JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 5, NO. 1, 2020

PRECLINICAL RESEARCH

Sonodynamic Therapy Suppresses Neovascularization in Atherosclerotic Plaques via Macrophage Apoptosis-Induced Endothelial Cell Apoptosis

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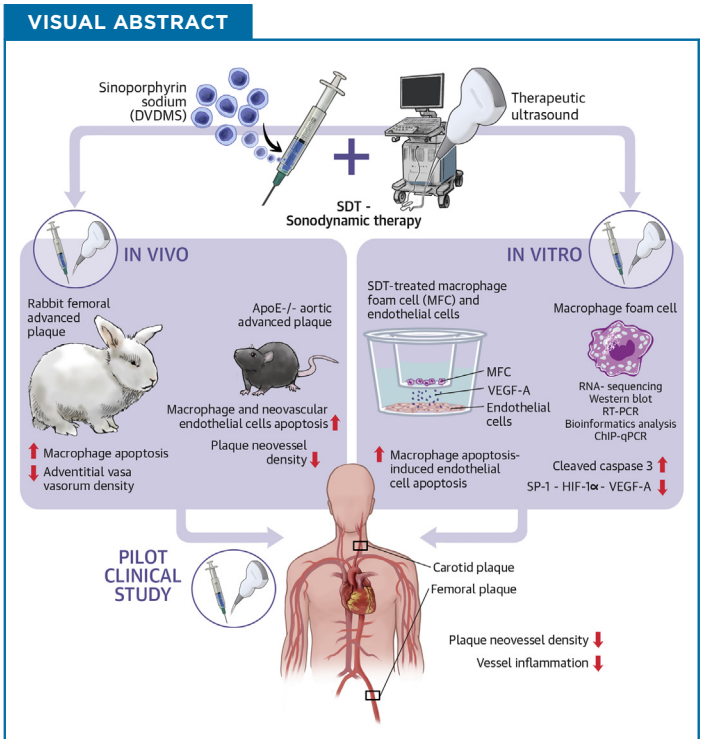
VISUAL ABSTRACT



Yao, J. et al. J Am Coll Cardiol Basic Trans Science. 2020;5(1):53–65.

HIGHLIGHTS

- **DVDMS-SDT reduces neovascularization in late-stage atherosclerotic lesions in both rabbit and mouse models.**
- **DVDMS-SDT enhances macrophage foam cell apoptosis, which in turn induces neovessel endothelial cell apoptosis and inhibits its proliferation, migration, and tubulogenesis, termed apoptosis-induced apoptosis.**
- **Mechanistically, DVDMS-SDT induces macrophage foam cell apoptosis via mitochondrial-caspase pathway, which activates caspase 3 to cleave SP-1, leading to the reduction of HIF-1α and VEGF-A.**
- **In the pilot translational study, DVDMS-SDT reduces plaque angiogenesis and inhibits vessel inflammation.**

ISSN 2452-302X https://doi.org/10.1016/j.jacbts.2019.10.007

JACC: BASIC TO TRANSLATIONAL SCIENCE VOL. 5, NO. 1, 2020
JANUARY 2020:53–65

ABBREVIATIONS AND ACRONYMS

ALA = 5-aminolevulinic acid

ApoE = apolipoprotein E

ChIP = chromatin immunoprecipitation

DVDMS = sinoporphyrin sodium

DVDMS-SDT = sinoporphyrin sodium-mediated sonodynamic therapy

HIF = hypoxia inducible factor

HUVEC = human umbilical vein endothelial cells

MVE = normalized maximal video-intensity enhancement

SDT = sonodynamic therapy

SP = specificity protein

TBR = target-to-background ratio

VEGF-A = vascular endothelial growth factor A

SUMMARY

During atherosclerosis plaque progression, pathological intraplaque angiogenesis leads to plaque rupture accompanied by thrombosis, which is probably the most important cause of arteries complications such as cerebral and myocardial infarction. Even though few treatments are available to mitigate plaque rupture, further investigation is required to develop a robust optimized therapeutic method. In this study using rabbit and mouse atherosclerotic models, sinoporphyrin sodium (DVDMS)-mediated sonodynamic therapy reduced abnormal angiogenesis and plaque rupture. Briefly, DVDMS is injected to animals, and then the plaque was locally exposed to pulse ultrasound for a few minutes. Furthermore, a small size clinical trial was conducted on patients with atherosclerosis. Notably, a significant reduction of arterial inflammation and angiogenesis was recorded following a short period of DVDMS-mediated sonodynamic therapy treatment. This beneficial outcome was almost equivalent to the therapeutic outcome after 3-month intensive statin treatment.

Pathological angiogenesis is a crucial factor that leads to vulnerable atherosclerotic plaque rupture (1). Thus, inhibition of plaque neovascularization provides an important therapeutic strategy to minimize or even prevent vulnerable lesion disruption and subsequent atherothrombotic complications. Vascular endothelial growth factor A (VEGF-A), a well-known hypoxia-inducible factor (HIF) target gene, constitutes the main regulator of neovascularization (2). To date, VEGF-A inhibitors have been used as systemic medications that directly exert an effect on endothelial cells to inhibit angiogenesis in cancer chemotherapy. However, the use of current VEGF-A blockers results in severe or even fatal off-target effects, such as hypertension and thromboembolism, because of the disruption of endothelial homeostasis (3–5). Therefore, a site-specific treatment that indirectly targets neovessel endothelial cells may present a safer and more feasible alternative to suppress plaque neovascularization.

Pathological neovessels induce intraplaque hemorrhage and inflammatory signals that result in the infiltration of macrophages into atherosclerotic plaques (6). In turn, these macrophages secrete VEGF-A on HIF-1α activation to promote pathological angiogenesis, vascular permeability, and inflammatory cell recruitment (7). The mutual exacerbation of pathological neovascularization and macrophage-mediated inflammation triggers plaque rupture (1,8). Accordingly, suppressing macrophage-mediated inflammation may play an essential role in inhibiting plaque neovascularization.

SEE PAGE 66

Sonodynamic therapy (SDT) constitutes a noninvasive treatment that utilizes ultrasound to locally activate sonosensitizer, thereby stimulating the

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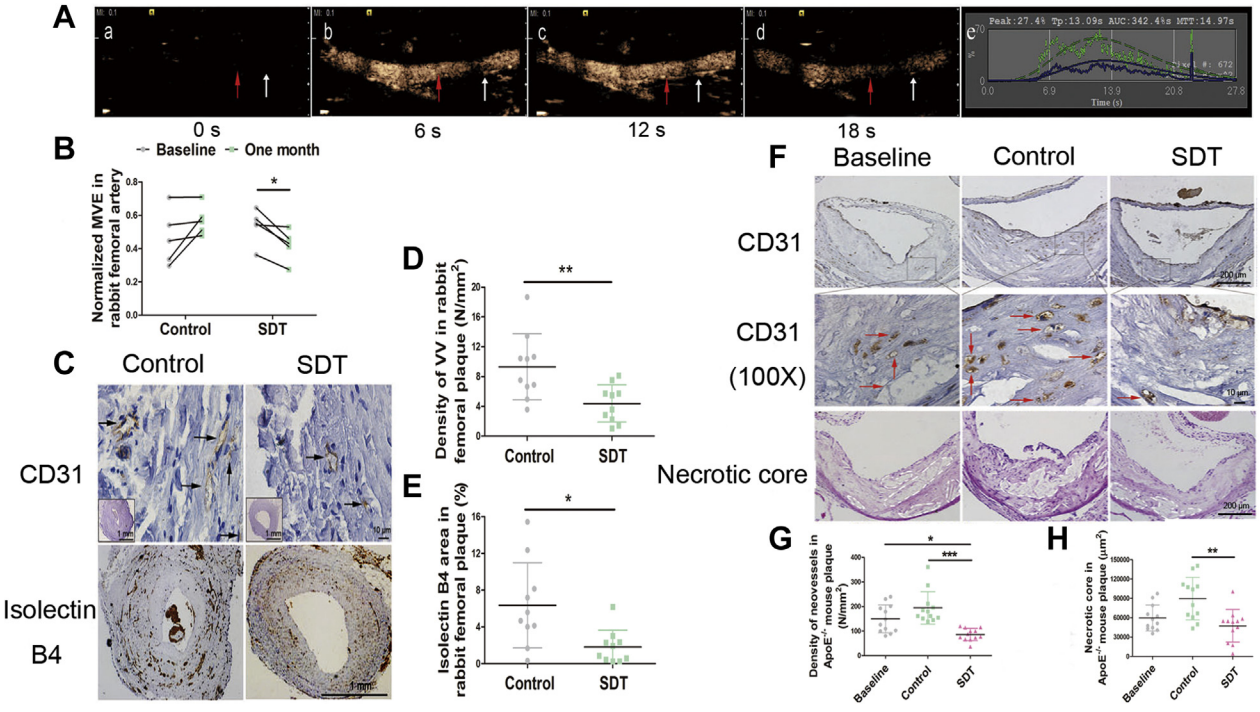
The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate. For more information, visit the *JACC: Basic to Translational Science* author instructions page.

Manuscript received July 29, 2019; revised manuscript received October 7, 2019, accepted October 7, 2019.

FIGURE 1 DVDMS-SDT Suppresses Neovascularization and Increases the Stability of Rabbit and ApoE$^{-/-}$ Mouse Advanced Atherosclerotic Plaque



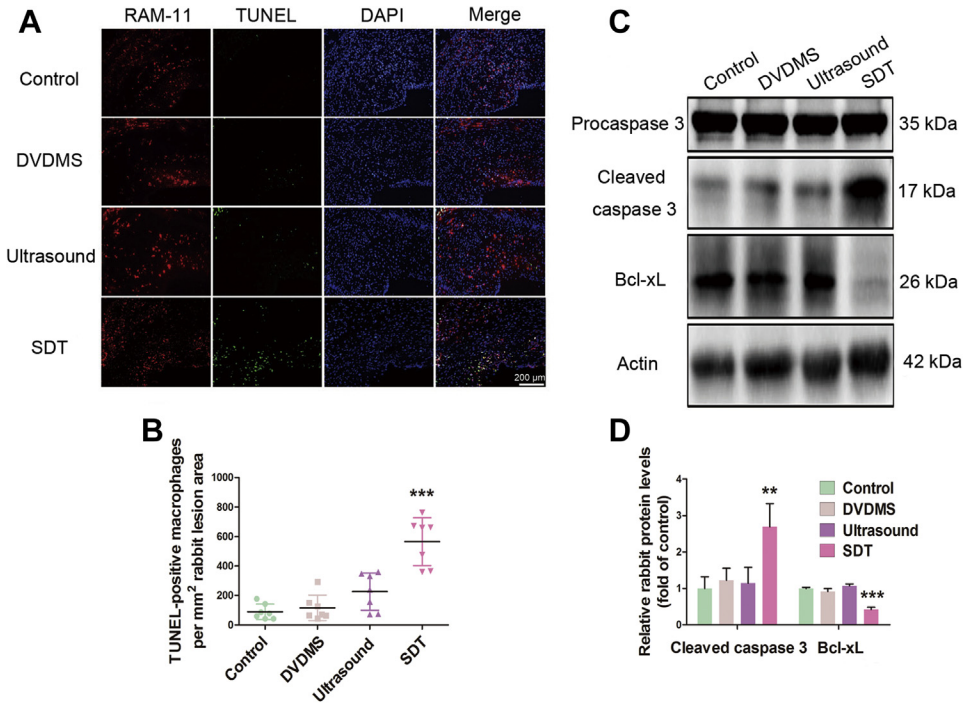
Sinoporphyrin sodium-mediated sonodynamic therapy (DVDMS-SDT) reduces abnormal adventitial vasa vasorum (VV) density at 1 month after treatment in rabbit femoral advanced plaque. **(A)** Representative consecutive contrast-enhanced ultrasound images and time-intensity curves. Prior to microbubble injection, the right femoral artery lumen **(red arrow)** and adventitia **(white arrow)** are dark and hypoechoic **(a)**, becoming visible at 6 s **(b)**. After 12 s, maximal microbubble penetration echo signal occurs in the adventitia **(c)**, up to 18 s **(d)**. **Green and blue** time-intensity curves represent lumen and adventitia microbubble concentration over time **(e)**. **(B)** Normalized maximal video-intensity enhancement (MVE) quantification (n = 5). Histopathological staining of plaque sections **(C)** and quantification **(D, E)** (n = 10). **Black arrows** indicate abnormal adventitial VV. DVDMS-SDT inhibits neovascularization at 1 month after treatment in mouse advanced plaque. Histopathological staining of the aortic plaque **(F)** and quantification **(G, H)** (n = 12). **Red arrows** indicate intraplaque neovessels. Area surrounded by **dashed lines** indicates the necrotic core. *p < 0.05; **p < 0.01; ***p < 0.001. ApoE = apolipoprotein E.

production of reactive oxygen species to modulate cell function or fate (9). We previously demonstrated that 5-aminolevulinic acid (ALA)-mediated SDT stabilizes plaques by inducing macrophage apoptosis and clearance without causing obvious off-target effects (10). However, the beneficial effect of SDT toward plaque neovascularization has not yet been defined. Additionally, the sonosensitizer precursor ALA by oral administration needs to be converted into sonosensitizer protoporphyrin IX through metabolism, meaning high dosage in clinical practice. Sinoporphyrin sodium (DVDMS), a novel sonosensitizer, triggered mitochondrial-caspase-dependent apoptosis in human esophageal cancer (ECA-109) cells and was observed to have higher brightness and singlet oxygen-generation efficiency than protoporphyrin IX (11). Therefore, in the present study, we utilized DVDMS as a sonosensitizer in rabbit and apolipoprotein E (ApoE)$^{-/-}$ mouse models of advanced plaque, as well as in patients with atherosclerosis, to investigate whether sinoporphyrin sodium-mediated sonodynamic therapy (DVDMS-SDT) produces significant protective effects on lesion stabilization by suppressing neovascularization. Moreover, human leukemia monocytic cell (THP-1) and mouse peritoneal macrophage-derived foam cells, human umbilical vein endothelial cells (HUVEC), and mouse aortic endothelial cells were utilized to elucidate the underlying mechanisms.

METHODS

ANIMAL EXPERIMENTS. The animal protocol was approved by the Ethics Committee of Harbin Medical University. All experiments were performed in accordance with ethical guidelines set out by the United Kingdom Animals (Scientific Procedures) Act 1986 and Directive 2010/63/EU of the European

FIGURE 2 DVDMS-SDT Induces Macrophage Apoptosis at Day 3 After Treatment in the Rabbit Femoral Advanced Plaque



(A, B) Representative sections and quantification of terminal uridine nick-end labeling (TUNEL)-positive macrophages (rabbit ant-mouse [RAM]-11$^+$ TUNEL$^+$ 4′,6-diamidino-2-phenylindole [DAPI]$^+$) in plaque from different groups (n = 7). **(C, D)** Representative Western blot of femoral advanced plaques immunoblotted and probed with antibodies against procaspase 3, cleaved caspase 3, and B-cell lymphoma–extra large transmembrane molecule (Bcl-xL) proteins. Cleaved caspase 3 and Bcl-xL protein expression relative to that of actin was quantified by densitometry. **$p < 0.01$, and ***$p < 0.001$ versus control. Abbreviations as in Figure 1.

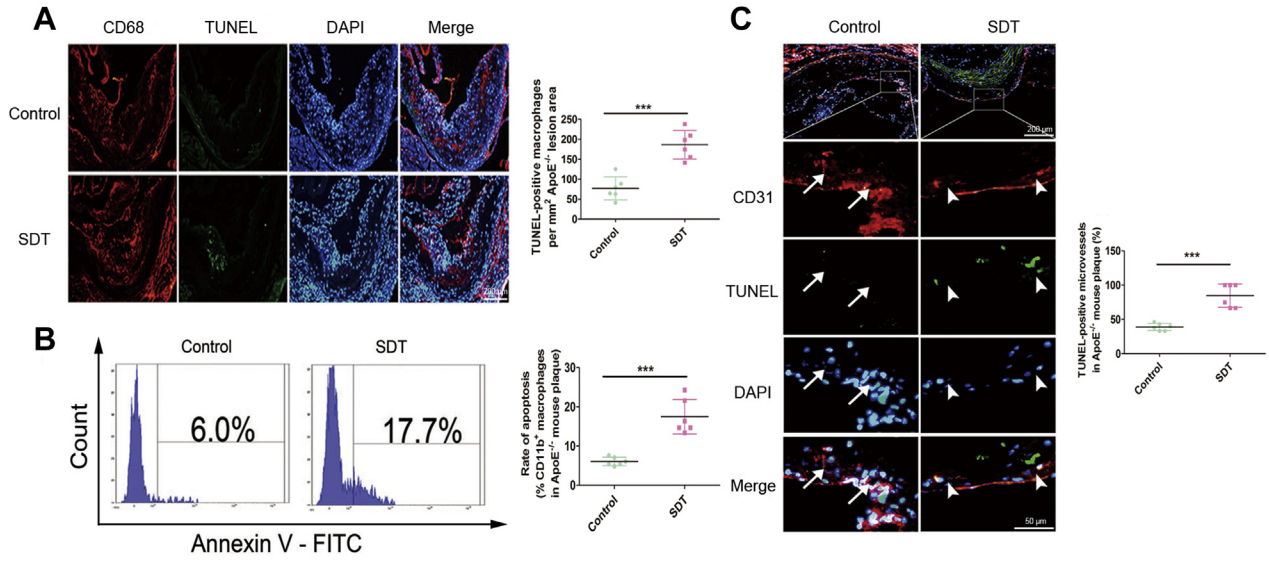
Parliament on the protection of animals used for scientific purposes. Studies conformed to the *Guide for the Care and Use of Laboratory Animals* published by the U.S. National Institutes of Health. Adult male New Zealand White rabbits (Solarbio Bioscience and Technology Co., Ltd., Shanghai, China) and male ApoE$^{-/-}$ mice (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) (C57BL6 background) were housed in the Animal Care Facilities of the First Affiliated Hospital of Harbin Medical University. Expanded methods are available in the Supplemental Appendix.

CELL EXPERIMENTS. THP-1 cells, mouse peritoneal macrophages, primary HUVEC (Cyagen Biosciences Inc., Guangzhou, China), primary mouse aortic endothelial cells, and human embryonic kidney cells (HEK-293, catalog no. CRL-1573, ATCC, Manassas, Virginia) were cultured in a humidified atmosphere with 5% CO_2 at 37°C as described in the Supplemental Appendix.

CLINICAL STUDY. This clinical pilot study was conducted at the First Affiliated Hospital of Harbin Medical University between February 2016 and May 2017 in compliance with the principles of the Declaration of Helsinki and according to Good Clinical Practice guidelines. In the stage 1 study, of 42 subjects who were initially screened, 24 subjects received SDT treatment with complete data documented. In the stage 2 study, of 32 subjects who were initially screened, 14 subjects were allocated into either SDT group or age- and sex-matched control group; 14 had evaluable ultrasound images; and 12 had evaluable fludeoxyglucose F 18-positron emission tomography-computed tomography images at the end of the study. The study protocol was described in the Supplemental Appendix.

STATISTICAL ANALYSIS. All quantitative data are expressed as the mean ± SD. The statistical analysis was performed using GraphPad Prism (version 6.0, GraphPad Software, La Jolla, California). A normality test (Shapiro-Wilk) was performed to determine whether the data were normally distributed. If data were normally distributed, the Student's unpaired

FIGURE 3 DVDMS-SDT Induces Macrophage and Neovascular Endothelial Cell Apoptosis at Day 3 After Treatment in ApoE$^{-/-}$ Mouse Advanced Plaque



(A) Representative aortic sections and quantification of TUNEL-positive macrophages in plaque (n = 7). **(B)** Representative cytograms and quantification of the proportion of apoptotic macrophages in plaque (n = 6). **(C)** Representative sections and quantification of the proportion of TUNEL-positive microvessels in plaque (n = 7). **Arrows and arrowheads** indicate TUNEL-negative and TUNEL-positive microvessels, respectively. ***p < 0.001. FITC = fluorescein isothiocyanate; other abbreviations as in Figures 1 and 2.

Student's t-test was used to determine the significant difference between 2 groups. One-way analysis of variance followed by Dunnett or Tukey post hoc testing, or 1-way or 2-way analysis of variance with repeated measures followed by Dunnett or Sidak post hoc testing were used to determine the significant difference between multiple groups. All qualitative data are expressed as frequencies (proportions); Fisher exact test was performed to analyze the qualitative data. All $p < 0.05$ were considered statistically significant.

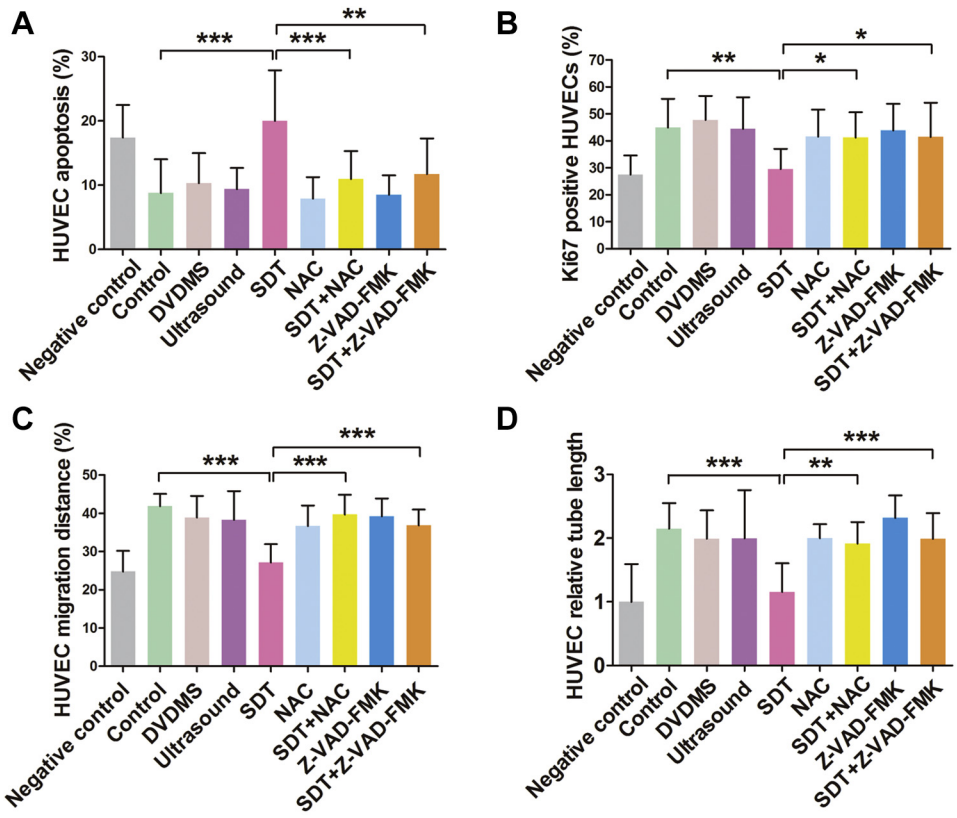
RESULTS

DVDMS-SDT SUPPRESSES NEOVASCULARIZATION AND INCREASES THE STABILITY OF RABBIT AND MOUSE ADVANCED ATHEROSCLEROTIC PLAQUE. Therapeutic ultrasound was utilized on rabbit plaques 4 h following injection of different concentrations of DVDMS. We determined 4 mg/kg DVDMS was the optimal dosage for SDT to obtain the greatest inhibitory effect on plaque progression 1 month after treatment (Supplemental Tables 1A and 1B). Four weeks after DVDMS-SDT treatment, the normalized maximal video-intensity enhancement (MVE), as a marker for neovessel density assessed by contrast-enhanced ultrasonography, was reduced by 20% compared with baseline (**Figures 1A and 1B**). In addition, DVDMS-SDT substantially reduced the plaque rupture rate compared with that of the control group (10% vs. 70%) (Supplemental Table 1C). Consistent with this result, histopathological analysis showed that DVDMS-SDT markedly reduced abnormal vasa vasorum density, erythrocyte membrane content (**Figures 1C to 1E**), macrophages, and proliferating cell nuclear antigen-positive cells in the plaque (Supplemental Table 1D).

With the same dosage of 4 mg/kg DVDMS required for SDT to inhibit plaque progression 4 weeks after treatment (Supplemental Table 2A), DVDMS-SDT treatment remarkably suppressed the neovessel density and necrotic core size in the mouse advanced plaque (**Figures 1F to 1H**).

DVDMS IS EXCLUSIVELY UPTAKEN BY LESION RESIDENT MACROPHAGES AND DVDMS-SDT ENHANCES MACROPHAGE AND NEOVASCULAR ENDOTHELIAL CELL APOPTOSIS. To clarify the cell-specific response on DVDMS-SDT, we observed that 4 h after injection, DVDMS exclusively accumulated in macrophages in rabbit advanced femoral plaque rather than in arterial smooth muscle cells and endothelial cells or in normal femoral arteries (Supplemental Figures 1A to 1I). DVDMS distribution in the advanced plaques of ApoE$^{-/-}$ mice was consistent with that found in rabbits

FIGURE 4 DVDMS-SDT Enhances THP-1–Derived Foam Cell and Subsequent Apoptosis and Inhibits Proliferation, Migration, and Tube Formation of HUVEC



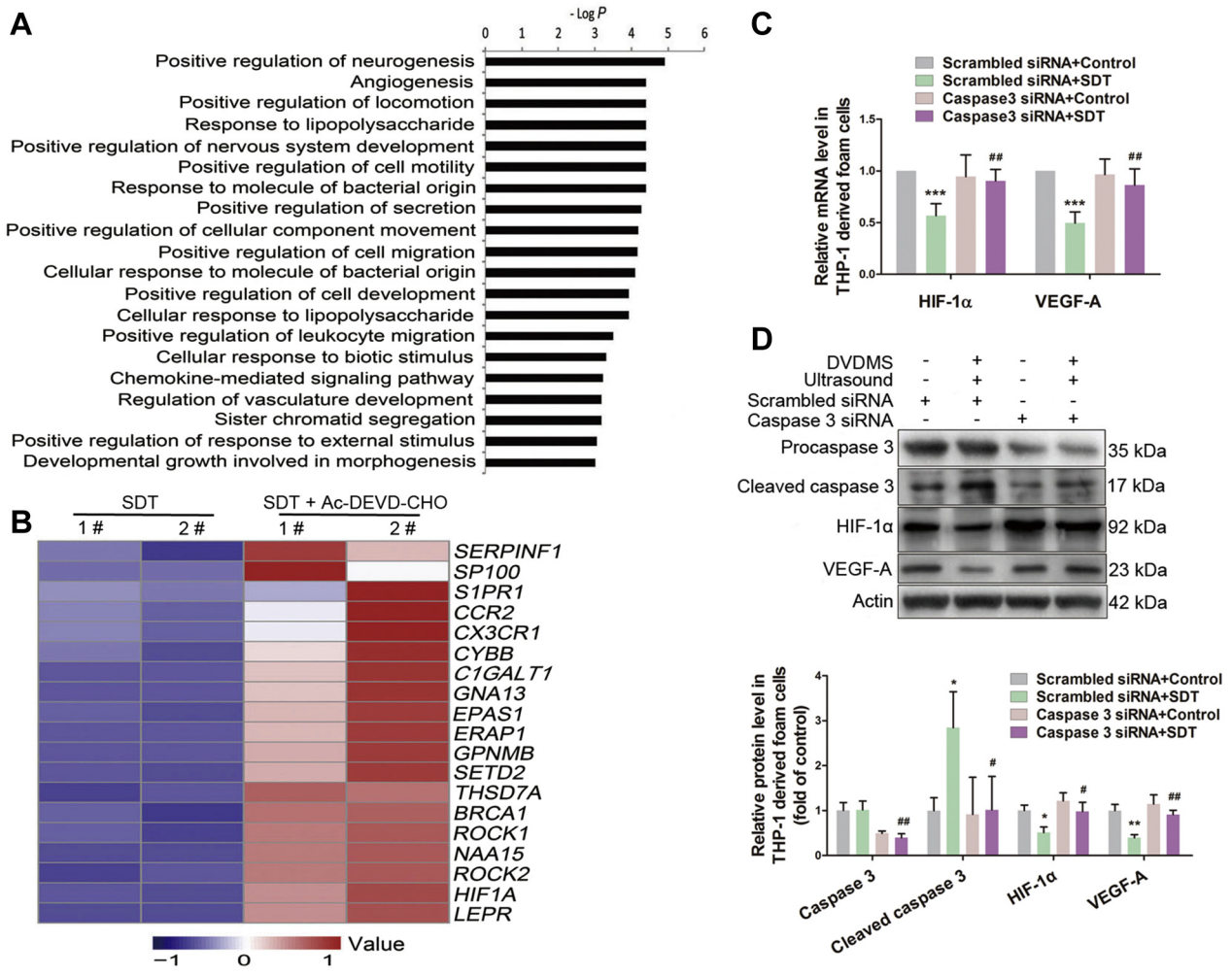
THP-1-derived foam cells were pre-treated with N-acetyl-L-cysteine (NAC) or Z-VAD-FMK for 1 h, followed by DVDMS-SDT treatment. Cells were transferred to the upper chamber of a coculture plate and cocultured with human umbilical vein endothelial cells (HUVEC) (lower chamber) for 48 h; alternatively, culture medium substituted in the upper chamber was used as the negative control. Quantification of HUVEC apoptosis **(A)**, proliferation **(B)**, migration at 6 h after scratching **(C)**, and tube formation **(D)** (n = 4, 5, 4, and 4). Data were from 3 independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. THP-1 = human leukemia monocytic cell; Z-VAD-FMK = methyl (3S)-5-fluoro-3-[[(2S)-2-[[(2S)-3-methyl-2-(phenylmethoxycarbonlamino)butanoyl]amino]propanoyl]amino]-4-oxopentanoate; other abbreviations as in Figure 1.

(Supplemental Figures 1J to 1M). Notably, at day 3 after DVDMS-SDT, the number of apoptotic macrophages in the rabbit plaque increased approximately 5-fold (**Figures 2A and 2B**). The protein level of cleaved caspase 3 increased 1.7-fold, whereas that of the B-cell lymphoma-extra large transmembrane molecule decreased 60% (**Figures 2C and 2D**). Consistently, mouse lesion site macrophage apoptosis (**Figures 3A to 3B**) and neovascular endothelial cell apoptosis (**Figure 3C**) were significantly increased compared with those in control mice.

CO-CULTURE CELL STUDY REVEALS NEOVASCULAR ENDOTHELIAL CELL APOPTOSIS IS TRIGGERED BY DVDMS-SDT–INDUCED FOAM CELL APOPTOSIS. We applied in vitro study to reveal the mechanisms of suppressed neovascularization. First, with optimized parameters, we discovered significant foam cell apoptosis, as well as caspase 3 activation on DVDMS-SDT (Supplemental Figures 2A to 2D, 3A to 3D, and 4). Then with the same parameter, we incubated HUVEC with 0.2 μmol/l DVDMS for 6 h and found no significant alterations of HUVEC viability, proliferation, migration, or tubulogenesis, meaning that DVDMS-SDT does not cause dysfunction of normal vascular endothelial cells (Supplemental Figure 5).

Due to the lesion resident macrophages as dominant target cells on DVDMS-SDT, we further utilized cell coculture system to observe the macrophage-endothelial cell interaction to address the mechanisms of neovascular endothelial cell apoptosis. As expected, the intracellular fluorescence intensity of DVDMS in HUVEC was only 10% of that in

FIGURE 5 DVDMS-SDT Inhibits HIF-1α and VEGF-A Expression on Activation of Caspase 3 in THP-1–Derived Foam Cells



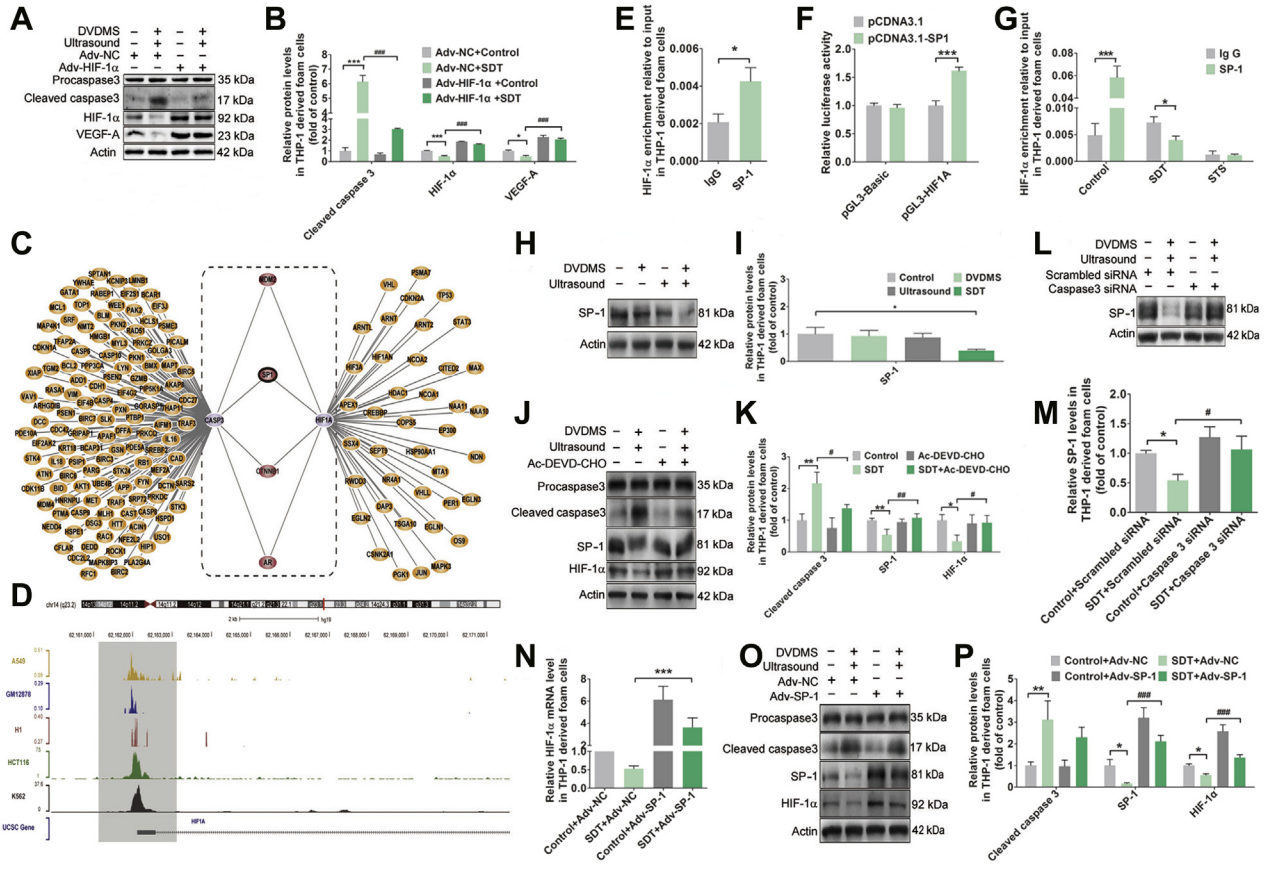
(A) Transcription expression profiling in DVDMS-SDT– and DVDMS-SDT+Ac-DEVD-CHO–treated cells assessed by ribonucleic acid sequencing. Significantly enriched gene ontology term biological processes were analyzed. **(B)** Heat map of differently expressed genes in DVDMS-SDT– and DVDMS-SDT+Ac-DEVD-CHO–treated cells associated with angiogenesis. **(C)** Quantitative real-time polymerase chain reaction analysis of the messenger ribonucleic acid (mRNA) expression of hypoxia inducible factor (HIF)-1α and vascular endothelial growth factor A (VEGF-A) (n = 5). **(D)** Immunoblotting analysis of caspase 3, HIF-1α, and VEGF-A protein levels and quantification. *$p < 0.05$, and **$p < 0.01$, and ***$p < 0.001$ versus scrambled small interfering ribonucleic acid (siRNA) + control. $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ versus scrambled siRNA + SDT. Abbreviations as in **Figure 1**.

THP-1-derived foam cells (Supplemental Figures 2E and 2F). Consistently, the fluorescence intensity of DVDMS in mouse peritoneal macrophage-derived foam cells after incubation with 0.7 μmol/l DVDMS for 5 h was increased by approximately 9-fold, whereas that of DVDMS in mouse aortic endothelial cells was not increased (Supplemental Figures 3E and 3F).

But interestingly, when DVDMS-SDT-treated macrophage foam cells were cocultured with vascular endothelial cells, the apoptotic rate of endothelial cells increased dramatically on foam cell apoptosis (**Figure 4A**, Supplemental Figure 6A), whereas proliferation (**Figure 4B**, Supplemental Figure 6B), migration (**Figure 4C**, Supplemental Figure 6C), and tubulogenesis (**Figure 4D**, Supplemental Figure 6D) decreased significantly.

DVDMS-SDT ACTIVATES CASPASE 3 AND SUBSEQUENT HIF-1α AND VEGF-A REDUCTION IN FOAM CELLS VIA SP-1 CLEAVAGE. In addition to triggering an apoptotic cascade, caspase 3 also plays important

FIGURE 6 Caspase 3 Activated by DVDMS-SDT Cleaves SP-1 Leading to the Inhibition HIF-1α Expression in THP-1-Derived Foam Cells

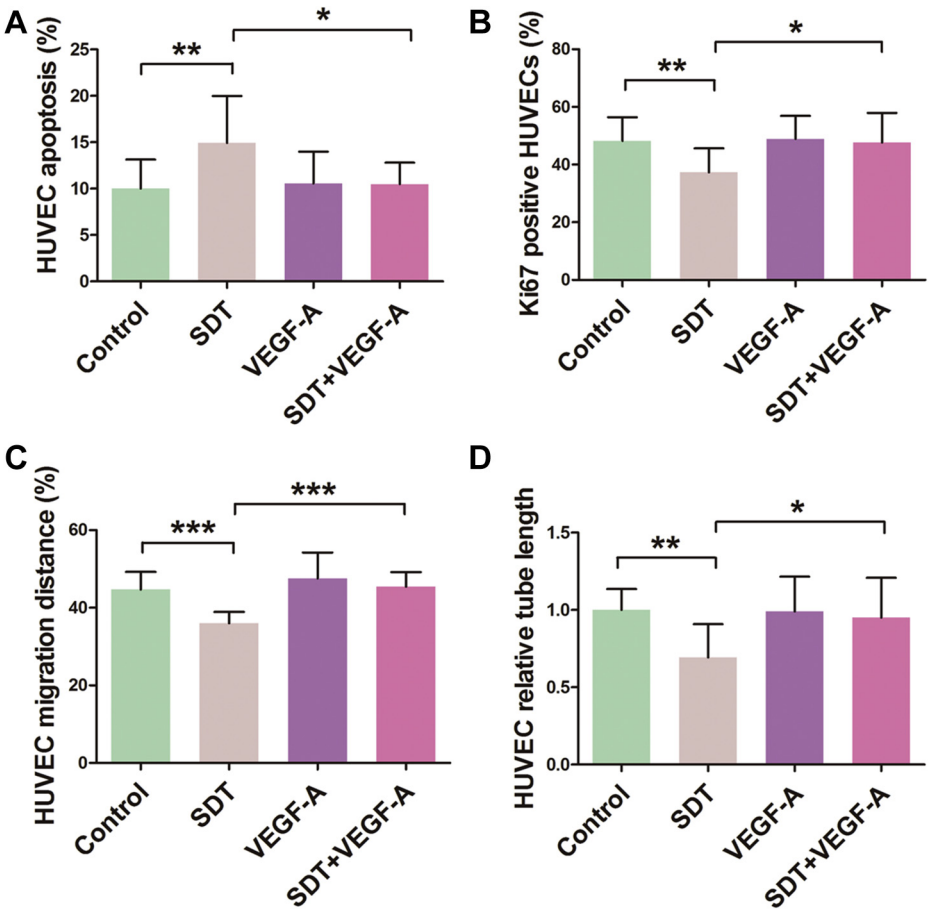


Immunoblotting of caspase 3, HIF-1α, and VEGF-A **(A)** and quantification **(B)**. **(C)** Caspase 3–HIF-1α interaction network. **Purple nodes** = nodes of interest of caspase 3 and HIF-1α; **orange nodes** = neighboring nodes of caspase 3 and HIF-1α; **red nodes** = caspase 3 substrates; **outer black circle** = transcription factor regulating *HIF1A*; **lines** = protein interactions. **Dashed box** = 4 proteins (MDM2, SP-1, CTNNB1, AR) associate CASP3 and HIF-1A. **(D)** UCSC (University of California, Santa Cruz) Genome Browser screenshot showing genomic coordination and UCSC gene and transcription factor specificity protein 1 (SP-1) chromatin immunoprecipitation (ChIP)-sequencing tracks (A549, GM12878, H1, HCT116, and K562 cell lines) of *HIF1A* from ENCODE. Shadowed regions: *HIF1A* promoter. **(E)** ChIP-quantitative polymerase chain reaction (PCR) in THP-1–derived foam cells (n = 3). **(F)** Luciferase promoter reporter assays in HEK293 cells (n = 5). **(G)** ChIP-quantitative PCR analyses of SP-1–*HIF1A* promoter binding (n = 3). **(H to M, O, P)** Caspase 3, SP-1, and HIF-1α immunoblotting and quantification. **(N)** *HIF1A* mRNA quantitative real-time PCR (n = 5). *p < 0.05; **p < 0.01; ***p < 0.001. #p < 0.05; ##p < 0.01; ###p < 0.001. Adv = adenovirus; NC = negative control; pcDNA = plasmid cytomegalovirus promoter deoxyribonucleic acid vector; pGL3 = polygalacturonase 1 beta-like protein 3; STS = staurosporine; other abbreviations as in Figures 1 and 5.

roles in tissue differentiation and regeneration (12). However, the regulatory role of caspase 3 in angiogenesis has not been identified. To address the molecular mechanism underlying apoptotic macrophage foam cell-induced endothelial cell apoptosis on DVDMS-SDT treatment, we performed ribonucleic acid sequencing and bioinformatics analysis and found that 720 genes were significantly altered (p = 0.032) in DVDMS-SDT-treated THP-1-derived foam cells compared with DVDMS-SDT plus caspase 3 inhibitor Ac-DEVD-CHO-treated cells. Gene ontology enrichment analysis revealed that the second most affected functional pathway was angiogenesis (Figure 5A). The heat map showed that DVDMS-SDT down-regulated a number of genes associated with angiogenesis signaling including HIF-1A, which was blocked by caspase 3-specific inhibitor Ac-DEVD-CHO (Figure 5B). Studies have well established that HIF-1α promotes VEGF-A expression (13,14). In the present study, we confirmed that DVDMS-SDT reduced both messenger ribonucleic acid and protein levels of HIF-1α and VEGF-A (Figures 5C and 5D) by activating caspase 3, which was reversed by HIF-1α overexpression (Figures 6A and 6B).

However, we found no direct regulatory effect of caspase 3 on HIF-1α. Therefore, we hypothesized that

FIGURE 7 Effects of VEGF-A Stimulation on Cocultured THP-1–Derived Foam Cells and HUVEC

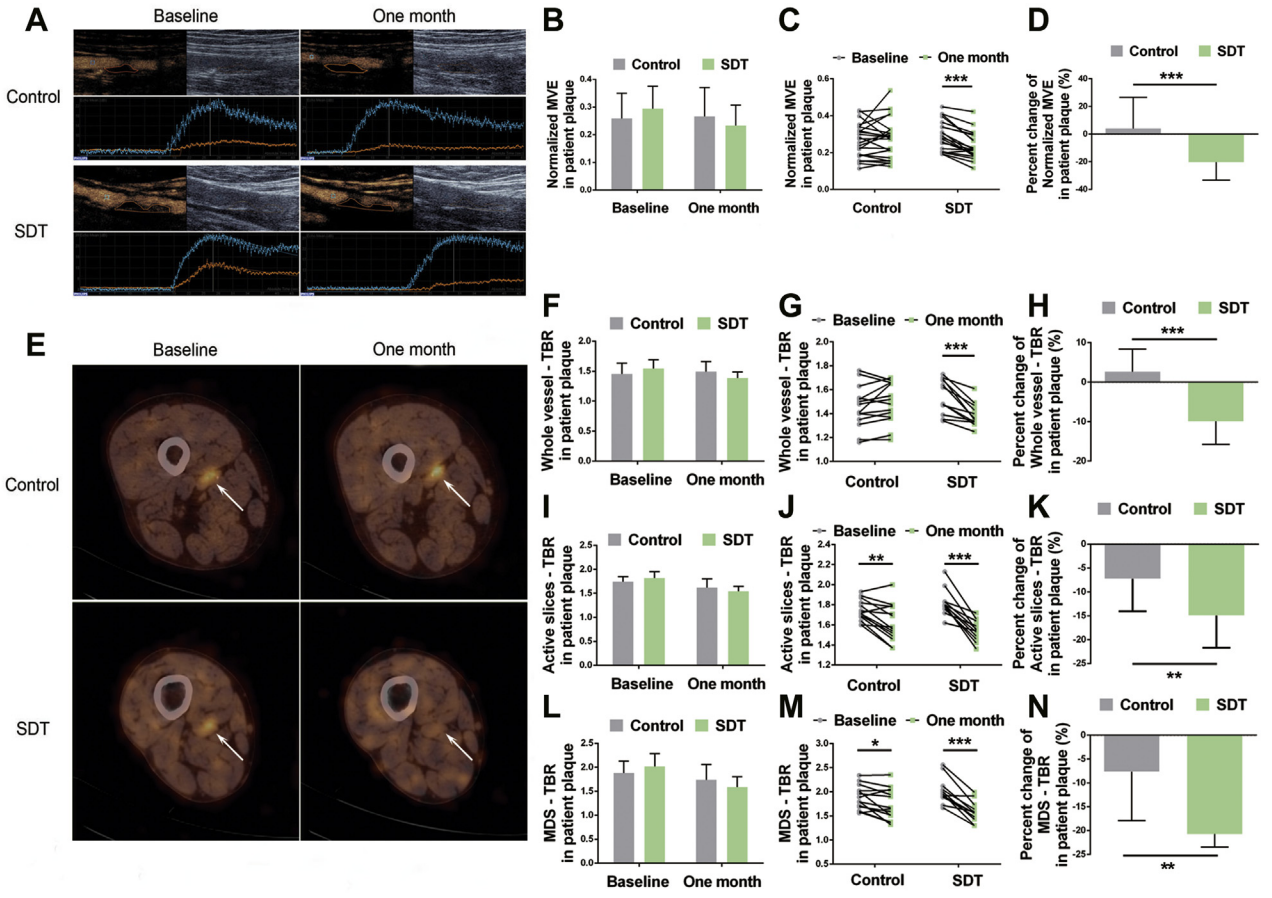


Co-culture of DVDMS-SDT–treated THP-1–derived foam cells and HUVEC were stimulated with 5 ng/ml VEGF-A for 48 h. Then, the apoptotic rate (n = 4) **(A)**, proliferation (n = 5) **(B)**, migration at 6 h after scratching (n = 4) **(C)**, and tube formation (n = 4) **(D)** of HUVEC were detected. Data on the graph were from 3 independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Abbreviations as in Figures 1, 4, and 5.

caspase 3 activation by DVDMS-SDT may cleave a transcription factor that promotes HIF-1α expression. We identified 4 proteins (androgen receptor, CTNNB1, specificity protein 1 [SP-1], and MDM2) that mediate caspase 3 and HIF-1α interactions by detecting caspase 3- and HIF-1α-related subnetworks from the Human Protein Reference Database Protein-Protein Interaction network (**Figure 6C**). The transcription factor SP-1 is a well-recognized substrate protein of caspase 3 (15). The chromatin immunoprecipitation (ChIP)-sequencing data of SP-1 in the ENCODE (Encyclopedia of DNA Elements) project showed a significant enrichment of SP-1 in the promoter regions of *HIF1A* in many human cell lines (**Figure 6D**). Therefore, our finding validated that SP-1 bound to the *HIF1A* promoter region (**Figure 6E**) and promoted *HIF1A* transcription in THP-1–derived foam cells (**Figure 6F**). Subsequent ChIP-quantitative polymerase chain reaction showed that the interaction of SP-1 with the *HIF1A* promoter diminished significantly on DVDMS-SDT treatment (**Figure 6G**). In addition, caspase 3 activated by DVDMS-SDT cleaved SP-1, but not androgen receptor, CTNNB1, and MDM2 (Supplemental Figure 7), leading to reduced HIF-1α expression (**Figures 6H to 6M**); which was reversed by SP-1 overexpression (**Figures 6N to 6P**).

VEGF-A ABOLISHED NEOVASCULAR ENDOTHELIAL CELL APOPTOSIS INDUCED BY APOPTOTIC FOAM CELLS ON DVDMS-SDT. Due to the significant reduction of VEGF-A levels in DVDMS-SDT–treated macrophages, we added 5 ng/ml VEGF-A in

FIGURE 8 DVDMS-SDT Reduces Plaque Angiogenesis and Arterial Inflammation in Patients



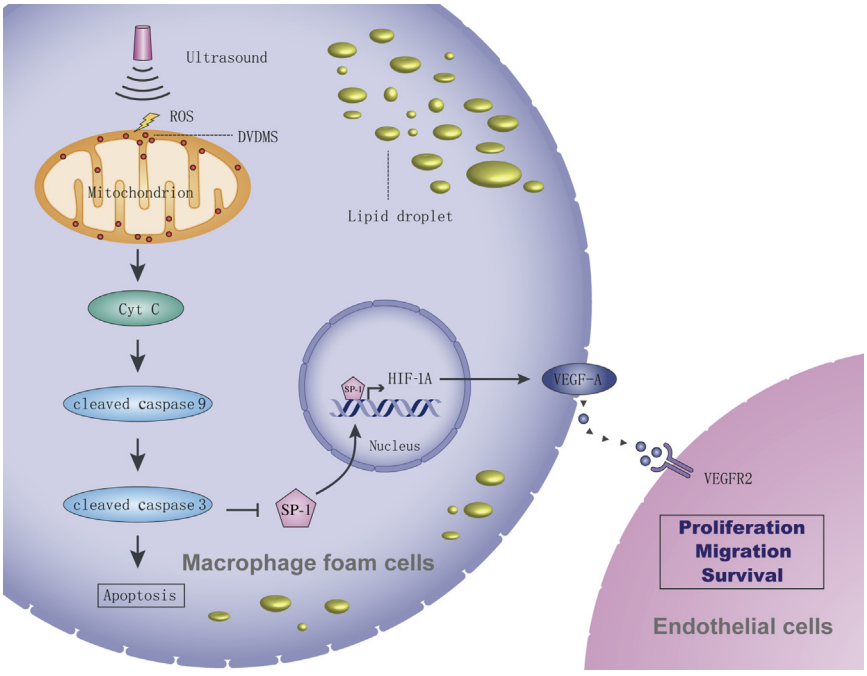
Representative contrast-enhanced ultrasonography images of femoral plaque **(A)** and quantification of normalized maximal video-intensity enhancement (MVE) at baseline and 1 month after DVDMS-SDT treatment **(B to D)**. **Orange and cyan curves** represent the time-intensity curve of the plaque and lumen, respectively. Representative cross-sectional fused fludeoxyglucose F 18–positron emission tomography–computed tomography images of the femoral artery at the mid-thigh level **(E)** and quantification of whole vessel target-to-background ratio (TBR) **(F to H)**, active slice TBR **(I to K)**, and most-diseased segment (MDS) TBR **(L to N)** at baseline and 1 month after DVDMS-SDT treatment. **White arrows** indicate the femoral artery. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Abbreviations as in Figure 1.

cocultured macrophage foam cells and endothelial cells and found that DVDMS-SDT–induced endothelial cell apoptosis and DVDMS-SDT–suppressed proliferation, migration, and tubulogenesis were significantly reversed by VEGF-A incubation (Figure 7, Supplemental Figure 8).

DVDMS-SDT REDUCES PLAQUE ANGIOGENESIS AND ARTERIAL INFLAMMATION IN PATIENTS. Our pilot clinical study consisted of 2 stages, shown in Supplemental Figures 9A and 9B, respectively. Subject demographic data and characteristics at baseline in stage 1 and stage 2 studies are shown in Supplemental Tables 3A and 3C.

In the stage 1 study, no significant difference in blood test parameters was observed after DVDMS-SDT treatment (Supplemental Table 3B). In the stage 2 study, the normalized MVE in plaque at 1 month after DVDMS-SDT was reduced by 20.4% as shown by contrast-enhanced ultrasonography analysis (Figures 8A to 8D) compared with baseline. One month after treatment, fludeoxyglucose F 18–positron emission tomography–computed tomography analysis showed that the whole vessel-target-to-background ratio (TBR) in the control group increased by 2.6% and decreased by 10% in the DVDMS-SDT group, whereas the active slice TBR decreased by 7.3% and 15% in the respective groups. In turn, the respective most-diseased segment TBR data decreased by 8% and 20% (Figures 8E to 8N).

FIGURE 9 Summary of DVDMS-SDT Suppression of Neovascularization in Advanced Atherosclerotic Plaque



DVDMS-SDT–induced augmentation of reactive oxygen species (ROS) production in mitochondria of macrophage foam cells leads to cytochrome C (Cyt C) release. Cytochrome C activates caspase 9, which subsequently activates caspase 3, resulting in macrophage foam cell apoptosis and cleavage of SP-1. Then, the lack of SP-1 induces the down-regulation of HIF-1α and VEGF-A expression, leading to the inhibition of proliferation, migration, and survival of endothelial cells. Abbreviations as in Figures 1, 5, and 6.

DISCUSSION

Despite vast knowledge and efforts toward controlling neovascularization in atherosclerosis to prevent plaque rupture (16), crucial challenges remain to effectively inhibit angiogenesis without causing off-target effects. In the present study, we examined the role of DVDMS-SDT in angiogenesis inhibition, based on our previous in vivo data that ALA-SDT significantly stabilized atherosclerotic plaques and inhibited lesion development (10). Therefore, we used 2 animal models generated via different methods of inducing atherosclerotic plaque formation, to mimic endothelial dysfunction or lipid metabolism disorder–promoted atherosclerosis in humans. Notably, on DVDMS-SDT treatment with much lower dosage of sonosensitizer (0.2 mg/kg) compared with ALA (20 mg/kg) as we used in former study (10), both the rabbit and ApoE$^{-/-}$ mouse models manifested similar and highly consistent phenotypes with markedly decreased neovasculature, along with enhanced apoptosis of macrophages and neovascular endothelial cells in the regressed lesions.

Because DVDMS is taken up exclusively by plaque resident macrophages but not by arterial smooth muscle or endothelial cells, we proposed that the macrophage apoptosis in response to DVDMS-SDT may stimulate neovascular endothelial cell apoptosis to inhibit plaque neovascularization. Previously, Xiong et al. (17) demonstrated that the number, length, and area of capillary-like structures were significantly reduced after exposure of cultured HUVEC to DVDMS (0.05 μmol/l for 3 h) plus 90-s continuous ultrasound (2 W/cm^2, 1.1 MHz). However, we found that DVDMS (0.2 μmol/l for 6 h) plus 5-min pulse ultrasound (0.7 W/cm^2, 1 MHz, duty cycle: 10%) had no direct effect on HUVEC viability, which further strengthened the study rationale that applied DVDMS-SDT protocol in the present study results in a minimal damage to normal arterial endothelial cells with significant enhancement in plaque stability.

Based on the established close spatiotemporal association between tissue macrophages and sprouting vessels (18), we used a coculture system of macrophages and vascular endothelial cells to mimic the plaque microenvironment. Consequently, we report the novel observation that macrophage foam cell apoptosis in response to DVDMS-SDT markedly promoted endothelial cell apoptosis through a reduction in VEGF-A levels. Interestingly, Winnik et al. (19) reported that systemic VEGF inhibition exacerbated atherosclerosis in ApoE$^{-/-}$ mice on high fat diet, which is different from our results. We consider the phenotype difference is due to different vascular biofunction and pathological reaction on systemic or local impact of VEGF-A.

Next, we attempted to address the molecular signaling pathway in macrophages following DVDMS-SDT to reveal the underlying mechanisms for subsequent endothelial apoptosis through ribonucleic acid sequencing and bioinformatics analyses on DVDMS-SDT-treated foam cells with or without apoptosis inhibition. We found that angiogenesis represented the second most responsive signaling pathway, with HIF-1α identified being involved in this process. HIF-1α constitutes a key transcription factor regulating hypoxia responses including cell apoptosis in atherosclerotic plaque; notably, it is also expressed in macrophages regulating their function (20).

Moreover, we found that DVDMS-SDT-activated caspase 3 cleaves SP-1 to inhibit the expression of HIF-1α, as well as VEGF-A reduction in macrophage foam cells, which is consistent with the results of other studies that activated caspase 3 by different apoptotic inducers, cleaving SP-1 on the aspartic acid 183 or 584 site (21,22). VEGF-A plays an important role in maintaining endothelial cell viability by binding to its receptor VEGFR2 (3). Hence, VEGF-A reduction may induce neovascular endothelial cell apoptosis (23). Although HIF-1α is known to bind to the promoter of caspase 3 to regulate its expression (24), the means by which caspase 3 inhibits HIF-1α expression requires further investigation. Our results revealed hypoxia-HIF-1α-caspase 3-SP-1-HIF-1α as a novel feedback signaling pathway, although the specific caspase 3 site of action on SP-1 is not known yet. It is therefore necessary to further analyze the cluster of genes and other metabolic pathways in our ribonucleic acid sequencing database to achieve a more comprehensive understanding of the role of DVDMS-SDT in atherosclerotic plaque stability.

Based on the promising findings from our in vivo and in vitro studies, we also conducted a small size pilot study in patients with atherosclerosis. Notably, a significant reduction of neovascularization and arterial inflammation was recorded following a short period of DVDMS-SDT treatment. This beneficial effect was almost equivalent to the therapeutic outcome after 3-month intensive statin treatment (25) but occurred earlier than the statin-mediated effect. Moreover, our regular follow-up screening shows no side effects consequent to DVDMS-SDT administration to date. The findings of the present study therefore suggest that DVDMS-SDT alone or in combination with a reduced statin dosage may potentially produce lesion-specific therapeutic effects in patients with vulnerable atherosclerotic plaques.

STUDY LIMITATIONS. The present small-scale pilot study was neither randomized nor double-blinded. We are currently in the process of assessing the safety and efficacy profile of DVDMS-SDT on atherosclerotic plaques in larger randomized, double-blinded, controlled trials; the results of these trials will be reported in due course (SMART-PAD [Sonodynamic Therapy Manipulates Atherosclerosis Regression Trial on Patients With PAD and Claudication], NCT03457662; SMART-C [Sonodynamic Therapy Manipulates Atherosclerosis Regression Trial on Patients With Carotid Atherosclerotic Plaques], NCT03382249).

CONCLUSIONS

As shown in **Figure 9**, DVDMS-SDT successfully inhibits atherosclerotic plaque neovascularization by promoting macrophage apoptosis-induced endothelial cell apoptosis. Although this noninvasive treatment undoubtedly requires further development and refinement, it represents a promising clinical strategy to prevent vulnerable plaque from intraplaque hemorrhage or thrombotic complications.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Systemic VEGF-A inhibitors are widely used to inhibit tumor angiogenesis but are not therapeutically viable in patients with chronic atherosclerosis because long-term inhibition is associated with severe or even fatal cardiovascular side effects. DVDMS-SDT locally inhibits atherosclerotic plaque neovascularization via macrophage apoptosis-induced endothelial cell apoptosis without any obvious off-target effects and has potential therapeutic value.

TRANSLATIONAL OUTLOOK: Randomized controlled trials are needed to establish the safety and efficacy of DVDMS-SDT toward atherosclerotic plaque neovascularization.

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KEY WORDS apoptosis-induced apoptosis, atherosclerotic plaque, endothelial cell, macrophage, neovascularization, sonodynamic therapy

APPENDIX For supplemental methods, references, figures, and tables, please see the online version of this paper.