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Subcutaneous administration of casein attenuates atherosclerotic progression in male apoE$^{-/-}$ mice fed with high-fat diet

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Abstract

The impact of casein on atherosclerotic lesion progression remains controversial. In this study, we tested the effect of casein on atherosclerotic development and its potential mechanisms in male apolipoprotein E knockout (apoE$^{-/-}$) mice fed with high-fat diet (HFD). Male apoE$^{-/-}$ mice fed with HFD were randomized into HFD group (subcutaneous injection with 0.5 ml of 0.9% sodium chloride daily, $n = 6$) and HFD + Casein group (subcutaneous injection with 0.5 ml of 10% casein daily, $n = 6$). Body weight was recorded at baseline and once a week thereafter. After 12 weeks of treatment, plasma lipid and inflammatory markers, and histological characterization of atherosclerotic plaques in the aortic arch and aortic sinus were analyzed. There was no significant difference in weight gain between the two groups after 12 weeks of treatment. Plasma levels of total cholesterol (TC), triglyceride (TG), and low-density lipoprotein cholesterol (LDL-C) were significantly lower, while high-density lipoprotein cholesterol (HDL-C) level tended to be higher in the HFD + Casein group compared with the HFD group. The positive percentages of atherosclerotic lesions in aortic arch and aortic sinus as well as collagen deposition in aortic sinus plaques were significantly lower in the HFD + Casein group compared with the HFD group. Plasma levels of interleukin (IL)-1β and granulocyte-macrophage colony-stimulating factor (GM-CSF) were also significantly lower in the HFD + Casein group compared with the HFD group. In conclusion, subcutaneous administration of casein attenuates atherosclerotic lesion progression, possibly through decreasing fibrosis and inflammatory responses in male apoE$^{-/-}$ mice fed with HFD.

KEYWORDS
atherosclerosis, Casein, fibrosis, inflammation

1 | INTRODUCTION

Atherosclerosis progression could be modified by various dietary proteins, either positively or negatively (Torres, Guevara-Cruz, Velazquez-Villegas, & Tovar, 2015). As an important source of protein in the diet, the physiological role of milk protein has attracted increasing research interest beyond its nutritional value. It was reported that angiotensin-I-converting enzyme (ACE) inhibitory peptides existed in the amino acid sequences of milk protein (Saito, 2008), which could improve vascular elasticity and reduce

blood pressure in spontaneously hypertensive rats (SHR) or hypertensive adults (Cadee et al., 2007; Sanchez et al., 2011; Xu, Qin, Wang, Li, & Chang, 2008). It was also found that milk protein intake could improve hyperglycemia by stimulating insulin secretion in individuals with metabolic syndrome or type 2 diabetes mellitus (T2DM) (McGregor & Poppitt, 2013; Pasin & Comerford, 2015). Moreover, milk protein was shown to exert antiobesity effects in diet-induced obese male C57BL/6J mice (Lillefosse et al., 2013). In addition, a meta-analysis of prospective cohort studies suggested that consumption of milk or milk products was negatively correlated with incidence of stroke (Elwood, Pickering, Givens, & Gallacher, 2010).

Casein comprises approximately 80% of the total milk protein (Dalziel, Young, McKenzie, Haggarty, & Roy, 2017), which is not only naturally present in dairy, but also popularly utilized as a protein supplement in its purified form of various nutrition products. Casein is often applied as an animal source of protein in experimental research as well. Till now, the role of casein in human health, especially on the lipid metabolism remains an issue of debate. Huff, Roberts, and Carroll (1982) found that casein enhanced triglyceride (TG) levels in plasma and atherosclerotic lesions development in the aortic arch region in male New Zealand white rabbits. Similarly, Damasceno et al. (2001) reported that casein feeding contributed to increasing plasma cholesterol and TG concentrations and the area of aorta atherosclerotic lesions in adult male New Zealand rabbits, while Ijaz et al. (2018) showed that casein diet for 12 weeks did not affect the lipid profile in male C57BL/6J mice when consumed with either low-fat diet (LFD) or high-fat diet (HFD). Another study conducted by Meinertz, Nilausen, and Faergeman (1990) suggested that the low-cholesterol or cholesterol-enriched casein diets could improve plasma lipid concentrations in healthy participants.

It is well known that inflammation is a key factor in the progression of atherosclerosis (Hedin & Matic, 2018). It was demonstrated that chronic systemic inflammation, characterized by the increase of serum levels of C-reactive protein (CRP) and proinflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin (IL)-6, was closely related to pathogenesis of multiple metabolic disorders including insulin resistance, T2DM, obesity, and dyslipidemia in both humans and animal models (Burhans, Hagman, Kuzma, Schmidt, & Kratz, 2018; Cox, West, & Cripps, 2015; Lira, Rosa Neto, Antunes, & Fernandes, 2014; Lontchi-Yimagou, Sobngwi, Matsha, & Kengne, 2013). Studies over the last several decades suggested that milk proteins had immunomodulatory effects but the results were mixed. It was reported that κ-casein suppressed lymphocyte proliferation induced by T- and B-cell mitogens (Cross & Gill, 2000). Panagiotakos, Pitsavos, Zampelas, Chrysohoou, and Stefanadis (2010) found a decreased level of inflammatory markers associated with the consumption of dairy products among healthy adults. However, Pal and Ellis (2010) demonstrated that casein supplementation had no significant influence on plasma inflammatory markers in overweight and obese individuals. This finding is in accordance with the study conducted by Bohl, Bjornshave, Gregersen, and Hermansen (2016) which

showed that casein protein did not alter circulating inflammatory markers in abdominally obese adults. Till now, the effect of casein on atherosclerotic progression and its potential mechanism are not fully understood. In this study, we tested the impact of casein on the atherosclerotic development in apolipoprotein E knockout (apoE$^{-/-}$) mice fed with HFD (1.25% cholesterol).

2 | MATERIALS AND METHODS

2.1 | Animals and study groups

Six-week-old male apoE$^{-/-}$ mice on C57BL/6 background were purchased from The Animal Center of Beijing University and kept under standard laboratory conditions (12 hr light cycle, temperature 25°C), with free access to standard chow and drinking water. After 2 weeks of acclimatization, the mice were weighed and randomly divided into HFD group ($n = 6$, subcutaneous injection with 0.5 ml of 0.9% sodium chloride daily) and HFD + Casein group ($n = 6$, subcutaneous injection with 0.5 ml of 10% casein daily) and fed with HFD for 12 weeks. Male mice were chosen in this study to avoid the potential impact of periodic sex hormone fluctuation on atherosclerosis. Subcutaneous injection was used to evaluate the effect of casein in a fixed dose range. This study was approved by the Animal Care Committees of Wuhan Fourth Hospital. Experimental protocol was based on the ARRIVE guidelines and guidelines from the National Institutes of Health for animal care and use (NIH Publications No. 85–23, revised 1996).

2.2 | Harvesting of tissue

Body weight was recorded at baseline and once a week thereafter for 12 weeks. After 12 weeks of treatment, the mice were anesthetized with pentobarbital sodium (40 mg/kg) after 6 hr of fasting. Blood samples were collected from retro-orbital plexus into heparin-coated tubes and then centrifuged to separate plasma. Both the heart and whole aorta were rapidly removed. All samples and tissues were snap-frozen and stored at −80°C until analysis.

2.3 | Measurements of lipid and inflammatory markers

The plasma lipid profiles of total cholesterol (TC), TG, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) were measured using commercially available kits (Nanjing Jiancheng Bioengineering Institute, A111-1, A110-1, A113-1, A112-1, Nanjing, China). The absorbance was recorded at 510 nm for TC and TG, and 550 nm for LDL-C and HDL-C.

Plasma levels of IL-1β, IL-6, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were analyzed using the ELISA kits (Neobioscience, EMC111, Shenzhen, China) according to the manufacturer's protocols. Plasma CRP level was measured with the

ELISA kit (Elabscience, Wuhan, China) following the manufacturer's instructions. The absorbance was recorded at 450 nm.

2.4 | Histological characterization of aortic arch and aortic sinus

Method used to evaluate the atherosclerosis in this model was identical as the method we used previously (Gao et al., 2015). The aortic arch was dissected and opened longitudinally. The atherosclerotic lesions of aortic arch were quantified by en face Oil Red O staining and the percentage of Oil Red O positive area was measured using Image-Pro Plus software (IPP, Media Cybernetics, Inc., Rockville, MD, USA).

Each heart was cut along with a horizontal plane between the lowest tips of the right and left atria. The upper portion was snap-frozen in O.C.T. compound (Tissue-Teck, 4,583, Torrance, U.S.A). Serial cryosections (7 μm thick) of the aortic sinus, spanning the three cusps of the aortic valves, were cut. Atherosclerotic lesions and collagen contents in plaques were determined by Oil Red O staining and Masson's Trichrome staining, respectively. Images were captured by microscope (Leica, DMi8, Germany) with 40 magnifications, and the stained area was measured using IPP software.

2.5 | Statistical analysis

Data were expressed as mean ± standard deviation (SD). Normal distribution was assessed by the Kolmogorov–Smirnov test. Unpaired Student's t test was used to evaluate statistical differences between groups. A value of $p < .05$ was considered statistically significant. All analyses were performed using SPSS 21.0 software (SPSS Inc., Illinois, USA).

3 | RESULTS

3.1 | Body weight and plasma lipid parameters of HFD group and HFD + Casein group

Baseline and final body weight as well as body weight gain were similar between the HFD group and HFD + Casein group (Table 1, Figure 1). Plasma levels of TC, TG, and LDL-C were significantly lower, while HDL-C level tended to be higher in the HFD + Casein group compared with the HFD group (Table 1).

3.2 | Histological characterization of aortic arch and aortic sinus of HFD group and HFD + Casein group

Histological characterization of atherosclerotic plaques in the aortic arch and aortic sinus was shown in Figure 2. Atherosclerotic lesion area was defined as the Oil Red O stained positive area. The positive percentages of atherosclerotic lesion area in aortic arch and aortic sinus were significantly lower in the HFD + Casein group

TABLE 1 Body weight and plasma lipid parameters of HFD group and HFD + Casein group

	HFD group (n = 6)	HFD + Casein group (n = 6)
Baseline body weight (g)	26.08 ± 1.46	24.67 ± 1.82
Final body weight (g)	31.37 ± 1.86	30.62 ± 2.13
Body weight gain (g)	5.28 ± 1.62	5.95 ± 3.07
TC (mmol/L)	39.93 ± 7.98	17.04 ± 4.82**
TG (mmol/L)	3.79 ± 1.09	2.07 ± 0.79*
LDL-C (mmol/L)	8.88 ± 1.60	3.49 ± 1.98**
HDL-C (mmol/L)	1.59 ± 0.75	2.58 ± 0.80

Note: ApoE$^{-/-}$ mice were fed with high-fat diet without (HFD group) or with 10% casein injections (HFD + Casein group). The body weight was measured once a week for 12 weeks. After 12 weeks of treatment, blood samples were collected and the plasma lipid profiles were measured. HFD, high-fat diet; TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.
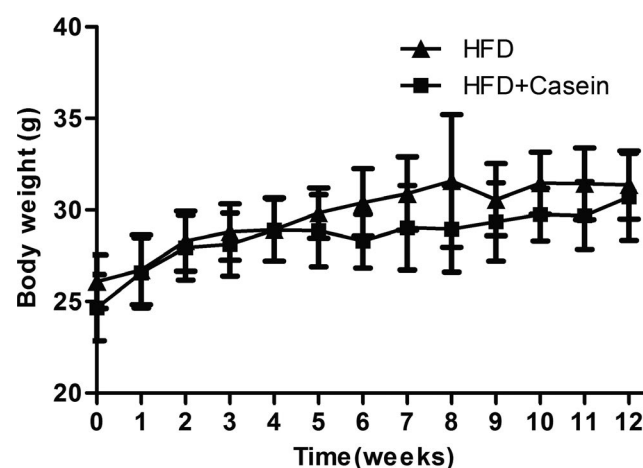
*$p < .05$;

**$p < .01$.

FIGURE 1 Body weight at each week for 12 weeks. ApoE$^{-/-}$ mice were fed with high-fat diet for 12 weeks without (HFD group) or with 10% casein injections (HFD + Casein group). The body weight was measured once a week for 12 weeks

compared with the HFD group (Figure 2a,b). Collagen deposition in atherosclerotic lesions was expressed as the Masson's Trichrome stained positive area. The positive percentage of collagen deposition area in aortic sinus plaques was also significantly reduced in the HFD + Casein group compared with the HFD group (Figure 2c).

3.3 | Plasma levels of inflammatory markers of the HFD group and HFD + casein group

As shown in Figure 3, plasma levels of IL-1β and GM-CSF were significantly lower, and IL-6 and CRP tended to be lower in the HFD + Casein group compared with the HFD group.
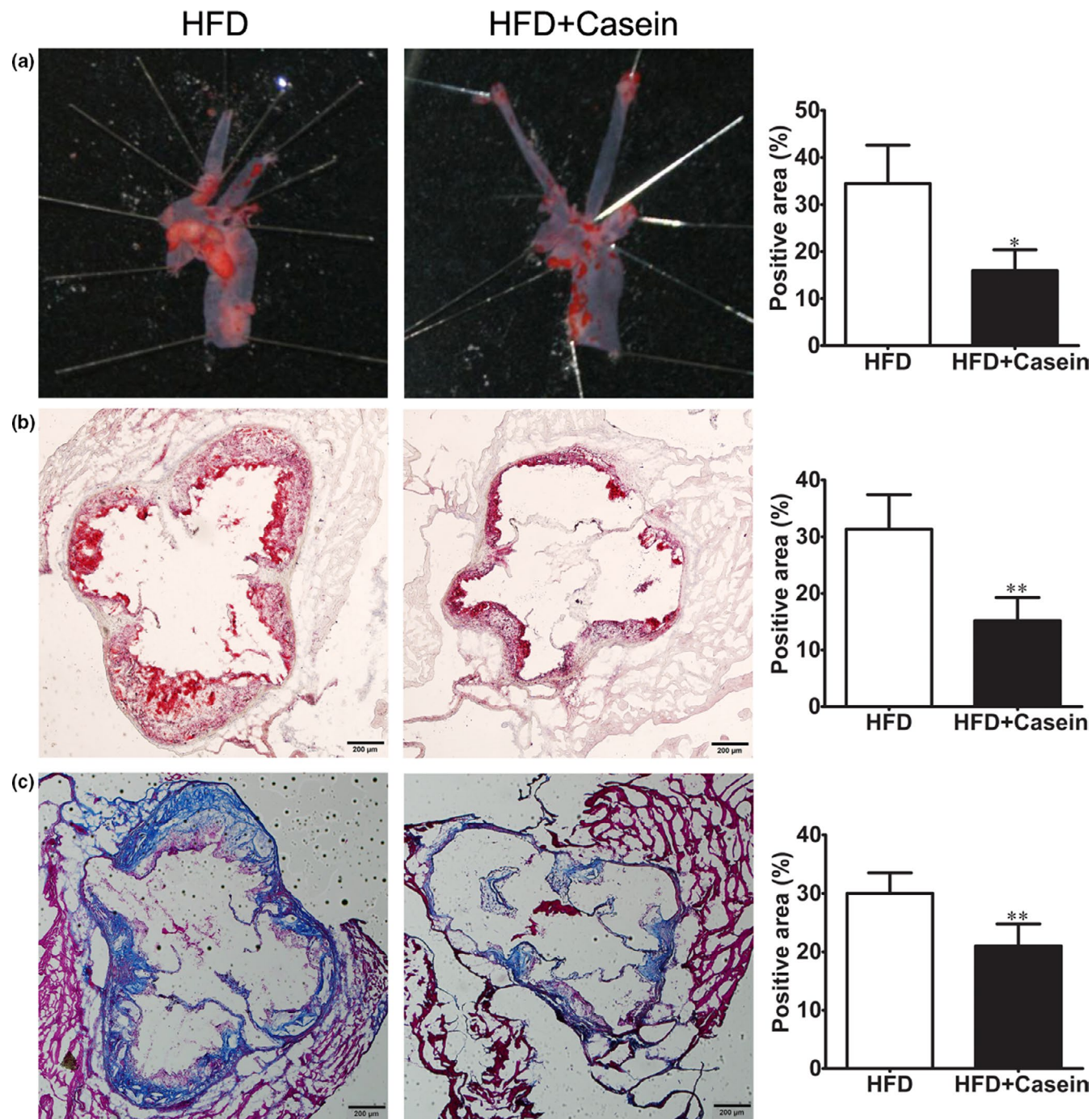
FIGURE 2 Histological characterization of atherosclerotic plaques in the aortic arch and aortic sinus. ApoE$^{-/-}$ mice were fed with high-fat diet for 12 weeks without (HFD group) or with 10% casein injections (HFD + Casein group). Atherosclerotic plaque areas in the aortic arch and aortic sinus were expressed as positive percentage of the Oil Red O stained area (×40) (A and B, respectively). Collagen deposition in atherosclerotic lesions was expressed as positive percentage of the Masson's Trichrome stained area over the total plaque area (×40) (C). *$p < .05$, **$p < .01$

4 | DISCUSSION

The major findings of this study are as follows: (1) 10% casein treatment decreases plasma TC, TG, and LDL-C concentration and alleviates aortic atherosclerosis in male apoE$^{-/-}$ mice fed with HFD. (2) Collagen deposition in aortic sinus plaques and plasma levels of inflammatory markers as IL-1β and GM-CSF are reduced after casein intervention. These results indicate that 10% casein treatment slows

down atherosclerotic lesion progression, possibly by decreasing fibrosis and inflammatory responses in male apoE$^{-/-}$ mice fed with HFD.

4.1 | The influence of casein on blood lipid level in male apoE$^{-/-}$ mice fed with HFD

Atherosclerosis remains the major cause of ischemic vascular diseases. Since the underlying pathological process of ischemic
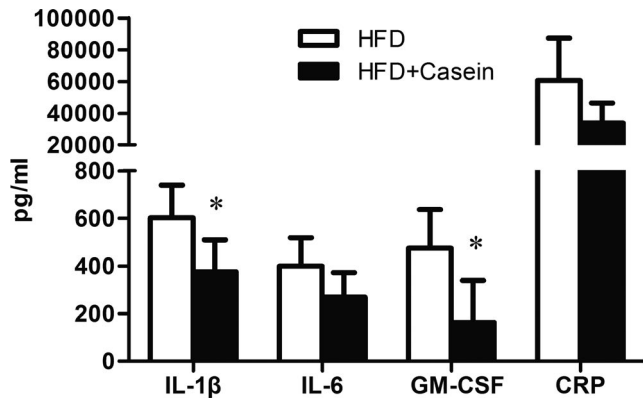
FIGURE 3 Inflammatory markers analysis in plasma. ApoE$^{-/-}$ mice were fed with high-fat diet for 12 weeks without (HFD group) or with 10% casein injections (HFD + Casein group). Blood samples were collected, and the plasma inflammatory markers were measured. *$p < .05$

vascular diseases is intravascular fat accumulation leading to atherosclerosis, early intervention in diet and other risk factors might affect the progression of atherosclerosis. It was reported in most studies that casein increased serum cholesterol levels and played a role in the development of atherosclerosis as early as in the 1980s (Huff et al., 1982; Terpstra, Harkes, & van der Veen, 1981). On the contrary, our study demonstrated that casein alleviated hypercholesterolemia in male apoE$^{-/-}$ mice fed with HFD, which was in line with previous report by Meinertz et al. (1990). The main reasons might be as follows:

First, an explanation for the discrepancy between our results and those of other authors might be the different species used. The hypercholesterolemia potential of casein would mainly be expressed in species (like the rabbit) with a low intestinal phosphatase activity and with a high glycine conjugation of bile acids. Van der Meer, De Vries, and Van Tintelen (1988) reported that dietary casein increased phosphate absorption and decreased fecal excretion of bile acids in male New Zealand white rabbits, thus increased the free concentration of bile acids and consequently stimulated the reabsorption of steroids from the gut. Eventually, this might cause an increase in serum cholesterol concentration because of the well-known suppression of hepatic apoB/E receptors (Brown & Goldstein, 1983).

Second, gender appeared to play a role on blood lipid levels and the development of atherosclerosis. Terpstra, Van Tintelen, and West (1982) investigated the effect of dietary casein on the serum concentration of cholesterol in male and female lean Zucker strain rats. The increase of cholesterol level in the serum was observed in the female rats fed the casein diet for 14 weeks during the experiment, whereas the male rats fed the casein diet exhibited a lower serum cholesterol level at the end of the experiment than at the beginning. Similar findings were reported by Fillios, Naito, Andrus, Portman, and Martin (1958) who found that after albino rats of the Sprague–Dawley strain were fed casein diet for a period of 17 weeks, serum cholesterol levels of female rats were twice as high as that of the corresponding males. Atherosclerotic lesions were

reported to be larger and more advanced in young female than male apoE$^{-/-}$ mice because of sex differences in immune mechanisms with activated T cells responding to oxidized LDL in female mice even in the absence of exogenous estrogens, while no such effect was seen in male mice (Caligiuri, Nicoletti, Zhou, Tornberg, & Hansson, 1999). These studies indicated that female animals were more predisposed to developing hypercholesterolemia and atherosclerosis in response to casein diet than their male counterparts.

Third, serum lipid level is quite sensitive to the protein level in the diet. A study in female lean Zucker strain rats showed that the hypercholesterolemic effect of dietary casein could be enhanced by increasing the proportion of this protein in the diet from 20% to 50%. (Terpstra et al., 1982) Furthermore, Huffman and Jones (1956) observed that the level of serum cholesterol in male weanling rats of the Sprague–Dawley stock fed 12.5% casein for 25 weeks was significantly lower than in those fed 25%, 9%, or 7.5% and tended to be lower than in those fed 18.75%, which revealed that reduction or elevation of dietary casein beyond a modest concentration (12%–18%) might lead to ultimate elevation of serum cholesterol. This observation was supported by Fillios et al. (1958) who reported that inadequate (5%) or excessive (40% or 60%) levels of dietary casein were found to contribute to an increased degree of serum cholesterol in male albino rats of the Sprague–Dawley strain. Regulation of plasma TG level is primarily mediated by lipoprotein lipase (LPL) attached to the capillary endothelium which catalyzes the hydrolytic cleavage of TG into fatty acids (Kersten, 2014). A study in male Wistar rats confirmed that tissue LPL activities were decreased with the consumption of a low-protein diet containing 50 g/kg protein compared with a balanced diet containing 200 g/kg protein (Boualga, Bouchenak, & Belleville, 2000).As a consequence, inadequate levels of dietary protein followed by decreased LPL activity could increase the levels of TG in plasma. These studies explained that too high or too low proportion of casein in dietary could increase the levels of serum lipids.

Taken together, the results from above studies might partially explain inconsistent effects of casein on serum cholesterol levels. Therefore, it was reasonable for 10% casein to reduce blood lipid levels in male apoE$^{-/-}$ mice fed with HFD in this study. Our study provides a potential way to alleviate hyperlipidemia induced by HFD in some individuals through administration of appropriate casein proportion.

4.2 | Potential mechanisms of casein affecting atherosclerotic progression

Vascular fibrosis, involving accumulation of extracellular matrix proteins particularly collagen, is associated with atherosclerosis. Previous study showed that vascular fibrosis contributed to vascular remodeling and scar formation (Touyz, 2005). Arterial stiffening or remodeling was recognized as an important consequence of vascular fibrosis to promote the development of atherosclerosis (Shirwany & Zou, 2010). The results of present study demonstrated that atherosclerotic plaque area and collagen deposition in the aortic root were significantly decreased

after casein treatment, suggesting that casein reduced the progression of vascular fibrosis and thereby attenuated atherosclerotic development in male apoE$^{-/-}$ mice fed with HFD.

The induction of inflammation provides the important link between hyperlipidemia and atherosclerosis. During the past decades, extensive observations supported a central role for inflammation in all phases of atherosclerotic process, including early atherogenesis, lesion progression, and final thrombotic complications (Pant et al., 2014). In a variety of animal models of atherosclerosis, signs of inflammation occur hand in hand with incipient lipid accumulation in the artery wall. Once adherent to the endothelium, the blood-derived leukocytes migrate into the intima at sites of lesion formation, participate in and perpetuate a local inflammatory response. The activated macrophage differentiated from entering monocytes could produce proteolytic enzymes rendering the plaque's protective fibrous cap thin, weak, and susceptible to rupture, which triggers the thrombus that causes most acute complications of atherosclerosis. Accumulating evidence indicated that elevated circulating inflammatory markers reflected the local inflammatory process for atherosclerosis and predicted adverse cardiovascular events (Pant et al., 2014). Our study showed that the reduction of plaque area in the aortic arch and aortic sinus was accompanied with lowering of plasma inflammatory markers in male apoE$^{-/-}$ mice following casein injection, which indicated the beneficial cardiovascular protective effect of casein on atherosclerotic development by decreasing inflammatory responses.

4.3 | Study limitations

The influences of casein on atherosclerotic development are varied in different gender and doses. This study only demonstrated that casein (subcutaneous injection with 0.5 ml of 10% casein daily for 12 weeks) attenuated atherosclerotic development in male apoE$^{-/-}$ mice fed with HFD (1.25% cholesterol). Future studies are warranted to explore dose dependent effect of casein on atherosclerotic progression and its potential mechanism in both male and female apoE$^{-/-}$ mice. It is to note that present study gave casein to mouse by subcutaneous injection, which is different from previous studies and is also different from casein's daily oral consumption habits; therefore, the results of subcutaneous injection cannot be compared with the results of previous oral consumption.

In conclusion, our study finds that 10% casein treatment decreases lipid concentrations in male apoE$^{-/-}$ mice fed with HFD. Casein administration is associated with attenuated atherosclerotic lesion progression, possibly by decreasing fibrosis and inflammatory responses in male apoE$^{-/-}$ mice fed with HFD.

5 | ETHICAL REVIEW

This study was approved by the Animal Care Committees of Wuhan Fourth Hospital.

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