined by VDU, which included 32 wild-type, 68 heterozygous (ApoE-/+) mutant and 8 homozygous (ApoE−/−) mutant dogs. According to age, wild-type and ApoE-/+ dogs were divided into two subgroups: young (6–15 months) and adult dogs (18–29 months). All homozygous dogs were young dogs. Dogs were feed with normal diet. The plasma lipid levels were tested. The diameter of the common carotid artery (CCA), internal carotid artery (ICA), external carotid artery (ECA), abdominal aorta and common iliac artery (CIA) and the intima-media thickness (IMT) of the CCA and abdominal aorta were measured by VDU. The artery sections of ApoE−/− and control dogs were analyzed by histological analysis.
Results: The plasma triglycerides (2.5–3 fold), total cholesterol (4–5 fold) and LDL levels (35–40 fold) of ApoE−/− dogs were higher than those of the wild-type and ApoE-/+ dogs. Compared with the wild-type and young ApoE-/+ dogs, the IMT of CCA and aorta in ApoE−/− dogs were increased ($p<0.05$). The occurrence of atherosclerosis in ApoE−/− dogs was higher than that in ApoE-/+ dogs (50% *vs.* 10.3%, $p=0.013$) and the occurrence time was earlier. Histology confirmed that the aorta, carotid arteries and CIA had atherosclerotic lesions in ApoE−/− dogs.
Conclusions: The ApoE-deficient dogs were a reliable animal model of atherosclerosis. VDU is an optimal *in vivo* noninvasive method for evaluating atherosclerosis phenotypes in large animal models.

Introduction

Atherosclerosis is a progressive inflammatory disease characterized by the accumulation of lipids in the arterial vessel wall [1]. Atherosclerotic cardiovascular disease and stroke have been the first leading causes of death in humans worldwide [2]. The use of animal models of atherosclerosis is an essential tool to explore the molecular mechanisms behind atherosclerotic plaque formation and progression, as well as the occurrence of plaque rupture and its associated cardiovascular and cerebrovascular events. Moreover, animal models allow us to assess novel pharmacological treatments that can prevent or slow down the onset of atherosclerosis [3].

Over the past decades, mice have been widely used as an atherosclerosis model. ApoE (apolipoprotein E) deficient mice and LDL (low-density lipoprotein) receptor deficient mice have high plasma LDL levels and show the phenotype of atherosclerosis [4–6]. The mouse model plays an important role in demonstrating the molecular mechanisms of atherosclerosis. However, due to the difference between mice and humans in the anatomy and size of arteries, lipid profile, metabolism and the immune system, direct translation from mouse studies to humans is problematic [1,7].

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Generating a large animal model with atherosclerotic lesions more similar to humans is a new tool to further illustrate the mechanism of atherosclerosis, screen the pharmacological targets and even develop endovascular procedures [7].

In 2018, our study group successfully generated ApoE-deficient dogs (Canis familiaris) via a combination of embryo injection of CRISPR/Cas9 with somatic cell nuclear transfer [8]. In this study, the characteristics of atherosclerosis distribution patterns on the main arteries and the intima-media thickness alterations along with aging in ApoE-deficient dogs were further investigated by means of vascular duplex ultrasonography (VDU). Our study provided an *in vivo* noninvasive VDU method for evaluating atherosclerosis phenotype in a large animal model and established fundamental data for further application of the animal model.

Materials and methods

Experimental animals

A total of 108 beagle dogs were examined, which included 32 wild-type dogs (25 males, 7 females), 68 heterozygous (ApoE-/+) mutant dogs (42 males, 26 females) and 8 homozygous (ApoE−/−) mutant dogs (8 males, 0 females). According to age, the wild-type and ApoE-/+ mutant dogs were further divided into two subgroups: 6–15 months (young dogs) and 18–29 months (adult dogs). The age of all 8 homozygous dogs was within 6–15 months (young dogs).

All experimental animals were housed individually in 2 × 0.9 × 1.5 m (length × width × height) cages and exposed to a 12: 12 light-dark cycle (lights on at 0600 h). The humidity was 40–60% and the temperature was between 22 °C and 24 °C. They were allowed to have a 1-h free time in an open field twice a day, and were fed at 0900 h and 1700 h with a regular commercial dog diet (28% crude protein, 9.5% fat, 6.2% crude ash, 1.55% calcium, and 1.84% phosphorus; Bomei, Xingtai, China). Water was available from an automatic watering device. All experimental protocols and animal welfare were approved by the Animal Care and Use Committee of Beijing Sinogene Biotechnology Co., Ltd. (No. XNG-IAC-20200401) and the experiments conformed to the relevant regulatory standards.

The electrocardiogram and blood pressure are monitored by professional veterinarians during the anesthesia. Before anesthesia, the animals were tracheal intubated to prevent suffocation caused by respiratory secretions during anesthesia. The purpose of this study is to continuously monitor the time of the appearance of atherosclerosis phenotypes in animals and the growth and development of the plaques. Therefore, this experiment had no humane endpoints.

Generation of ApoE deficient dogs

ApoE deficient beagle dogs were generated by Cas9/sgRNA editing according to our previous study [8]. Estrus staging, mating, zygote collection, cytoplasmic injection, embryo transfer and pregnancy diagnosis were carried out following previous reports [8]. Briefly, a high gene editing efficiency sgRNA targeting the exon 3 of dog ApoE was selected (CCGGGTGGCAGACTGGCCAGCCC). A total of 65 zygotes were collected and microinjected with Cas9 mRNA and ApoE sgRNA. The injected embryos were transferred into 13 donor-recipient female dogs. Among them, 7 recipients were pregnant to term and gave birth to 13 puppies. PCR products amplified from the genome of all 13 puppies were sequenced. Among these 13 puppies, 2 were confirmed to have genetic mutations in the ApoE locus by DNA sequencing. One (numbered #161207) of the 2 gene-target puppies was male and was named "Apple" with a single mutation pattern of 34 bp deletion and 17 bp insertion at the target locus. All offspring were carried from "Apple" and wild-type females through natural mating, and they were genotyped for ApoE mutations with DNA extracted from the tail within one week after birth. The primers used to examine mutations were F1: GCAGGCTGGAAGATGAAGGTT, R1: GAGTCAGAGGGGTCCATAGCC, F2: CCTGGACCAGGGAGGCT and R2: CCAGGGCTGGCCAGTCT.

Blood test

Peripheral venous blood samples of fasting dogs were collected for biochemical tests. In this study, plasma lipid levels, including triglycerides, total cholesterol, apolipoprotein A1(ApoA1) and apolipoprotein B (ApoB, BioSino Bio-Technology & Science Inc. Beijing, China), LDL and high-density lipoprotein (HDL, Hitachi Chemical Diagnositics Inc. Tokyo, Japan) were tested by diagnostic kits. In addition, blood alanine transaminase (ALT), aspartate transaminase (AST, BioSino Bio-Technology & Science Inc. Beijing, China) and high-sensitivity C reactive protein (HsCRP, DiSys Diagnostic Systems GmbH, Holzheim, Germany) were measured by test kits.

Vascular duplex ultrasonography (VDU)

VDU was performed under general anesthesia with iv. propofol (4 mg/kg). A Hitachi Ascendus Ultrasound System (Hitachi, Inc., Tokyo, Japan) with a 4–8 MHz microcurvilinear array probe and a 3–7 MHz linear array probe and a Philips CX-50 ultrasound System (Philips Inc., Bothell, USA) were used to examine these dogs. The examinations were performed by experienced sonographers with more than 15 years of experience. The examinations followed up the vascular ultrasound protocols, previously described by Pellerito and Polak [9] and the vascular ultrasound examination guidelines published by the Chinese Medical Doctor Association of Ultrasonography [10]. The diameters of the common carotid artery (CCA), internal carotid artery (ICA), external carotid artery (ECA), abdominal aorta and common iliac artery (CIA) were measured in longitudinal axis view under two dimensional imaging. The most significant segments of atherosclerotic lesions were obtained by scanning with different beam directions. The IMT was obtained at the site with the maximum IMT along the artery wall yet did not reach the criteria for plaques. Plaques were defined as a focal increase in the IMT of 50% or greater compared with the surrounding wall thickness [11]. The IMT of the CCA and abdominal aorta of all involved dogs were routinely measured. The IMTs of the ICA, ECA and CIA were measured only when there was obvious IMT thickening. In addition, the peak systolic velocity (PSV) and end diastolic velocity (EDV) of these arteries were measured and the angle between the Doppler beam and the blood flow was set to be less than or equal to 60°. The sonographers were blinded to the genotypes of the examined dogs.

Histological analysis of arteries

An ApoE−/− dog (N0.191020) and a control dog were euthanized, and the arteries were removed and fixed for 48 h in 4% paraformaldehyde prepared in 1 × PBS (Na_2HPO_4 8 mM, NaCl 136 mM, KH $_2PO_4$ 2 mM, KCl 2.6 mM). Paraformaldehyde-fixed artery segments were embedded in paraffin, and 4-μm sections were stained with hematoxylin and eosin. Moreover, the artery sections were immunohistochemically stained with antibodies against α-smooth muscle actin for smooth muscle cells (ZM-0003, Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd). In addition, the aorta was opened and stained with oil red O solution. All

staining images were acquired with a Nikon Eclipse Ci microscope.

Statistical analysis

Statistical Package for Social Sciences (SPSS version 22.0) and GraphPad Prism 8.0 software were used for the statistical analysis.

The sample size of each group was determined by preliminary experiments. The mean IMTs of bilateral CCA of three young wild-type dogs and ApoE−/− dogs were used as the parameters for calculating the sample size (0.28±0.029 mm *vs.* 0.48±0.127 mm). The number ratio of ApoE−/− dogs to young wild-type dogs was set to 1:2. The calculated minimum sample size of ApoE−/− dogs was 8. The minimum young wild-type dogs was 16.

Numerical values were shown as the mean ± SEM. One-way ANOVA was used to compare the numerical variables among the groups and Tukey's multiple comparisons test was further used to compare the groups with each other. Fisher's exact test was used to compare the occurrence rate of the atherosclerosis phenotype between the ApoE-/+ and ApoE−/−groups. A $p<0.05$ was considered statistically significant.

Results

The blood test of ApoE deficient dogs

The age of young dogs in wild-type, ApoE-/+ and ApoE−/− groups had no difference and the ages of adult dogs in the wild-type and ApoE-/+ groups were similar.

As shown in Table 1, the plasma triglycerides (the mean values of it increased 2.5-3-fold), total cholesterol (the mean values of it increased 4-5-fold) and LDL levels (the mean values of it increased 35-40-fold) of ApoE−/− dogs were significantly higher than those of wild-type and ApoE-/+ dogs (all $p<0.05$). The HDL levels of ApoE−/− dogs were similar to those of wild-type and ApoE-/+ dogs ($p>0.05$). The lipid levels of ApoE-/+ dogs were not different from those of wild-type dogs ($p>0.05$). In addition, the AST of ApoE−/− dogs was higher than those of ApoE-/+ dogs (the mean values of it increased by 43%–50%, $p<0.05$). HsCRP levels were similar among the five groups ($p>0.05$).

The diameters and intima-media thickness of the arteries of ApoE-deficient dogs

In this study, we found that the diameters of the ECA of dogs were obviously larger than those of the ICA. As growth progressed, the diameters of CCA, ECA, CIA and aorta gradually increased in wild-type and ApoE-/+ dogs. Only in the ApoE-/+ dogs, did the diameters of ICA increase with growth. The diameters of all tested arteries in ApoE−/− dogs were not different from those of young wild-type and ApoE-/+ dogs (Fig. 1 and Supplementary Table 1).

However, compared with the young wild-type and ApoE-/+ dogs, the IMT of CCA (the mean values of it increased by 37%–77%) and aorta (the mean values of it increased by 55%–78%) in young ApoE−/− dogs were significantly increased ($p<0.05$). In addition, the IMT of the CCA and aorta in young ApoE−/− dogs was even larger than that of the adult wild-type dogs (the mean values of it increased by 50%–59%, $p<0.05$, Fig. 1 and Supplementary Table 1).

The occurrence of atherosclerosis in ApoE deficient dogs

We did not find any atherosclerosis phenotype in wild type dogs by ultrasound. Among the 68 ApoE-/+ dogs, 7 dogs (4 males and 3 females) showed atherosclerosis phenotype, with a maximum age of 29 months and a minimum age of 18 months. Among the 8 ApoE−/− dogs, 4 dogs (all males) showed an atherosclerosis phenotype, with a maximum age of 15 months and a minimum age of 9 months. The occurrence of atherosclerosis in ApoE−/− dogs was significantly higher than that in ApoE-/+ dogs (50% *vs.*10.3%, $p=0.013$) and the occurrence time of the atherosclerosis phenotype was earlier. The atherosclerotic lesions of these dogs in detail were listed in Table 2.

Vascular ultrasound images of an ApoE−/− dog (No. 191020) showed obvious atherosclerotic lesions (including IMT thickening and atherosclerotic plaques) in the carotid artery, CIA and abdominal aorta (Fig. 2). One month after VDU examination, the limbs of the dog showed severe gangrene (Fig. 3). Due to severe progressive gangrene and not recovered by medicines, the ApoE−/− dog (No. 191020) was euthanized for histological analysis. The of aorta arch, thoracic aorta and abdominal aorta in the ApoE−/− dogs showed obvious atherosclerosis compared with a wild type of the same age and sex. HE staining of the carotid arteries and CIA showed significant atherosclerotic plaques (Fig. 3), which were consistent with the findings of ultrasound. In addition,

Table 1
Age and blood biochemistry test of dogs with the three genotypes.

	Wild type young (N=19)	Wild type adult (N=13)	ApoE-/+ young (N=32)	ApoE-/+ adult (N=36)	ApoE−/−young (N=8)	F	*P*
Age (month)	8.95±0.52	23.15±1.19	7.81±0.23	20.7±0.81	9.63±1.70	88.26	<0.001
ALT(IU/L)	36.6±2.77	45.7±7.2	37.8±2.31	34.9±1.49	43.6±8.11	1.633	0.177
AST(IU/L)	34.3±2.20	37.1±6.79	32.6±0.89	30.2±1.27	49.0±9.35[a,b]	3.894	0.006
Triglycerides (mmol/L)	0.53±0.08	0.59±0.09	0.45±0.026	0.42±0.04	1.36±0.41[a,b,c,d]	10.23	<0.001
Total cholesterol (mmol/L)	4.85±0.18	5.08±0.23	4.29±0.15	4.57±0.13	20.8±3.13[a,b,c,d]	82.28	<0.001
HDL (mmol/L)	3.89±0.19	4.49±0.14	3.85±0.092	3.94±0.099	3.70±0.32	2.875	0.025
LDL(mmol/L)	0.34±0.04	0.46±0.10	0.37±0.050	0.32±0.040	13.44±2.63[a,b,c,d]	82.6	<0.001
ApoA-1(g/L)	0.44±0.03	0.28±0.01	0.34±0.009	0.42±0.018	0.44±0.04	9.549	<0.001
ApoB (g/L)	0.02±0.01	0.015±0.002	0.02±0.002	0.01±0.001	0.02±0.002	1.654	0.167
HsCRP(mg/L)	0.05±0.015	0.05±0.017	0.06±0.010	0.06±0.015	0.04±0.011	0.222	0.926

Note: ALT: alanine transaminase; AST: aspartate transaminase; LDL: low density lipoprotein; HDL: high density lipoprotein; ApoA1: apolipoprotein A1; ApoB: apolipoprotein B. HsCRP: high-sensitivity C reactive protein.
F values and *p* values: One-way ANOVA analysis among the five groups.
Tukey's multiple comparisons test.
[a] Compared with young ApoE-/+ group, $p<0.05$.
[b] Compared with adult ApoE-/+ group, $p<0.05$.
[c] Compared with young wild-type group, $p<0.05$.
[d] Compared with adult wild-type group, $p<0.05$.

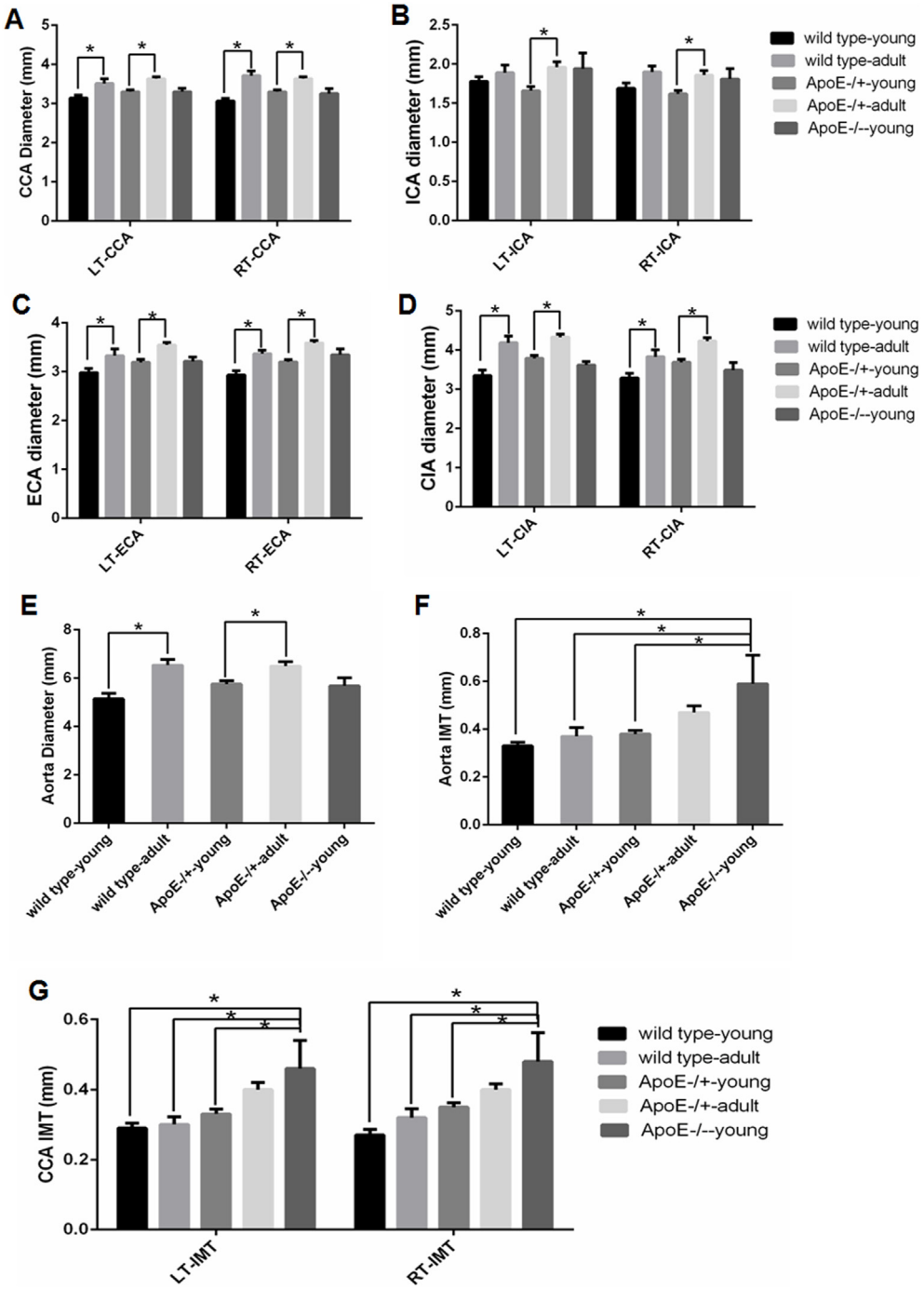

Fig. 1. Comparison of the diameter of the CCA (A), ICA (B), ECA (C), CIA (D) and abdominal aorta (E) as well as the IMT of the abdominal aorta (F) and CCA (G) among dogs with the three genotypes.
Note: LT: left side; RT: right side; CCA: common carotid artery; ICA: internal carotid artery; ECA: external carotid artery; CIA: common iliac artery; IMT: intima-media thickness. *P<0.05.

immunohistochemistry staining showed that the atherosclerotic lesions had smooth muscle cells migration and proliferation (Fig. 3), which was similar to the lesions of humans.

Discussion

In vivo evaluation and validation of atherosclerosis phenotype is important to study the progression of atherosclerosis and its underlying mechanisms in large animal model, is essential to dynamic observation of drug effects during drug development process, and is help to evaluate the efficacy of endovascular procedures [7]. As a noninvasive, economic, repeatable method, VDU is an ideal tool to evaluate the atherosclerosis phenotype in large animal models. In this study, we found that the incidence rate of the atherosclerosis phenotype in ApoE−/− dogs was much higher than that in ApoE-/+ dogs and that the occurrence time was earlier. The atherosclerosis was distributed on the main arteries, including the CCA, ICA, ECA, aorta and CIA, which are also the vulnerable sites for

Table 2
Detailed atherosclerosis phenotype of ApoE deficient dogs.

No.	genotype	age (month)	sex	atherosclerosis distribution	IMT and plaques information
191020	ApoE−/−	15	male	aorta, carotid artery and CIA	Bi-CCA IMT 1.0 mm, Aorta IMT 1.3 mm Plaques: RCCA (3.3 × 1.1 mm), LICA (5.2 × 0.9 mm) hypoechoic plaques; RECA (8.6 × 1.2 mm and 8.9 × 1.0 mm) isoechoic plaques. Bi-ICA,RECA and Bi-CIA stenosis <50%
200510	ApoE−/−	9	male	right carotid artery	RCCA IMT 0.6 mm, Plaques: RCCA (5.8 × 0.7 mm), RICA (4.9 × 0.7 mm) isoechoic plaques.
191077	ApoE−/−	15	male	RCIA	RCIA IMT 0.5 mm
200210	ApoE−/−	12	male	LCIA	LCIA IMT 0.6 mm
191009	ApoE-/+	18	male	Aorta	Plaques: aorta (6.1 × 0.8 mm) isoechoic plaque.
191006	ApoE-/+	18	female	RCCA, aorta, and LCIA	Plaques: RCCA (3.5 × 0.7 mm and 3.9 × 0.8 mm), aorta (8.2 × 1.1 mm), LCIA (3.5 × 0.9 mm) isoechoic plaques
181011	ApoE-/+	29	male	carotid artery and LCIA	RCCA IMT 0.6 mm, LCIA IMT 0.9 mm LCIA (6.4 × 0.9 mm) isoechoic plaques
181016	ApoE-/+	28	female	LECA	LECA IMT 0.4 mm
181014	ApoE-/+	29	male	RCCA and aorta	RCCA IMT 0.5 mm, aorta IMT 0.7 mm
181007	ApoE-/+	29	female	Aorta	aorta IMT 0.6 mm
181021	ApoE-/+	29	male	RCCA	RCCA IMT 0.5 mm

atherosclerosis in humans. By a relatively large sample study, these findings further confirmed that the ApoE-deficient dogs were a reliable animal model of atherosclerosis.

Although the Echocardiography Committee of the Specialty of Cardiology, American College of Veterinary Internal Medicine published a recommendation for standards in transthoracic two-dimensional echocardiography in dogs and cats in 1993 [12], there were few large-sample studies related to the normal reference values of diameter and IMT of main arteries in dogs. By using the wild-type dogs as a normal control, we first established the normal reference values of diameter and IMT of the main arteries during the young (6–15 months) and adult periods (18–29 months) and found that along with growth, the diameters of CCA, ECA, CIA and aorta gradually increased in wild-type dogs. In addition, the IMT of CCA in wild type beagle dogs was approximately 0.3 mm, which was similar to the Tian's study on carotid proliferative plaque formation in a canine model of chronic hypertension (beagle dogs, n=5 for each group, age 12–15 months) [13].

Then, we found that compared with the young wild type dogs and ApoE-/+ dogs, the IMT of the CCA and aorta in young ApoE−/− dogs was significantly increased. In addition, the IMT of the CCA and aorta in young ApoE−/− dogs was even larger than that of adult wild-type dogs. It is well known that IMT thickening is the early stage of atherosclerosis that can be measured accurately by ultrasound. Moreover, ApoE−/− dogs demonstrated the atherosclerotic plaques similar to that of human plaques, as evaluated by ultrasound, which was also confirmed by pathological results. All these findings showed that ApoE deficient dogs can demonstrate not only an early stage of atherosclerosis (IMT thickening) but also advanced atherosclerotic plaques (with amount smooth muscle cells accumulation) similar to humans, and the phenotype might be more obvious along with aging.

With respect to the atherosclerosis distribution, the location of the atherosclerosis in ApoE and LDL receptor-deficient mice mostly occurred in the aortic root with fewer lesions on the carotid artery and iliac artery, which are locations prone to atherosclerosis in humans [14]. As demonstrated in this study, the atherosclerosis distribution pattern in ApoE-deficient dogs was on CCA, ICA, ECA, aorta and CIA, even in a dog (No. 191020) with lumen stenosis <50% in the ICA、ECA and CIA, which is more similar to the human lesion pattern.

In 2018, Fang et al. reported the establishment of an ApoE-deficient model in miniature pigs [15]. There were several differences between the present dog model and the miniature pig model. First, the plasma lipid level elevated folds were different. The miniature pigs only exhibited moderately increased plasma cholesterol levels with a regular chow diet (with total cholesterol and LDL levels elevated approximately 2-fold compared with the wild-type control). However, the present ApoE-deficient dogs exhibited severely increased plasma total cholesterol (4-5-fold) and LDL levels (35-40-fold), with the latter increased fold comparable to the that in miniature pigs fed a high-fat diet. As we know, establishing a spontaneous animal model of atherosclerosis under a regular chow diet is more economical, especially in large animal models. Second, Fang et al. only described the atherosclerotic lesions distributed at the coronary artery and aorta. While in this study, by using VUS and confirmed by histology, we found that the lesions not only distributed in the aorta but also in the carotid artery and iliac artery, which are locations prone to atherosclerosis in humans. Third, the sample number of this study was larger than that of Fang. et al.'s study (wild-type n=8, and ApoE−/− miniature pigs n=4).

More importantly, the atherosclerosis model of dogs will provide large enough vessels and appropriate lesions for novel endovascular procedure validation, noninvasive measurements of atherosclerotic lesions, and invasive angiogram or measurements. Actually, dogs have been used to establish several experimental models of cardiovascular and cerebrovascular diseases, such as chronic hypertension, stroke, mechanical thrombectomy in stroke and carotid dissecting aneurysms [13,16–18].

High levels of plasma triglycerides, total cholesterol and LDL levels were considered to be involved in the development of atherosclerotic lesions in ApoE−/− dogs. Unlike humans, HDL is the most abundant lipoprotein in wild-type dogs [19]. The baseline plasma triglycerides and total cholesterol measured in this study were in consistent with the Tian's study [13]. However, in this present study, the LDL level in ApoE −/− dogs was approximately 35-40-fold that in wild-type dogs and became the main lipoprotein in plasma. In addition, the triglycerides and total cholesterol almost were elevated 2.5-3- fold and 4-5-fold, respectively, in ApoE −/− dogs.

It should be noted that, although the total cholesterol and LDL

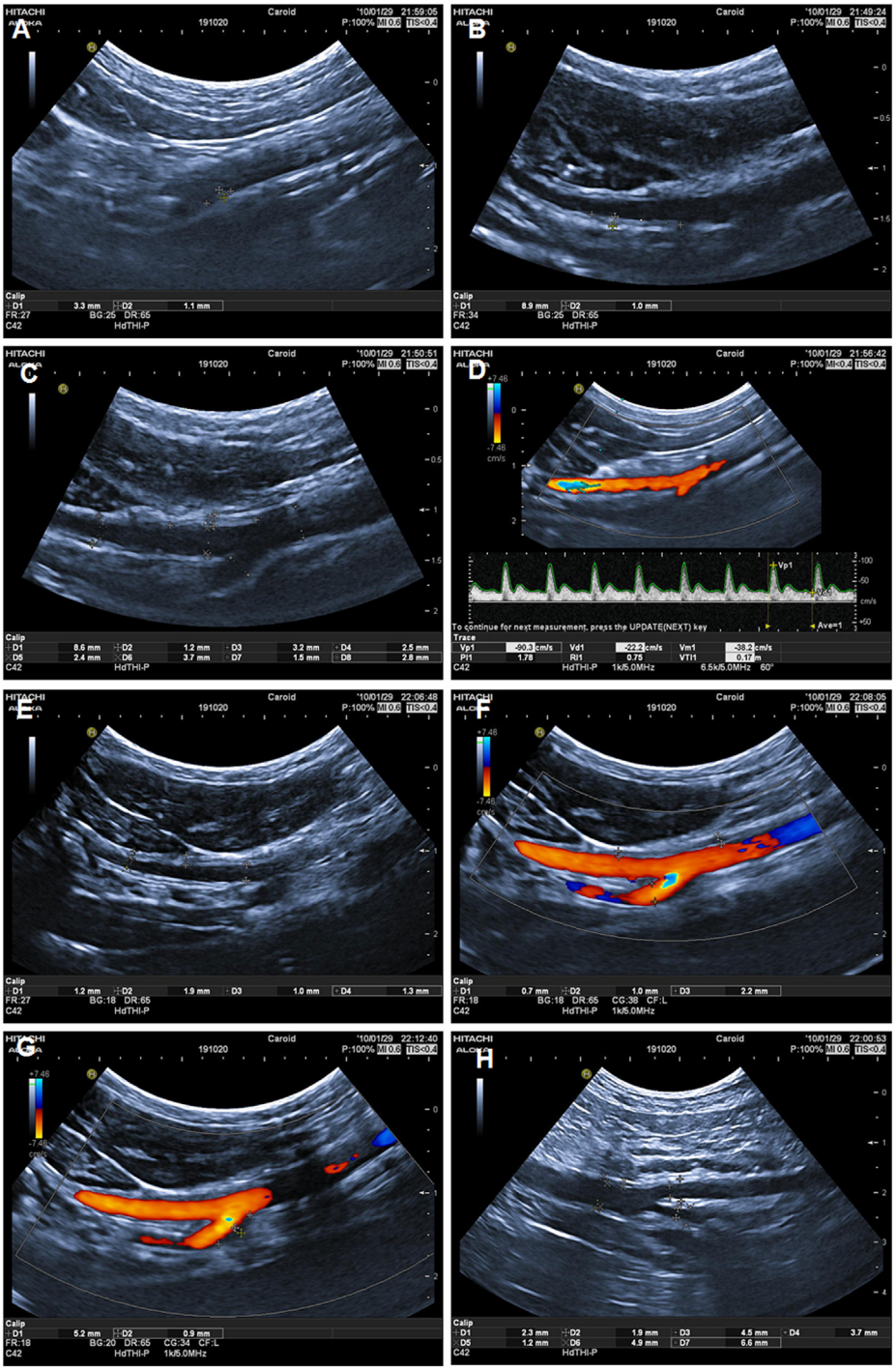

Fig. 2. Vascular duplex ultrasound images of an ApoE−/− dogs (No. 191020). (A–C) 2D image of right carotid artery. (A) Hypoechoic plaque (3.3 × 1.1 mm) at the posterior wall of the right CCA. (B) An isoechoic plaque (8.9 × 1.0 mm) at the posterior wall of the distal of right ECA. (C) An isoechoic plaque (8.6 × 1.2 mm) at the anterior wall of the distal of right ECA. The residual diameter and the original diameter of the proximal right ECA were 2.4 mm and 3.7 mm, respectively. The residual diameter and the original diameter of the distal right ECA were 1.5 mm and 2.8 mm, respectively. (D) The spectrum of the right ECA showed a PSV of 90.3 cm/s and EDV of 22.2 cm/s (E) 2D image of left carotid artery. The IMT and diameter of the proximal left ECA were 1.2 mm and 1.9 mm. The IMT and diameter of the distal left ECA were 1.0 mm and 1.3 mm, respectively. (F) The color Doppler images of left ECA. The IMT of left CCA was 1.0 mm. (G) A hypoechoic plaque (5.2 × 0.9 mm) at the posterior wall of the distal of left ICA. (H) 2D image of abdomen aorta and CIA. The IMT of the abdominal aorta was 1.2 mm. The residual diameter and original diameter of the aorta were 4.9 mm and 6.6 mm, respectively. The residual diameter and original diameter of the left CIA were 1.9 mm and 2.3 mm, respectively. The residual diameter and original diameter of the right CIA were 3.7 mm and 4.5 mm, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

levels in ApoE-/+ dogs were not different from those of wild-type dogs, a very small portion of these heterozygous dogs (10.3%) still showed an atherosclerotic phenotype, although this phenotype occurred later and the portion was significantly lower than that of ApoE −/− dogs. The IMT of the CCA and aorta of ApoE-/+ dogs was slightly higher than those of wide-type dogs, but had no statistical significance, which might due to the sample size. The incidence of the atherosclerosis phenotype in ApoE-/+ dogs needs further

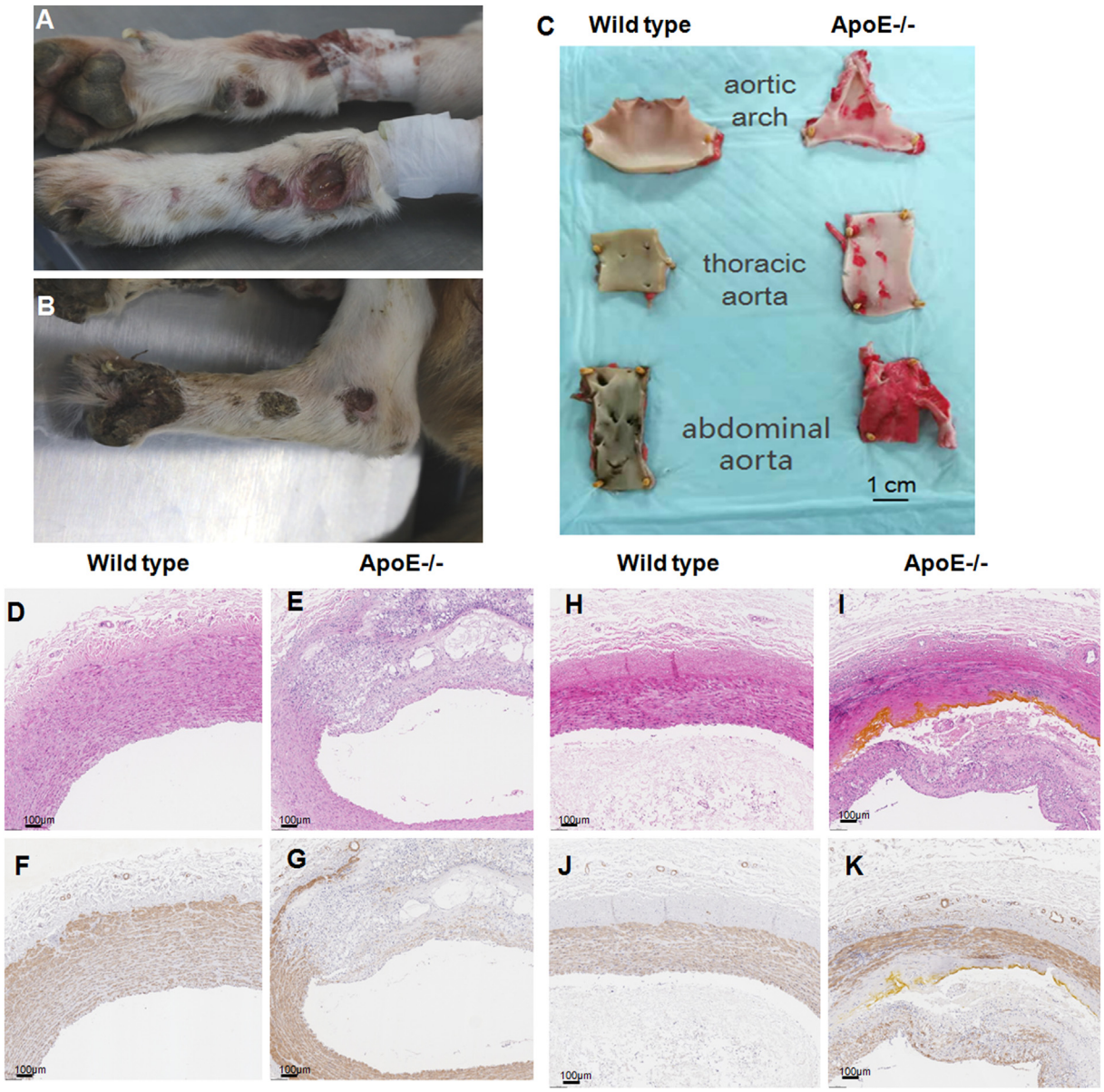

Fig. 3. Photos of limbs, specimens of aortas and immunohistochemistry analysis carotid arteries and common iliac arteries of an ApoE−/− dog (No. 191020) and a wild-type control dog. (A) The bilateral anterior limbs showed obvious ulcer. (B) The left posterior limb showed gangrene. (C) Oil red O staining of the aorta arch, thoracic aorta and abdominal aorta in an ApoE−/− dog showed obvious atherosclerosis compared with a wild-type dog of the same age and sex. (D) and (F) HE staining and α-smooth muscle actin immunohistochemical staining of carotid arteries of a wild-type dog. (E) and (G) HE staining and α-smooth muscle actin immunohistochemical staining of carotid arteries in an ApoE−/− dog showed significant atherosclerotic plaque. (H) and (J) HE staining and α-smooth muscle actin immunohistochemical staining of common iliac arteries arteries of a wild-type dog. (I) and (K) HE staining and α-smooth muscle actin immunohistochemical staining of common iliac arteries in an ApoE−/− dog showed significant atherosclerotic plaque. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

investigation, such as giving these heterozygous dogs with a high-fat diet to evaluate whether they can show higher plasma lipid levels and higher proportion of atherosclerosis. In addition, we found that the HDL level of adult ApoE-/+ dogs with atherosclerotic lesions was lower than that of adult ApoE-/+ dogs without atherosclerotic lesions (3.45±0.19 mmol/L *vs.* 4.06±0.10 mmol/L, t=2.597, p=0.014), whereas the other plasma lipids such as triglycerides, total cholesterol and LDL levels were similar (data not shown). Moreover, ApoE is an important mediator of macrophage cholesterol efflux [20]. Whether HDL differences and/or macrophage cholesterol efflux capacities contribute to the occurrence of atherosclerosis phenotype in ApoE-/+ dogs still need further investigation.

The limitation of the study was the relatively small sample size of ApoE −/− dogs (n=8), compared with wild type and ApoE-/+ dogs, which could not be categorized into young and adult groups. We will still follow-up these dogs by VUS to observe the lesion progression and the continuously occurrence of an atherosclerosis phenotype along with aging. In addition, due to the relative small size of arteries, we did not evaluate the coronary arteries by ultrasound. However, in the autopsy of No.191020, we did find the atherosclerosis in the coronary artery (data not shown), which was similar to the findings in the pig models [15,21] and lesions occurred in human patients.

In conclusion, in this study, we evaluated the characteristics of the atherosclerosis phenotype and its panvascular distribution

patterns in ApoE deficient dogs fed with a regular diet. The results further confirmed that the ApoE deficient dog was a reliable animal model of atherosclerosis. In addition, this study provided an *in vivo* noninvasive ultrasound imaging method for evaluating atherosclerosis phenotypes and established the fundamental data for further application of the animal models.

Authorship contribution statement

Lingyun Jia: Investigation, Statistical analysis, Writing-original draft, Writing-review & editing. **Yuan Li:** Investigation, Writing-original draft, Writing-review & editing. **Yang Hua:** Conceptualization, Investigation, Writing-review & editing, Project administration. **Yumei Liu,** Investigation, Writing-review & editing. **Nan Zhang,** Investigation, Writing-review & editing. **Mingjie Gao** Investigation, Writing-review & editing. **Ke Zhang,** Investigation, Writing-review & editing. **Jingzhi Li,** Investigation, Writing-review & editing. **Jidong Mi**, Investigation, Writing-review & editing, Supervision. **Jianqi Zhang** Investigation, Writing-review & editing. **Shiyu Jiao, Investigation, Immunohistochemistry experiments.**

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.athplu.2021.12.001.

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