Article

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Creation of an Atherosclerosis Model Using Palmitic Acid and Oleic Acid in the Vascular Smooth Muscle Cells of Rats

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Background: Atherosclerosis (AS) is a chronic vascular inflammatory disease resulting from vascular endothelial injury and lipid deposition, closely linked to abnormal lipid metabolism within the body. The critical processes involved in atherosclerosis encompass lipid deposition, oxidation, metabolic disruptions, and inflammatory stimulation within the inner vessel wall. Lipid deposition emerges as a pivotal factor triggering these pathological changes, with vascular smooth muscle cells (VSMCs) playing a significant role in the development of AS. Therefore, the goal was to employ lipids, specifically palmitic acid (PA) and oleic acid (OA) solutions, to stimulate VSMCs and create an *in vitro* atherosclerosis model. This approach allows for the establishment of a rapid and efficient cell model for simulating atherosclerosis *in vitro*.
Methods: Primary vascular smooth muscle cells (VSMCs) were isolated and cultured from the thoracic aorta of healthy rats using the tissue-block method. VSMCs were identified through cell climbing slices and immunofluorescence. The growth of VSMCs was observed using light microscopy. The logarithmic growth phase of VSMCs was induced and stimulated by various concentrations of palmitic acid (PA) and oleic acid (OA) ranging from 0 to 650 µmol/L, with a gradient dilution of 50 µmol/L. VSMC activity was assessed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Intracellular lipid deposition was visualized through Oil Red O staining. The levels of total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) within VSMCs were quantified using commercially available kits.
Results: The optimal conditions for VSMC proliferation were determined to be an OA concentration of 500 µmol/L, a PA concentration of 300 µmol/L, and a culture duration of 48 hours. In comparison to the control group, the presence of lipid droplets within VSMCs became significantly evident following treatment with OA or PA. Furthermore, the levels of TC, TG, and LDL-C increased, while the HDL-C content decreased after treatment with OA or PA.
Conclusions: A research model for atherosclerosis (AS) and the early stages of cardiovascular events, specifically lipid deposition, was successfully established through the use of OA and PA solutions. This model has the potential to open up new research avenues for gaining a deeper understanding of the pathogenesis and progression of AS.

Keywords: palmitic acid; oleic acid; lipid metabolism; vascular smooth muscle cells

Introduction

Atherosclerosis (AS) is a lipid-metabolic disorder that affects large and medium-sized arteries, along with vasculitis [1,2]. Atherosclerotic cardiovascular diseases and cardiovascular diseases are associated with a significant global mortality rate [1,3].

The pathogenesis of AS is intricately linked to factors such as inflammation, lipid metabolism, oxidative stress, endothelial injury, the immune response, apoptosis, cellular molecular mechanisms, and hemodynamic changes [4,5]. Central to the development of AS are the accumulation and abnormal metabolism of lipids, foam cell buildup, and plaque formation [6]. This abnormal lipid accumulation can result in injury, dysfunction, and even the death of vascular endothelial cells [4,6,7]. Continuous uptake of oxidized low-density lipoprotein (ox-LDL) by macrophages (monocytes) and vascular smooth muscle cells (VSMCs) is a prominent feature. However, these cells are unable to effectively metabolize ox-LDL through LDL receptors, leading to lipid deposition and the “foamy” transformation of cells, which ultimately contributes to AS development [6,8]. Consequently, abnormal lipid deposition serves as an independent risk factor for AS.

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The formation of foam cells and plaques is intricately linked to macrophages and VSMCs [8]. VSMCs are often utilized for *in vitro* investigations of AS mechanisms due to their ready availability and close association with the occurrence and progression of AS. Palmitic acid (PA) and oleic acid (OA) are common fatty acids naturally occurring in the body and have been used to establish models for liver steatosis [9,10]. Research has demonstrated that PA serves as a phenotypic biomarker of AS [11,12], while the presence of OA in blood plasma is recognized as a risk factor for cardiovascular disease [12,13]. It is worth noting that the most commonly used reagents for inducing stimulation and establishing AS-related cell models are oxidized low-density lipoprotein (ox-LDL) [14,15] and lipopolysaccharides (LPS) [16,17]. However, few experiments have explored the utilization of PA and OA, two common dietary fatty acids, to simulate the creation of AS-related lipid deposition models and investigate the mechanisms underlying abnormal lipid metabolism during the early stages of AS lipid deposition. Hence, in this study, we employed two lipid reagents, PA and OA, to establish an AS model in rat VSMCs. This approach allows for the rapid and effective generation of a cell model for *in vitro* investigations of lipid deposition on the inner walls of blood vessels, presenting a novel method for the study of AS-related mechanisms.

Methods and Materials

Materials

The thoracic aortas were obtained from Male Sprague-Dawley rats (4–8 weeks; 150–200 g) at the Animal Center Laboratory of Beijing Shijitan Hospital, Capital Medical University, Beijing, China. We sourced the following materials from various suppliers: Dulbecco's modified Eagle's medium (DMEM)-high glucose from HyClone (Logan, UT, USA, SH30243.FS), phosphate-buffered saline (PBS, USA, 10010023), 0.25% tryptase-EDTA (USA, 25200056), fetal bovine serum (FBS, USA, 10093), penicillin, and streptomycin from Gibco (Grand Island, NY, USA, 15140122), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, China, Top0190), endotoxin-free bovine serum albumin (BSA, China, 9048-46-8), and cell climbing slices from Biotopped (Beijing, China). Additionally, we procured palmitic acid (PA), oleic acid (OA), and dimethyl sulfoxide from Millipore-Sigma (Burlington, MA, USA, P9767, O1008), assay kits for total cholesterol (TC, China, A111-1-1), triglycerides (TG, China, A110-1-1), high-density lipoprotein-cholesterol (HDL-C, China, A112-1-1), and low-density lipoprotein-cholesterol (LDL-C, China, A113-1-1) from Nanjing Jiancheng (China) for measuring lipid contents. Other materials, such as Triton X-100 (China, T8200), Oil Red O dye (China, G1262), 5% BSA blocking solution (SW3015), 4% paraformaldehyde (China, P1110), and 4′,6-diamidino-2-phenylindole (DAPI, China, C0065) were obtained from Solarbio (Beijing, China). Isopropyl alcohol was purchased from Beijing Chemical Company (Beijing, China). Furthermore, we acquired α-smooth muscle actin (SMA) antibody (catalog number, D4K9N) and XP® rabbit monoclonal antibody (19245) from Cell Signaling Technology (Danvers, MA, USA), along with fluorescent secondary antibodies from Yi Sheng (Beijing, China, 34213ES60). All other materials used for cell culture were obtained from Corning (Corning, NY, USA).

Isolation, Culture, and Passage of VSMCs

VSMCs were isolated from rat thoracic aorta using the tissue-block adherence method [18]. The procedure involved the following steps:

A segment of the thoracic aorta was carefully exposed, dissected free, and promptly placed in DMEM containing 5% penicillin and 5% streptomycin. Adipose tissue and connective tissue were removed under sterile conditions. After clearing the fat tissue surrounding the artery, it was longitudinally cut open using sterile instruments. The opened artery was then transferred to another cell-culture dish filled with DMEM containing 10% FBS. The inner surface of the artery was gently scraped 2–3 times using an ophthalmic curved tweezer (OCT, ZO012RB, Xinhua surgical instrument company, China) to remove endothelial cells. The artery was secured with OCT, and another OCT was used to compress and push along the short axis, creating a small break in the vessel. Vascular fractions were then isolated as described, and the tunica adventitia was entirely separated and discarded. The fractions were cut into approximately 1 mm-sized squares, transferred to a 25 cm^2 cell-culture flask, and placed in a 37 °C thermostatic incubator (Thermo Scientific company, USA, 3120) in an atmosphere containing 5% CO_2 for 4–6 hours. The flask was inverted, and DMEM containing 20% FBS, 1% penicillin, and 1% streptomycin was carefully added. The small fragments were fully immersed in the medium and incubated statically for 5 days. Once the cell confluence reached 80–90%, the cells were passaged for the second or third generations. It's important to note that the extracted cells were tested for mycoplasma to ensure their quality and safety.

VSMCs Identified by Immunofluorescence

The VSMCs obtained were cultured on cell climbing slices. They were washed with PBS and fixed in 4% paraformaldehyde. Cells were permeabilized by 0.3% Triton X-100 and blocked with 5% BSA blocking solution for 1 hour. Subsequently, they were incubated with α-SMA antibody (1:200) overnight at 4 °C. The following day, cells were labeled with a fluorescent secondary antibody (1:400 dilution), allowed to incubate for 1 hour, and washed. Nuclei were stained with DAPI for 10 minutes and washed. Slices were processed for immunofluorescent microscopy using a CSU-W1 SoRa microscope from Nikon, Japan.

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Preparation of PA Solution and OA Solution

The molecular weights of PA and OA were determined in the following manner [9,10].

To prepare these solutions, 0.0307 g of PA powder and 19.04 µL of OA stock solution were individually dissolved in 0.1 mol/L of NaOH. The saponification process was carried out in a water bath at 75 °C for 30 minutes, resulting in a final PA concentration of 40 mmol/L and an OA concentration of 20 mmol/L. Additionally, BSA powder was dissolved in PBS, and the solution was clarified by centrifugation (133 ×g for 20 minutes at room temperature, using a Centrifuge 5430 from Eppendorf, Germany). The OA and PA solutions were mixed with the BSA solution and thoroughly combined. Subsequently, they were passed through a sterile membrane filter with a pore size of 0.22 µm and then diluted in DMEM complete medium. The final concentrations of PA and OA ranged from 100 to 650 µmol/L, including increments at 100, 200, 250, 300, 400, 350, 450, 500, 550, 600, and 650 µmol/L.

Cell Viability

Cell viability was assessed using the MTT assay. Initially, 1000 cells were inoculated. Following treatment with either the OA or PA solution, 10 µL of MTT at a working concentration of 5 mg/mL was added to each well, and incubation was allowed for 4 hours. The MTT solution was then removed, and 150 µL of DMSO was added to each well to dissolve the needle-like formazan crystals formed by viable cells. The absorbance was measured at 490 nm using the Multiskan Spectrum (A51119500C, Thermo Scientific company, USA), and the percentage of viable cells was calculated using the formula:

Cell viability = (Optical density (OD) value experimental group – OD value blank group) / OD value blank group × 100%

This assay was performed with 6 repetitions per group.

Oil Red O Staining

Oil Red O staining was employed to assess intracellular lipid accumulation. The Oil Red O staining solution was prepared by diluting Oil Red O in double-distilled H_2O (3:2) and then filtering it. VSMCs, which were seeded into six-well plates, were treated with the optimal concentrations of PA or OA for the appropriate duration. Following treatment, the cells were washed three times with PBS. They were then fixed with 4% paraformaldehyde for 30 minutes, followed by another round of washing. Subsequently, the cells were stained with freshly diluted Oil Red O solution for 2 hours. After the staining period, the stain was removed, and a brief wash with 60% isopropanol for 5-10 seconds was performed. Following this, the cells were washed twice with PBS and observed under an inverted cell observation microscope (NIB600, Yongxin, China).

Lipid Accumulation

Lipid contents were quantified using commercial kits. Following a 48-hour incubation in either OA or PA solutions, the cells were washed 2–3 times with PBS. Subsequently, the cells were harvested and lysed with 1% Triton X-100 for 10 minutes. The measurements of TC, TG, HDL-C, and LDL-C were conducted as per the kit instructions in 96-well plates, with three replicate wells set for each sample. The absorbance readings were taken using the Multiskan Spectrum (Thermo Scientific company, USA, A51119500C), following the manufacturer's instructions.

Statistical Analyses

Data analysis was performed using Prism 8.0 (GraphPad, La Jolla, CA, USA) and SPSS 23.0 (IBM, Armonk, NY, USA). All data were expressed as mean ± standard deviations (SD) from three independent experiments. For comparing differences between two independent groups, a two-tailed Student's *t*-test was applied. To assess differences among multiple groups, a one-way ANOVA was utilized. Post hoc Tukey's test was conducted for comparisons between groups. Statistical significance was defined as $p < 0.05$.

Results

Growth, Proliferation, and Identification of VSMCs

Starting from the fifth day of culture, VSMCs began to emerge from the edges of the tissue blocks. The majority of these VSMCs exhibited irregular polygonal shapes. By the tenth day of cultivation, nearly all VSMCs had extended from the tissue blocks, taking on a long and spindly appearance (Fig. 1). After fifteen days, VSMCs reached a confluence of 60–70%. VSMCs grown at a high density displayed a whirlpool-like morphology, while those grown at a lower density were closely intertwined, forming characteristic "hill and valley" formations [18]. To confirm their identity, VSMCs were identified through immunofluorescence staining, with approximately 90% of the cells exhibiting positivity for α-SMA, a specific marker for VSMCs (Fig. 2). Nuclei were stained using DAPI. It's worth noting that after VSMCs had been passaged more than three times, their purity remained high.

Effects of PA and OA on VSMC Activity

VSMCs were subjected to treatments with varying concentrations of OA or PA, as well as different culture durations, and their activity was assessed using the MTT assay. The results are presented in Fig. 3a,b, which illustrate VSMC activity concerning OA and PA concentrations, respectively. Comparatively, VSMC activity was significantly increased at an OA concentration of 500 µmol/L ($p < 0.0001$) (Fig. 3a) and a PA concentration of 300 µmol/L ($p < 0.0001$) (Fig. 3b) when contrasted with other groups. The impact of culture duration was also examined, showing that

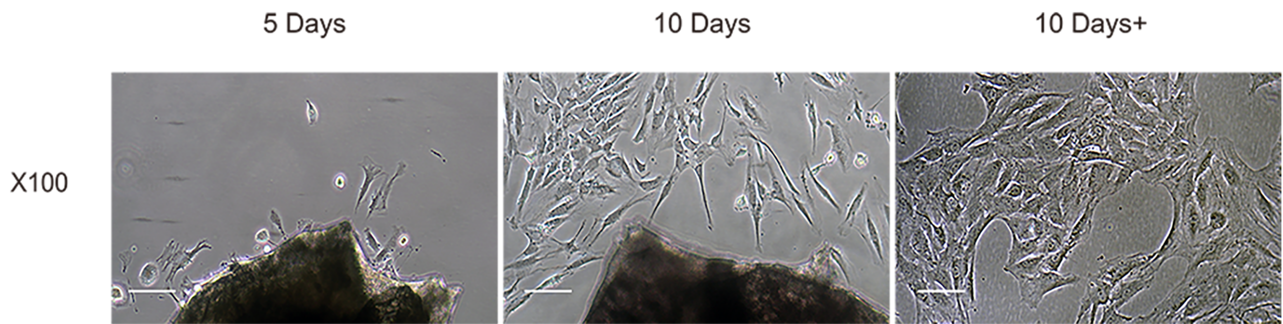

Fig. 1. Primary culture of vascular smooth muscle cells (VSMCs). VSMCs utilized the tissue-adhesion method to gradually extend from the tissue blocks, resulting in a spindly and triangular morphology. Scale bar = 200 µm.

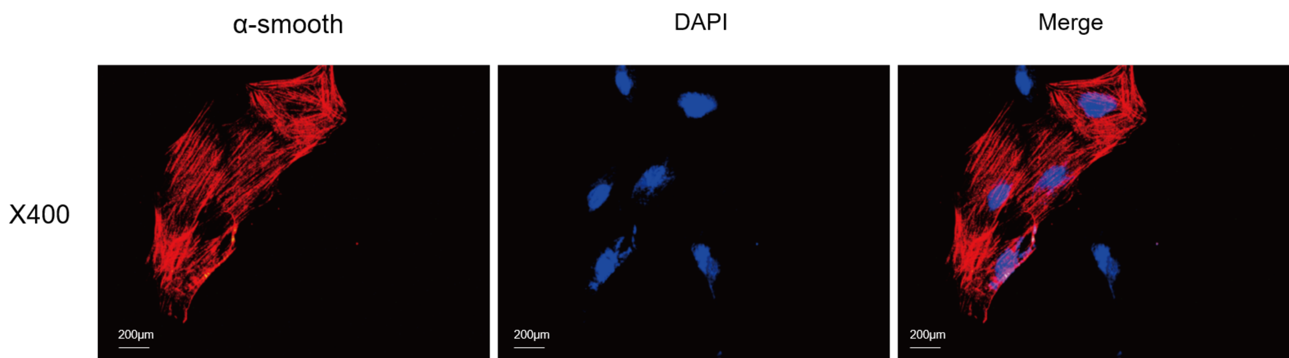

Fig. 2. VSMCs observed under fluorescence microscopy. Immunofluorescence staining demonstrated the expression of myofilament protein in VSMCs. The red fluorescence indicated positivity to the primary antibody, α-smooth muscle actin, with a 1:200 dilution. Nuclei of VSMCs were revealed by the blue fluorescence from 4′,6-diamidino-2-phenylindole (DAPI) staining. Scale bar = 200 µm.

as the culture duration extended to 48 hours, the percentage of viable VSMCs significantly increased. VSMC viability reached its peak when cultured with OA at 500 µmol/L ($p < 0.0001$) (Fig. 3c) or PA at 300 µmol/L ($p < 0.0001$) (Fig. 3d) after 48 hours in comparison to other groups cultured for different durations. Hence, it was determined that the optimal conditions for VSMCs were an OA concentration of 500 µmol/L, a PA concentration of 300 µmol/L, and a culture duration of 48 hours.

OA and PA Stimulated Lipid Deposition in VSMCs

In Fig. 4, representative images depicting lipid accumulation in VSMCs under an OA concentration of 500 µmol/L, a PA concentration of 300 µmol/L, and a culture duration of 48 hours are presented. The Oil Red O staining, conducted at the 2-hour mark, revealed the accumulation of red lipid droplets within VSMCs when viewed at 200× magnification. A comparison with the control groups indicated that the use of 500 µmol/L of OA or 300 µmol/L of PA for 48 hours resulted in the formation of a greater number of intracellular lipid droplets when viewed at 400× magnification.

Intracellular Lipid Levels Increased after Treatment with OA or PA in VSMCs

To validate the establishment of our cell model, we continued to treat VSMCs with an OA concentration of 500 µmol/L and a PA concentration of 300 µmol/L for 48 hours. Subsequently, we measured the intracellular lipid levels, including TC, TG, LDL-C, and HDL-C (µmol/L). The results revealed a significant increase in the contents of TC and TG after treatment of VSMCs with OA or PA ($p < 0.01$ vs. the control group, $p < 0.05$ vs. the control group). Additionally, the PA group exhibited higher TC and TG contents than the OA group (see Fig. 5a,b).

Comparatively, the HDL-C content decreased after treatment with PA or OA in contrast to the control group ($p < 0.05$ vs. control group, $p < 0.01$ vs. control group) (Fig. 5c). The LDL-C content showed a slight increase in the OA group when compared to the control group ($p < 0.05$ vs. control group), but there was no significant difference between the PA group and the control group (Fig. 5d). In summary, these findings indicated that treating VSMCs with OA at 500 µmol/L or PA at 300 µmol/L for 48 h could establish a lipid model in VSMCs.

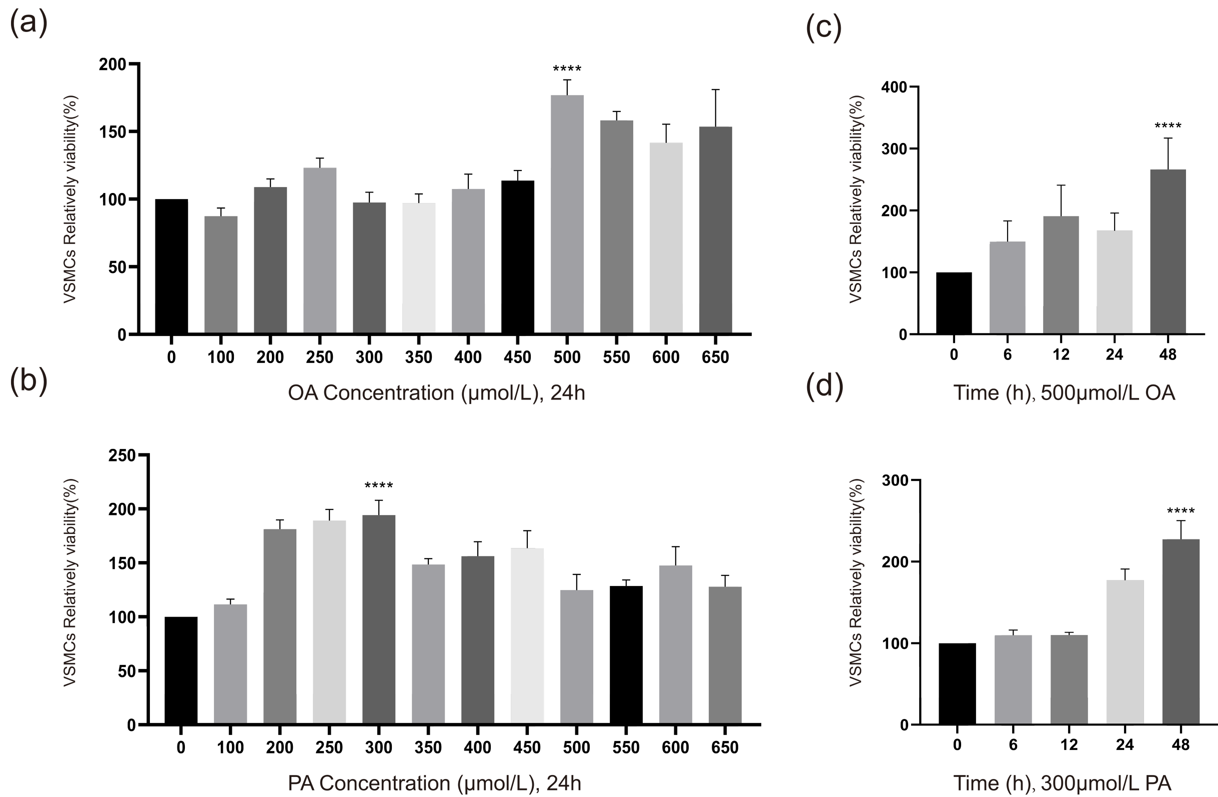

Fig. 3. Influence of oleic acid (OA) and palmitic acid (PA) on VSMC viability. VSMC viability under different concentrations (µmol/L) of OA (a) and PA (b), ****$p < 0.0001$ vs. control (0 µmol/L). VSMC viability under different culture duration under 500 µmol/L of OA (c) and 300 µmol/L of PA (d), ****$p < 0.0001$ vs. control (0 h). This assay was performed with 6 repetitions per group.

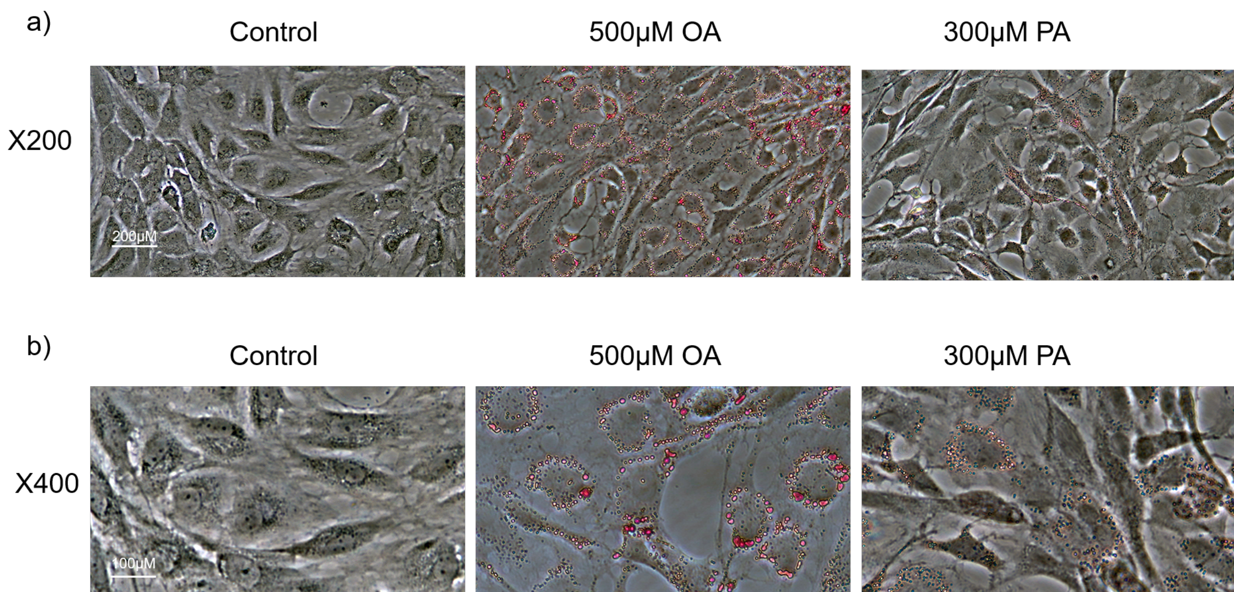

Fig. 4. Oil Red O staining of VSMCs. Using OA (500 µmol/L) and PA (300 µmol/L), the formation of live drops in VSMCs was separately observed at 200× (a) and 400× magnetization (b). µM = µmol/L. This assay was performed with 6 repetitions per group.

Discussion

Atherosclerosis (AS) is a multifactorial chronic inflammatory disease that primarily affects the walls of large and medium-sized arteries, thereby elevating the risk of adverse cardiovascular events. Inflammatory reactions are integral to the progression of atherosclerosis, myocar-

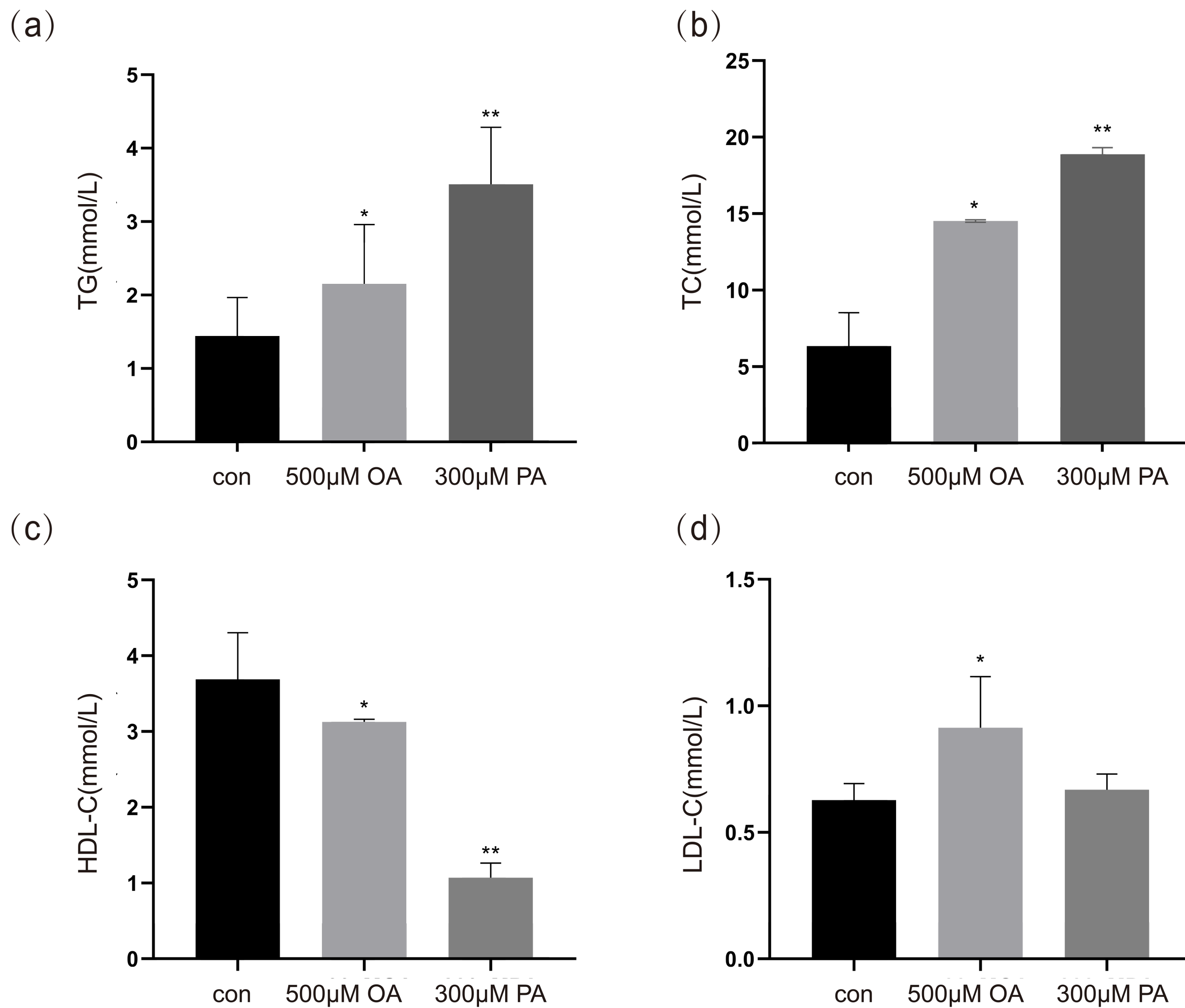

Fig. 5. Contents of total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-C), and low-density lipoprotein-cholesterol (LDL-C) after treatment with OA or PA in VSMCs for 48 h. VSMCs were treated with OA (500 μmol/L) or PA (300 μmol/L) for 48 h. The contents changes of TG (a), TC (b), HDL-C(c), LDL-C (d) were shown, *$p < 0.05$, **$p < 0.01$. This assay was performed with 6 repetitions per group.

dial lesions, and the onset of atherothrombotic or systemic complications [1,2,19,20]. When the inner arterial wall is subjected to stimuli such as dyslipidemia, endothelial injury, proinflammatory cytokines, chemokines, and atherosclerosis-associated adhesion molecules are released. These factors further lead to the adherence of white blood cells, fibrinogen, proteins, and red blood cells to the endothelial wall. Subsequently, macrophages migrate, and VSMCs proliferate under the influence of inflammation. They then uptake ox-LDL, transforming into foam cells, and contribute to the development of atherosclerotic fibrous plaques [21–23]. Hence, the abnormal deposition of lipids and the dysregulated metabolism of fatty acids result in dyslipidemia within arterial walls, thereby hastening the pathological deterioration of the aorta, an independent risk factor for AS [24]. It is essential to recognize that dyslipidemia, oxidative stress, endothelial dysfunction, inflammatory processes, hemodynamic abnormalities, and thrombosis stand as the principal risk factors driving the pathophysiological events of AS [6,21].

To determine the optimal conditions for growth and proliferation, we treated VSMCs with varying concentrations of OA and PA, along with different culture durations. Subsequently, we established an *in vitro* model to simulate the early phase of AS (lipid deposition). After conducting Oil Red O staining, lipid droplets within VSMCs became clearly visible under light microscopy. Treatment with OA or PA led to an increase in the contents of TG and TC were increased, HDL-C content, and LDL-C content. This indicated that lipid metabolism in VSMCs became aberrant following stimulation with OA or PA. OA and PA are common dietary fatty acids and have previously been employed to establish models for hepatic steatosis, high-fat diets, and high-glucose diets [25–27]. Abnormal fat accumulation

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and metabolism have been linked to conditions such as obesity, hyperlipidemia, and diabetes mellitus, along with other diseases [28,29]. Furthermore, these abnormalities are recognized as significant contributors to the development of AS. Consequently, there is a pressing need for a reliable *in vitro* cell model to investigate lipid deposition in vascular walls, thereby facilitating a deeper understanding of the mechanisms underlying atherosclerosis.

Upon treating VSMCs with OA or PA for 48 hours, we observed changes in their proliferation under light microscopy. The treatment groups displayed intracellular lipid droplets of varying sizes, distinct from those observed in the control group. Additionally, the morphology of VSMCs shifted from long, spindle-like shapes to round forms. Considering both cell growth status and lipid deposition, we determined that culturing VSMCs for 48 hours with an OA concentration of 500 µmol/L and a PA concentration of 300 µmol/L (Fig. 3) yielded a pronounced cell lipidation culture status (Fig. 4). It was evident that a higher number of lipid droplets correlated with increased contents of TC and TG. Interestingly, the establishment of our VSMC model required a lower concentration of PA compared to OA. Furthermore, we observed greater lipid accumulation when using PA as opposed to OA, resulting in a reduction in the content of HDL-C. In sum, our model effectively reflected the pathophysiological changes associated with atherosclerosis.

In light of the findings from previous studies [30–32], it is noteworthy that the key difference observed in this study lies in the state of VSMCs, which exhibited cell proliferation in response to 48-hour exposure to high-fat treatments, rather than undergoing cell apoptosis. Several factors contributed to this difference: (i) The concentrations of OA and PA employed in our study were lower than those used in other investigations. (ii) We utilized 5% BSA as the vehicle for the PA and OA solutions, which could have affected the cellular response. (iii) The relatively extended culture duration allowed VSMCs to adapt to the high-fat solutions, leading to a proliferation response. It is important to acknowledge the main limitations of our study, notably the lack of in-depth analysis regarding the apoptosis or proliferation of VSMCs.

Conclusions

We have successfully established a research model for AS and the early stages of cardiovascular events, specifically lipid deposition, through the use of OA and PA solutions. This model represents a valuable addition to research tools and offers a new avenue for elucidating the pathogenesis and progression of AS.

Availability of Data and Materials

The original contributions presented in the study are included in the article. Further inquiries can be directed to the corresponding author.

Author Contributions

FZ and GL designed the study and performed the experiments. FZ, GL, XL, YZ, WD, BZ, and DS analyzed the data, prepared figures and drafts of the paper. GL confirmed the knowledge content and approved the final draft. FZ, GL, XL, YZ, WD, BZ, and DS contributed to drafting and modifying the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The primary cell culture animals in this experiment were from a blank control group of another study in the same research group. The experimental animals were bled under deep anesthesia with 125–250 mg/kg tribromoethanol and euthanized through cervical dislocation, following relevant regulations of animal ethics. The Research Ethics Committee of the Beijing Shijitan Hospital of Capital Medical University approved the collection of tissue samples for research (sjtky11-1x-2020(101)).

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Conflict of Interest

The authors declare no conflict of interest.

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