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Primary Culture of Rat Aortic Vascular Smooth Muscle Cells: A New Method

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Background: Developing a simple and efficient method of obtaining primary cultured VSMCs is necessary for basic cardiovascular research.

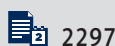
Material/Methods: The procedure of our new method mainly includes 6 steps: isolation of the aortic artery, removal of the fat tissue around the artery, separation of the media, cutting the media into small tissue blocks, transferring the tissue blocks to cell culture plates, and incubation until the cells reach confluence. The cells were identified as VSMCs by morphology and immunofluorescence. Then, VSMCs obtained by this new tissue explants method, the traditional tissue explants method, the enzyme digestion method, and A7r5 cell line were divided into 4 groups. The purity of cells was test by multiple fluorescent staining. Western blotting was used to investigate the phenotype of VSMCs obtained by different methods.

Results: Cells began to grow out at about 8 days and became relatively confluent within 16 days. Compared with VSMCs from the traditional tissue explants method and enzyme digestion method or A7r5 cell line, VSMCs obtained by our method showed higher purity and manifested a more "contractile" phenotype characteristic.

Conclusions: We have conquered the disadvantages in the previous primary culture methods and established a simple and reliable way to isolate and culture rat aortic VSMCs with high purity and stability.

MeSH Keywords: **Muscle, Smooth, Vascular • Phenotype • Primary Cell Culture**

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Background

Primary culture of rat aortic vascular smooth muscle cells (VSMCs) is extensively used *in vitro* to study the physiology and pathophysiology of cardiovascular diseases, including atherosclerosis (AS), hypertension, and aneurysm [1–3]. The 2 methods mainly used to obtain primary culture of VSMCs are tissue explants method and enzyme digestion method [4,5].

Ross first used the tissue explants method to isolate pig aortic VSMCs in 1971 [6,7]. The procedure mainly included 5 steps: isolation of the aortic artery, removal of the fat tissue around the artery, cutting the artery into small tissue blocks, transferring the tissue blocks to cell culture plates, and incubation until the cells reach confluency. This method to obtain primary cultured VSMCs has been widely used in many labs. However, the low purity was a serious disadvantage with the VSMCs obtained by this method. The artery is composed of adventitia, media, and intima, and all VSMCs are located in the media. Because this traditional method did not remove the adventitia wrapped around the media, the fibroblasts in the adventitia could grow out from the edge of tissue blocks when incubated in the cell culture plates. A few researchers found that these fibroblasts disappear naturally after several cell generations, and high purity of the VSMCs could be achieved easily only by normal incubation [8]. However, a study by Sartore found that in an altered external environment, fibroblasts transformed into myofibroblasts that also expressed SM-actin, leading to false-positive results in the VSMCs purity testing [9]. Therefore, to obtain high-purity VSMCs, it is necessary to obtain media without adventitia. Some researchers used tweezers, pulling or tearing the adventitia to separate it from the media, but this also had effect on the media and finally resulted in reduced activity of the media tissue blocks and even failure of primary cell culture.

Unlike skeletal or cardiac muscle cells that have undergone terminal differentiation, VSMCs of adult animals retain plasticity. The mature, quiescent, contractile phenotype VSMCs can dedifferentiate to the proliferative, synthetic phenotype in response to various physiological and pathological factors [10]. Differentiation status of VSMCs *in vitro* can be measured by testing the proliferation and migration ability and investigating the expression of smooth muscle-specific phenotype marker proteins, including smooth muscle actin (SM-actin), calponin, smooth muscle myosin heavy chain (SM-MHC), and osteopontin (OPN) [11]. Primary cultured VSMCs lose the capacity to contract and transform into synthetic phenotype VSMCs after several generations [12] and VSMCs used in most experiments need to be the contractile phenotype. Therefore, keeping the “contractile” phenotype for more generations is very important for the primary cultured VSMCs. However, the VSMCs obtained by enzyme digestion method showed “contractile” phenotype characteristic in just a few generations.

In the present study, we developed a simple and efficient method to obtain primary cultures of VSMCs by separating the adventitia from the media with the blunt back side of a pair of ophthalmic curved tweezers. We found the VSMCs obtained by our method not only had high purity, but also kept the “contractile” phenotype characteristic over many generations.

Material and Methods

Animals used in this study were treated in compliance with the Guide for Care and Use of Laboratory Animals and the animal care protocol was approved by the Animal Care and Use Committee of Shaoxing Hospital of Zhejiang University.

Materials

(1) Animals: Specific pathogen-free (SPF) Sprague-Dawley (SD) rats (50 days old, body weight 150–180 g), males and females, were obtained from the Animal Center laboratory of Zhejiang Province Institute of Medicine. (2) The rat VSMCs cell line A7r5 was purchased from Vasculife (Shanghai, China). (3) Reagents: Dulbecco’s modified Eagle’s medium (DMEM)-high glucose, PBS, 0.25% trypsin-EDTA, penicillin, and streptomycin were purchased from Jinuo Biotech Company (Hangzhou, China). Collagenase II and elastase were purchased from Sigma (USA), fetal calf serum (FBS) was from GIBCO (USA), MTT was from Emresco (USA), and DAPI was from Roche (USA). Antibodies against SM-actin, calponin, OPN, SM-MHC, and β -actin were purchased from Abcam (Cambridge, MA), and horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG antibody were purchased from Jackson ImmunoResearch Laboratories (USA). The other reagents for immunoblot assay were purchased from Beyotime (Jiangsu, China).

Cell isolation and identification

Under sterile conditions, anaesthetized SD rats were placed in the supine position and their chests were opened. The thoracic aorta was removed and transferred to a culture dish with cold (4°C) DMEM (Figure 1A). After removal of the fat tissue around the artery (Figure 1B), the artery was longitudinally cut and placed in another cell culture dish containing DMEM (Figure 1C). Then, we used a pair of ophthalmic curved tweezers to scrape the intima softly to get rid of endothelial cells (Figure 1D). Later, we did not separate the adventitia or directly cut the artery into small tissue blocks. Two pairs of ophthalmic curved tweezers were used: one to press the artery to fix it and another to separate the media from the artery by pressing and pushing the artery with its blunt back side (Figure 2). After half of the media was removed, the same method was used to get the other half. Then, the media was cut into approximately 1-mm squares and transferred into cell culture

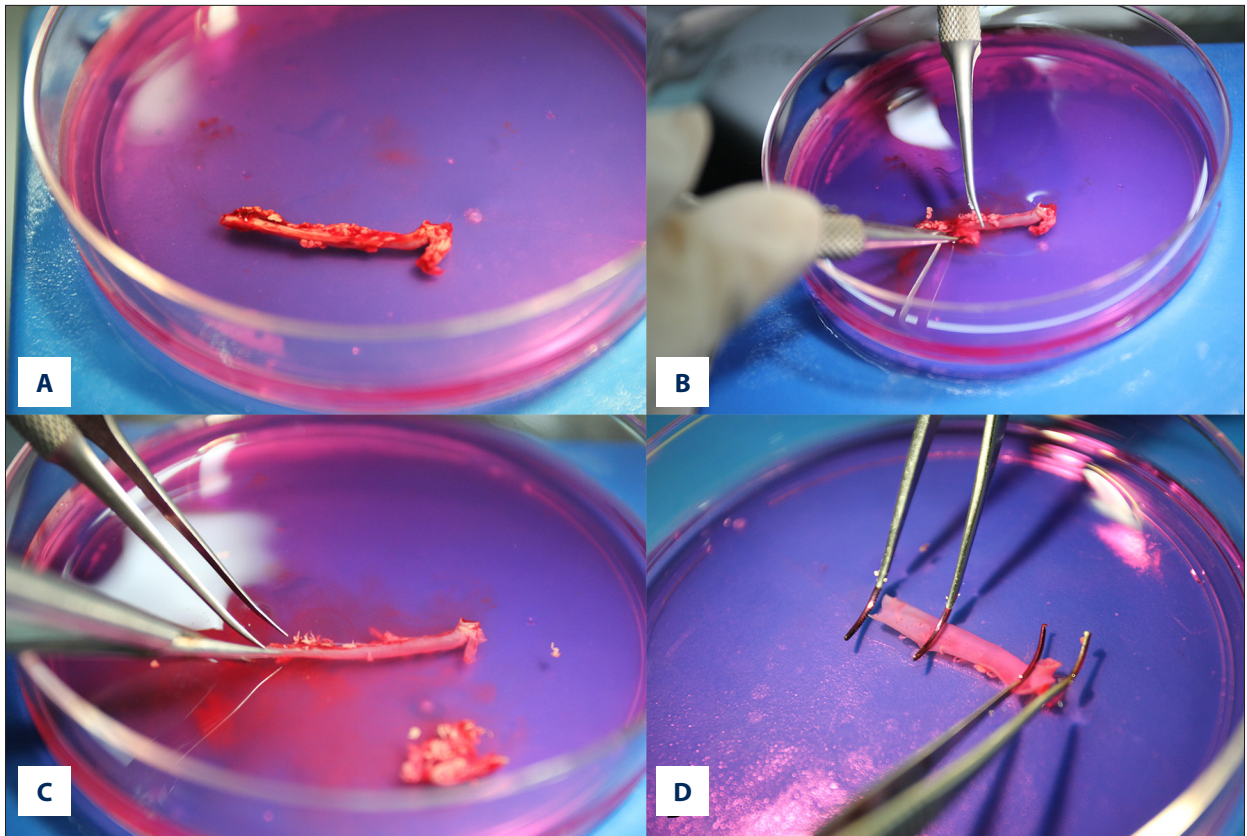


Figure 1. Steps for extract of rat thoracic aorta. (A) Isolation the thoracic aorta from a rat. (B) Eliminating the fat tissue around the artery. (C) The artery was longitudinally cut. (D) Scraping the intima softly to eliminate endothelial cells.

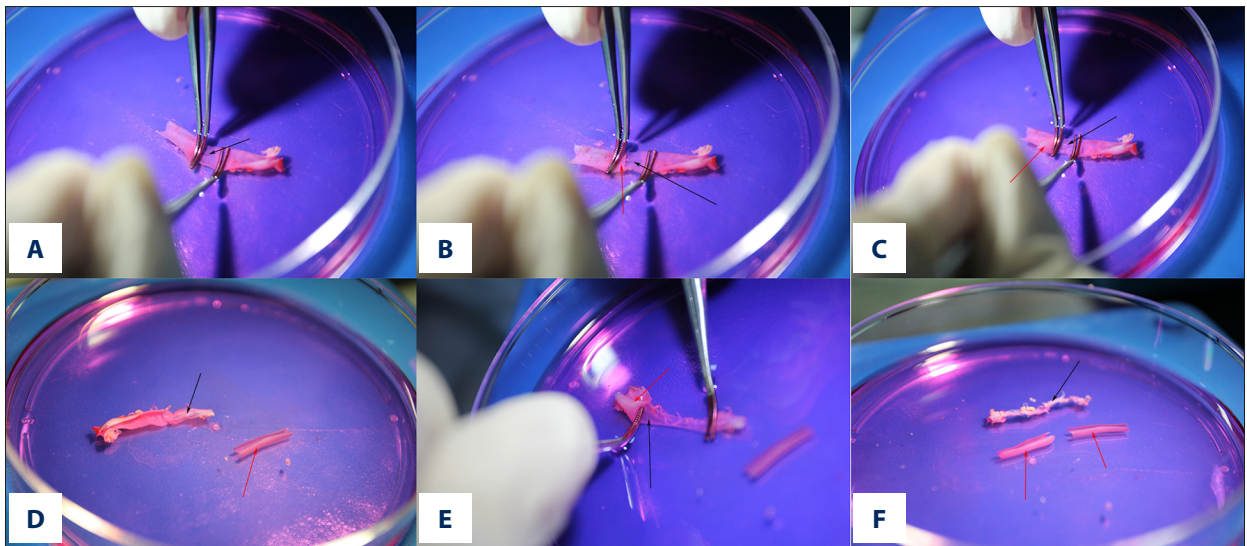


Figure 2. Steps for separation of the media. (A–C) A pair of ophthalmic curved tweezers was used to fix the artery by pressing the artery dorsally and another pair of ophthalmic curved tweezers was used to separate the media from the artery by pressing and pushing the artery dorsally. The media is indicated by the red arrow and the adventitia is indicated by the black arrow. The media (indicated in D, E, and F by the red arrow) was totally separated from the adventitia.

plates. The plates were placed in a cell culture chamber for about 4 h to let the small tissue blocks adhere to the plates. DMEM containing 20% FBS was carefully added and the tissue blocks were incubated in the cell culture chamber without disturbance for the first 5 days.

In the traditional tissue explants method, all the steps were similar, and after the fat tissue was removed, the artery was directly cut into small tissue blocks and transferred to cell culture plates without removal of the adventitia.

In the enzyme digestion method, after the fat tissue was removed, the aorta was digested with 1 mg/mL collagenase II and 100 µg/mL elastase at 37°C for 1 h. Later, the cells were pelleted and plated in DMEM with 20% FBS. The next morning, the cells were washed with PBS 3 times and the media were refreshed every 48 h.

The A7r5 cell line has been widely used *in vitro* to study the physiology and pathophysiology of VSMCs [13,14]. However, it has lost some VSMCs selectivity and has many differences from primary cultured VSMCs. Therefore, we chose the A7r5 cell line to compare its viability with primary cultured VSMCs.

The VSMCs obtained by the above methods (the new tissue explants method, the traditional tissue explants method, the enzyme digestion method, and A7r5 cell line) were identified through morphology and immunofluorescence detection of SM-actin. The purity of the VSMCs was tested through multiple fluorescent staining with DAPI and SM-actin antibody.

Immunohistochemistry

The second generations of VSMCs obtained by different methods were cultured on glass coverslips. Cells were washed with PBS and fixed in 4% paraformaldehyde. Cells were permeabilized by 0.1% Triton. Then, VSMCs were blocked with 10% goat serum in PBST and incubated with SM α -actin antibodies in PBST for 1 h. After washing, anti-mouse fluorescein isothiocyanate (FITC)-conjugated second antibody was incubated for 1 h and washed. Then, cells were incubated with DAPI (2 µg/ml) for 10 min and washed. Coverslips were then processed for immunofluorescent microscopy.

Western blot analysis

The third and seventh generations of VSMCs obtained by different methods were used. After incubation, cellular protein was obtained with the radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, Haimen, China) for Western blot analysis. BCA method (BCA Protein Assay kit, Beyotime Company, China) was used to detect the protein concentrations of the supernatant. The supernatants were then separated on SDS-PAGE

(10%) and transferred to polyvinylidene fluoride membranes. After that, the membranes were blocked with blocking buffer for 30 min at room temperature and then incubated with rabbit anti-SM α -actin, calponin, OPN, and SM-MHC monoclonal antibody (1: 1000 dilution), and mouse anti- β -actin monoclonal antibody (1: 10 000 dilution) overnight at 4°C. TBS-T was used to wash the membranes (3 times for 10 min), and the membranes were incubated with goat anti-rabbit IgG-HRP (1: 10 000 dilution), or goat anti-mouse IgG-HRP (1: 10 000 dilution) for 1 h at room temperature. The standard chemical luminescence method (Beyotime Company, China) was used to detect the antigen by exposing the membranes to Kodak X-Omat AR film. The resultant films were scanned on a gel imaging and analysis system and analyzed by Quantity One 4.4 (Bio-Rad, Hercules, CA).

Statistical analysis

All experiments were repeated 3 times. Data are expressed as means \pm SEM for all experiments. All statistical analyses were performed using SPSS 20.0. One-way analysis of variance (ANOVA) followed by Tukey's post hoc analysis was performed to compare between multiple experimental groups. The results were considered statistically significant at a P value of <0.05.

Results

Primary cultures of VSMCs and its identification

Observed with a phase-contrast microscope, cells began to grow out from the edge of the tissue blocks at about 8 days (Figure 3A), and became relatively confluent by approximately 16 days (Figure 3B). The primary culture of confluent VSMCs exhibited a typical spindle-shaped appearance with a characteristic "hill-and-valley" pattern (Figure 3C), and immunofluorescence showed 100% positive SM α -actin staining (Figure 3D).

Cell purity

Immunocytochemistry was used to test the expression of SM α -actin, which is a special marker for VSMCs. Then, DAPI was used to stain the nucleus. Because the DAPI combined with chromosomes non-selectively, the nucleus of all kinds of cells stained blue under the fluorescence microscope. The relationship between SM α -actin and nucleus reflected the purity of VSMCs. Compared with the cells obtained by the traditional tissue explants method, VSMCs obtained by our method showed higher purity ($P < 0.01$, Figure 4). The primary VSMCs obtained by enzyme digestion were less elongated, and showed a randomized actin filament distribution (Figure 4A).

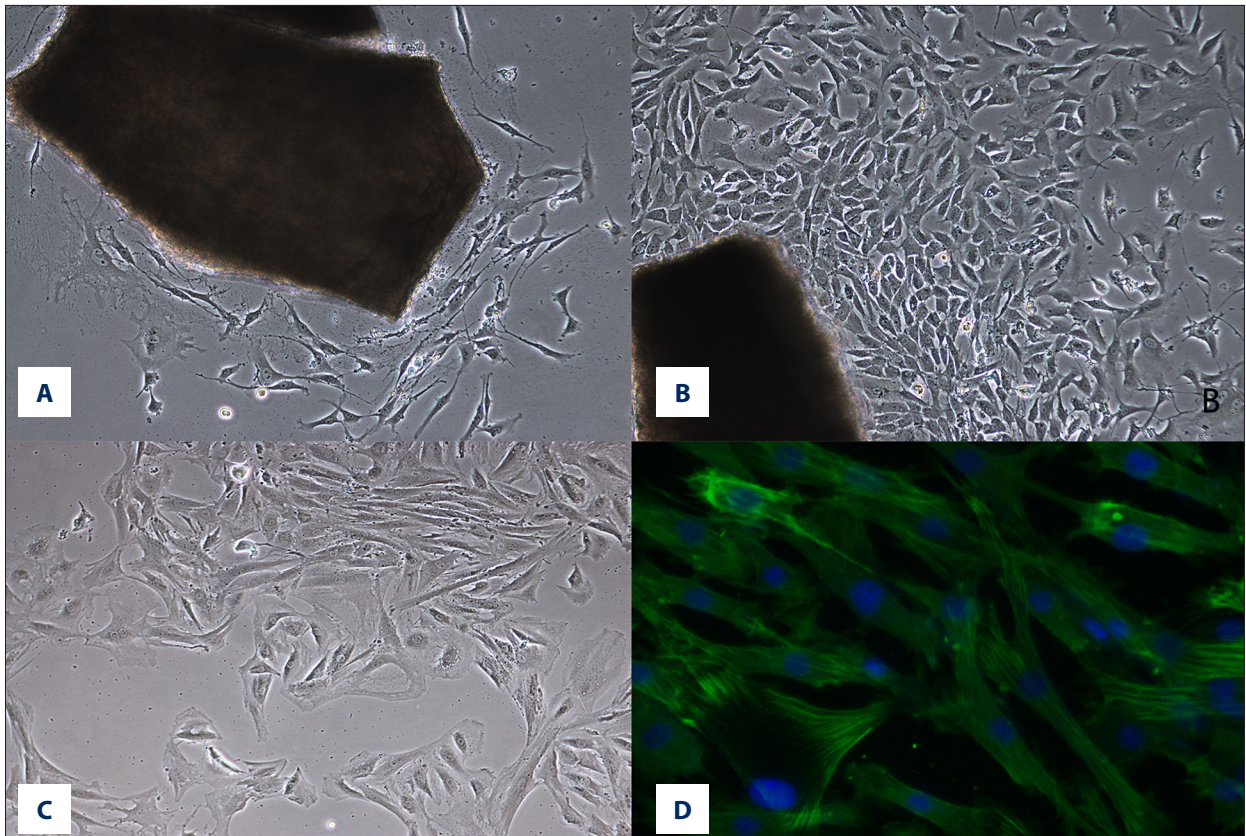


Figure 3. VSMCs identification by morphology and immunofluorescence. (A) Cells began to grow out from the edge of the tissue blocks at about 8 d ys. (B) Cells became relatively confluent by approximately 16 days. (C) Cells exhibited a typical spindle-shaped appearance with a characteristic "hill-and-valley" pattern. (D) Immunofluorescence showed 100% positive SM α -actin staining of the cells.

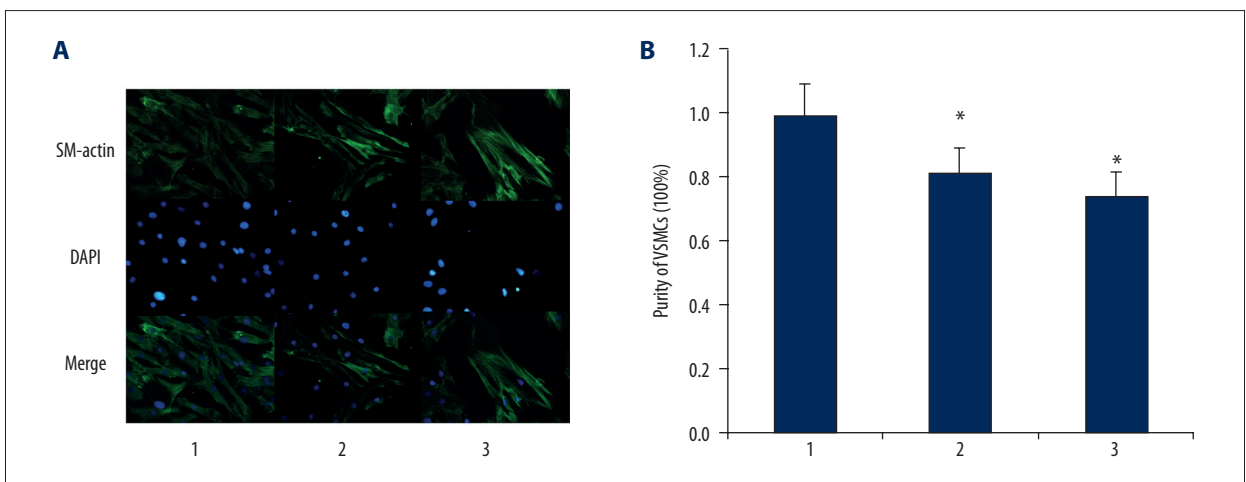


Figure 4. (A, B) VSMCs obtained by our method showed higher purity and elongation, with a regular actin filament distribution. VSMCs obtained by our method showed higher purity than the VSMCs isolated by traditional tissue explants. They were more elongated, with a regular actin filament distribution, than VSMCs isolated by enzyme digestion. 1 – VSMCs obtained by our method; 2 – VSMCs obtained by the traditional tissue explant method; 3 – VSMCs isolated by enzyme digestion.

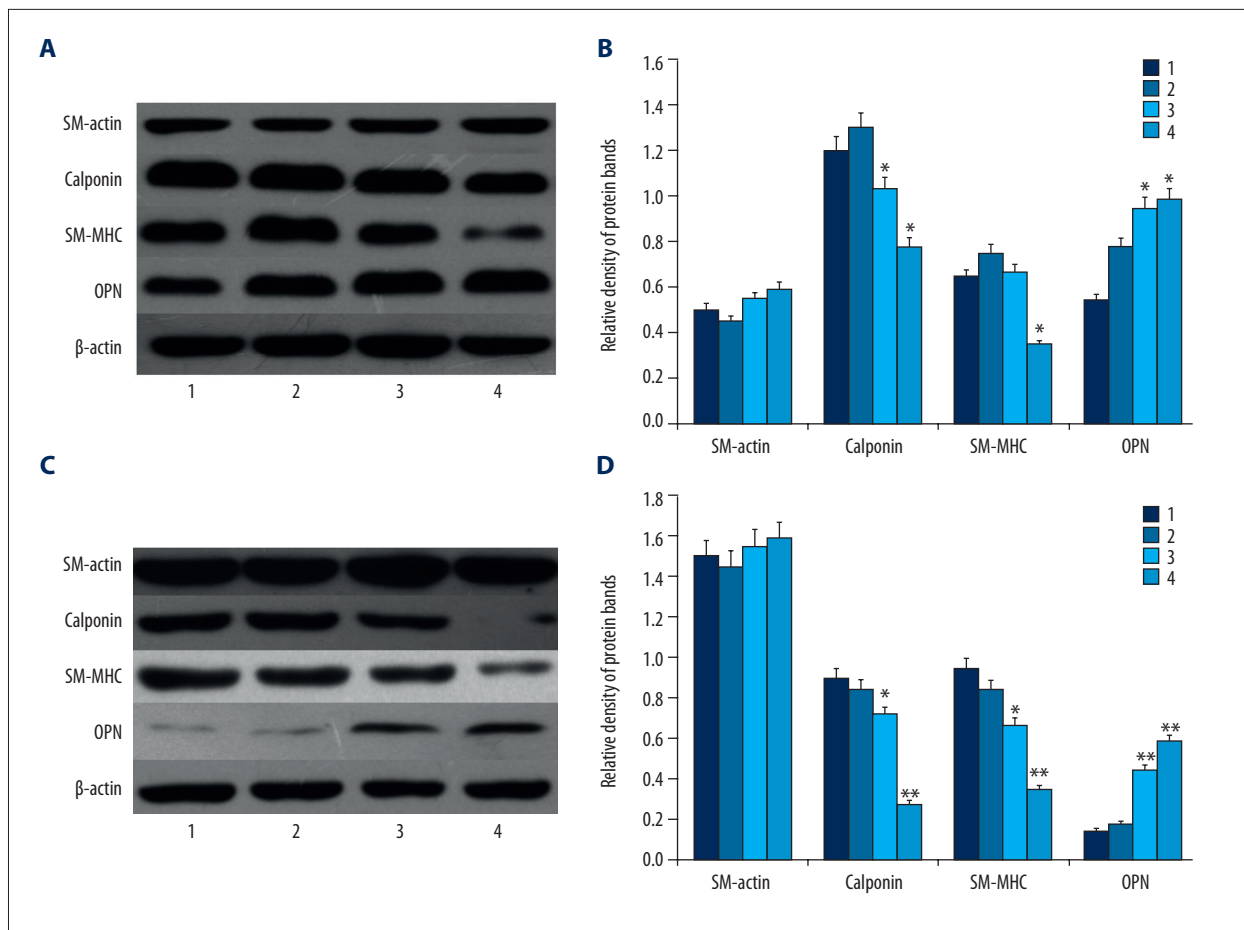


Figure 5. Expression of VSMCs phenotype-related proteins. The protein expression of SM-actin, Calponin, SM-MHC, and OPN was determined by Western blot (A, C). The histogram shows the means \pm SD of the densitometric scans of the different protein bands in the 3 experiments following normalization compared with β -actin or GAPDH (B, D). 1 – VSMCs isolated by our method; 2 – VSMCs isolated by the traditional tissue explant method; 3 – VSMCs obtained by enzyme digestion; 4 – A7r5 cell line. * $p < 0.05$ vs. 1; ** $p < 0.001$ vs. 1.

VSMCs phenotype identification

The phenotype of VSMCs obtained by the traditional tissue explants method, the enzyme digestion method, our new method, and A7r5 cell line were identified by testing the expression of VSMC phenotype-specific proteins SM α -actin, SM-MHC, calponin, and OPN. VSMCs from passage 3–7 are usually used for research; therefore, we identified the phenotype of VSMCs in passage 3 and passage 7. Our results showed that in the VSMCs of passage 3, the expression of calponin was decreased in the enzyme digestion and A7r5 cell line groups compared with the tissue explant groups. The “synthetic” phenotype-specific protein osteopontin (OPN) was increased in the enzyme digestion and A7r5 cell line groups ($P < 0.01$, Figure 5AB). This difference was further increased in the passage 7 VSMCs ($P < 0.01$, Figure 5CD). These results indicate that VSMCs obtained by the enzyme digestion method showed “contractile” phenotype characteristic in few generations and VSMCs obtained

by tissue explant method were much more stable than those obtained by enzyme digestion method or the A7r5 cell line.

Discussion

Despite the critical role of VSMCs in the development of cardiovascular diseases, very little effort has been made to develop an improved method for their primary culture. To overcome the disadvantages of traditional methods, it is necessary to obtain the media without adventitia. Studies so far were all focused on separating the adventitia from the media, and they found it inevitably affected the activity of tissue blocks because of the tight connection between media and adventitia. In the present study, we separated the media from the adventitia, not the adventitia from the media. We found it was easy to separate the media from the artery without hurting the activity of the media by using the blunt back side of a

pair of ophthalmic curved tweezers (Figure 2). This idea came from the “blunt dissection” technique commonly used in surgery to separate tissues, arteries, and nerves. As we expected, cells grow out from the edge of the tissue blocks and rapidly became relatively confluent. The purity of VSMCs obtained by this new method was nearly 100%, which was far higher than in the traditional tissue explants method.

VSMC undergo phenotypic modulation in response to extracellular signals and de-differentiate to the synthetic phenotype [15]. Collagenase induces VSMC proliferation and migration [16]. Because collagenase was the enzyme mainly used in the enzyme digestion method, the phenotype and functions of VSMCs obtained by this method are inevitably affected. Our results demonstrated that the $\text{SM}\alpha$ -actin was less elongated and irregularly distributed in the VSMCs obtained by enzyme digestion method. We used Western blot analysis to test the expression of VSMCs phenotype-specific markers in the third and seventh generation of VSMCs, and found that the expression of calponin were decreased in the VSMCs obtained by enzyme digestion method, and the expression of OPN increased. These results all indicate that collagenase affected the phenotype of VSMCs, and VSMCs obtained by enzyme digestion method retained the “contractile” phenotype for fewer generations than with the tissue explants method.

The A7r5 cell line is widely used *in vitro* to study the physiology and pathophysiology of VSMCs [13,14]. Some studies demonstrated this cell line as the “contractile” phenotype [17,18], while others showed the A7r5 cell line is “synthetic” phenotype VSMCs [19]. We tested the VSMCs phenotype-specific markers in the third and seventh generation of the A7r5 cell line and found that the cell line showed a more “synthetic” phenotype characteristic compared with the VSMCs obtained by the tissue explants method.

Many studies have been focused on the VSMCs phenotype switch as its critical role in the development of most cardiovascular disease [20–22]. Many research results depend on the kind of cells used. The ideal cells have the highly “contractile” phenotype characteristic and are not affected by cell passage. Our results show that VSMCs obtained by the tissue explants method are the best.

Conclusions

We established a simple and efficient method to isolate rat aortic VSMCs. The VSMCs showed a higher purity than the VSMCs obtained from the traditional tissue explants method and manifested more “contractile” phenotype characteristic and greater stability than VSMCs obtained by the enzyme digestion method or the A7r5 cell line.

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