Hydrogen sulphide-releasing aspirin enhances cell capabilities of anti-oxidative lesions and anti-inflammation

An-Sha Zhao1,2, *, Dan Zou1,2, *, Hao-Hao Wang1,2, Xiao Han1,2, Ping Yang1,2, Nan Huang1,2
1 Key Laboratory for Advanced Technologies of Materials, Ministry of Education, Chengdu, Sichuan Province, China
2 School of Material Science and Engineering, Southwest Jiaotong University, Chengdu, Sichuan Province, China

#These authors contributed equally to this work.
*Correspondence to: An-Sha Zhao, anshazhao@263.net.

INTRODUCTION

Once being considered as a poison gas for a long time till new researches discovered the endogenous H₂S effects on physiological and pathological processes. In virtue of H₂S’s effects on cellular redox imbalance and aspirin’s good anticoagulation property, exogenous H₂S donors, such as H₂S-releasing aspirin (ACS14), have been explored to attenuate side effects of aspirin on gastrointestinal mucosal damage. However, existing researches mainly focus on the antithrombotic effects. Considering H₂S role in angiogenesis and vascular-protection progress, we herein focused on if ACS14 further has the ability to attenuate oxidative lesion and inflammation in human umbilical vein endothelial cells (HUVECs) and macrophages. In this study, we synthesized ACS14 by 5-(4-methoxyphenyl)-1,2-dithiole-3-thione and o-acetylsalicylic acid (aspirin), and the obtained compounds showed the ability to release H₂S. Our data illustrated that both aspirin and ACS14 had good cytocompatibility, and could support the proliferation of HUVECs. And, ACS14 was found to be able to promote 1.6 folds increase compared to aspirin. H₂S released from ACS14 was detected inside cells, wherein H₂S fluorescence intensity increased twofold in 5 μM and 10 μM ACS14 groups than 1 μM group. Owing to reactive oxygen species inside cells being obviously decreased in ACS14 group, the apoptosis rate of HUVEC herein was reduced as low as 1.6% from 60% of blank group. Meanwhile, the tumour necrosis factor alpha release in macrophage was also declined by 15% in ACS14 groups than the others. Basically, the ACS14 we obtained had the cyto-protective and anti-inflammatory capabilities. Potential applications for vascular intima repair in atherosclerosis are further expected.

Key words: hydrogen sulphide; ACS14; oxidative lesion; inflammation; atherosclerosis; anticoagulation; endothelial cell; macrophage; H₂S donor

doi: 10.4103/2045-9912.266990

How to cite this article: Zhao AS, Zou D, Wang HH, Han X, Yang P, Huang N. Hydrogen sulphide-releasing aspirin enhances cell capabilities of anti-oxidative lesions and anti-inflammation. Med Gas Res. 2019;9(3):145-152.

Funding: This research was financially supported by the National Natural Science Foundation of China, No. 81771988 (to ASZ) and the National Natural Science Foundation of China, No. 81401522 (to ASZ).

Abstract

Hydrogen sulphide (H₂S) has been considered as a toxic gas for a long time till new researches discovered the endogenous H₂S effects on physiological and pathological processes. In virtue of H₂S’s effects on cellular redox imbalance and aspirin’s good anticoagulation property, exogenous H₂S donors, such as H₂S-releasing aspirin (ACS14), have been explored to attenuate side effects of aspirin on gastrointestinal mucosal damage. However, existing researches mainly focus on the antithrombotic effects. Considering H₂S role in angiogenesis and vascular-protection progress, we herein focused on if ACS14 further has the ability to attenuate oxidative lesion and inflammation in human umbilical vein endothelial cells (HUVECs) and macrophages. In this study, we synthesized ACS14 by 5-(4-methoxyphenyl)-1,2-dithiole-3-thione and o-acetylsalicylic acid (aspirin), and the obtained compounds showed the ability to release H₂S. Our data illustrated that both aspirin and ACS14 had good cytocompatibility, and could support the proliferation of HUVECs. And, ACS14 was found to be able to promote 1.6 folds increase compared to aspirin. H₂S released from ACS14 was detected inside cells, wherein H₂S fluorescence intensity increased twofold in 5 μM and 10 μM ACS14 groups than 1 μM group. Owing to reactive oxygen species inside cells being obviously decreased in ACS14 group, the apoptosis rate of HUVEC herein was reduced as low as 1.6% from 60% of blank group. Meanwhile, the tumour necrosis factor alpha release in macrophage was also declined by 15% in ACS14 groups than the others. Basically, the ACS14 we obtained had the cyto-protective and anti-inflammatory capabilities. Potential applications for vascular intima repair in atherosclerosis are further expected.

Key words: hydrogen sulphide; ACS14; oxidative lesion; inflammation; atherosclerosis; anticoagulation; endothelial cell; macrophage; H₂S donor

doi: 10.4103/2045-9912.266990

How to cite this article: Zhao AS, Zou D, Wang HH, Han X, Yang P, Huang N. Hydrogen sulphide-releasing aspirin enhances cell capabilities of anti-oxidative lesions and anti-inflammation. Med Gas Res. 2019;9(3):145-152.

Funding: This research was financially supported by the National Natural Science Foundation of China, No. 81771988 (to ASZ) and the National Natural Science Foundation of China, No. 81401522 (to ASZ).
on platelet aggregation via releasing H$_2$S which depressing gap junction intercellular communication, and also ACS14 exerts strong antithrombotic properties by impairing the activation of fibrinogen receptor. Another research additionally indicated that ACS14 could attenuate the high-glucose-induced oxidative stress on smooth muscle cells. Briefly, ACS14, based on mother aspirin, has good hemocompatibility that is required for treating atherosclerosis. In light of these findings, we herein further investigated the oxidative stress-protective ability and anti-inflammatory effect and synthesized ACS14 on the basis of ADT and o-acetylsalicylic chloride. Our priority aimed to get ACS14 and test the H$_2$S release behaviour, investigate its cytocompatibility, and examine to what extent ACS14 can protects cells against the pro-atherosclerotic environment induced by oxidative stress and inflammation.

**MATERIALS AND METHODS**

**Demethylation of anethol trithione**

ADT (Sigma, St. Louis, MO, USA) reacted with pyridine hydrochloride (Capot Chemical Co., Ltd., Shanghai, China) at 215°C for 40 minutes in a mol ratio of 1:5, and stirring was no stopped until at room temperature. Then, 1 M hydrochloric acid of 200 mL was added into and stirred continuously for 1 hour. Precipitates were obtained via filtering, washed with deionized water and got perfectly dried. Later, precipitates were mixed in ethyl acetate and heated in oil bath at 85°C. Organic phase was finally collected into beaker after cold treatment at 0°C for 1 hour, and precipitates were obtained in brownish red color. H$_2$S was put into separating funnel and respectively washed by 0.25 M hydrochloric acid, deionized water, 0.1 M iced sodium hydroxide. Organic phase was finally collected into beaker and got perfectly dried. Later, precipitates were mixed in ethyl acetate and heated in oil bath at 85°C. Once precipitates were completely dissolved, ligarine was added for precipitation. After cold treatment at −20°C for 1 hour, 5-(4-hydroxy-phenyl)-3H-1,2-dithiole-3-thione (ADTOH) was obtained in brownish red color, and analyzed by nuclear magnetic resonance spectroscopy (MRS) (Bruker, AVANCE III HD 400M, Madison, WI, USA) and mass spectrum (MS) (Dionex, Mass spectrometer ICS90, Sunnyvale, CA, USA).

**Synthesis of H$_2$S-releasing ACS14**

ADTOH, o-acetylsalicyloyl chloride (Sigma) and triethylamine (Capot Chemical Co., Ltd.) were in a mol ratio of 1:1.2:2. Both ADTOH and o-acetylsalicyloyl chloride were dissolved in tetrahydrofuran (Capot Chemical Co., Ltd.). ADTOH solution in two-neck flask was added with trimethylamine and stirred. O-acetylsalicyloyl chloride solution in dropping and stirred continuously for 1 hour. Precipitates were obtained via filtering, washed with deionized water and got perfectly dried. Later, precipitates were mixed in ethyl acetate and heated in oil bath at 85°C. Once precipitates were completely dissolved, ligarine was added for precipitation. After cold treatment at −20°C for 1 hour, 5-(4-hydroxy-phenyl)-3H-1,2-dithiole-3-thione (ADTOH) was obtained in brownish red color, and analyzed by nuclear magnetic resonance spectroscopy (MRS) (Bruker, AVANCE III HD 400M, Madison, WI, USA) and mass spectrum (MS) (Dionex, Mass spectrometer ICS90, Sunnyvale, CA, USA).

**Anti-oxidative lesions property**

Cell culture process was the same as above. Briefly, ECs were digested firstly, and 1 × 10$^5$ cells were seeded with ACS14 on culture plate for 24 hours. Then, culture media was removed and 400 μM H$_2$O$_2$ was added in each sample. Cells were re-cultured for 12 hours at 37°C, and 1 mL culture media with 10 μL acridine orange (Sigma) and 10 μL propidium iodide (Sigma) were added to stain living and apoptotic cells. Fluorescence microscopy was used to observe cells activities, and cell numbers were calculated in ImageJ Software (NIH, Bethesda, MD, USA).

**Anti-inflammation property**

Macrophages solution of 2 × 10$^4$ cells/mL were seeded on 24-well culture plates and cultured with ACS14 for 24 hours at 37°C with 5% CO$_2$. Then cells were re-cultured for 24 hours with fresh culture media after washing by PBS. Supernatant was collected for inflammation related factors detection by tumor necrosis factor alpha (TNF-α; Bioss Antibodies, Beijing, China) and interleukin-10 enzyme-linked immunosorbent assay kit (Bioss Antibodies), and cell viability was tested by cell counting kit-8 kit (Sigma) at 450 nm. Cells were stained by Rodamine123 (Sigma) and 4,6-diamino-2-phenylindole (Sigma) was used for staining. Images were taken by Fluorescence microscopy (Olympus IX51, Tokyo, Japan).  

**H$_2$S detection in cells**

HUVECs were cultured with each sample on coverslips for 24 hours, and then incubated with 250 μM H$_2$S fluorescent probe WSP-1 (Maokangbio, Shanghai, China) for 30 minutes at 37°C away from light. After washing by PBS, cells were imaged at 476 nm by fluorescence microscope, and the fluorescent intensity was measured by Image J software.
ROS detection in cells
HUVECs and macrophages were respectively seeded on coverslips and cultured with samples for 24 hours and then treated with 400 μM H₂O₂ for 12 hours. After washing by PBS, cells were incubated with 10 μM ROS fluorescent probe-dihydroethidium (Maokangbio, Shanghai, China) at 37°C for 30 minutes away from light. Solution was removed for detection. Cells were observed and photographed at 488 nm using fluorescence microscope, with the fluorescent intensity measured by ImageJ software.

Statistical analysis
Data were expressed as the mean ± standard deviation (SD). Two groups were compared via two-tailed Student’s t-tests, and groups more than two were analyzed via one-way analysis of variance. The probability values P < 0.05 was considered as significant differences. All statistical analysis was performed using SPSS 20.0 Software (IBM SPSS, Chicago, IL, USA).

RESULTS
Demethylation of ADT and synthesis of ACS14
To obtain demethylated products ADTOH, ADT was reacted with pyridine hydrochloride in a mol ratio of 1:5 at 215°C for 40 minutes (Figure 1A). Then, both raw material ADT and ADTOH were dissolved into CDC13 for ¹H MRS characterization. Besides the CDC13 peak at δ7.20–7.24, ADT (Figure 1B) and ADTOH (Figure 1C) classically showed the double proton peaks of benzene ring at δ6.9–7.0 and δ7.55–7.65, and a single peak of five-membered ring at δ7.35–7.4, which matched with each other perfectly. Furthermore, the data had shown the presence of methyl proton peak at δ3.8–3.9 in ADT, which disappeared in ADTOH after demethylation. MS was employed to confirm the existence and ratio of ADTOH in compounds. The results (Figure 1D) showed that the highest peak was located at 226.96, which was assigned to 225.96 ADTOH with one proton added. These data suggested that ADT had been demethylated successfully.

Demethylated product ADTOH was reacted with o-acetylsalicyloyl chloride to obtain ACS14 (Figure 2A), and ¹H MRS and MS was respectively applied to confirm the specific proton peaks and the compounds molecular mass. In Figure 2B, proton peaks at δ8.15–8.25 and δ7.6–7.8 were attributed to the benzene ring of o-acetylsalicyloyl chloride; peaks at δ7.15–7.23 and δ7.28–7.35 were assigned to the benzene ring of ADTOH; peaks at δ7.56–7.45 and δ2.25–2.35 were respectively ascribed to the five-membered ring of ADTOH and methyl group in o-acetylsalicyloyl chloride. The results had shown a weak peak shift due to structure change after reaction, but each peak can match with their chemical structures. Moreover, MS data in Figure 2C confirmed the ACS14 products via molecular mass. Peaks at 388.9964, 410.9794 and 426.9577 were respectively the molecular mass of ACS14-H, ACS14-Na and ACS14-K. In addition, ACS14 products accounted for more than 95% in the compounds. These data indicated that H₂S-releasing ACS14 was successfully synthesized and obtained in a high purity.

H₂S release
Here, we investigated the H₂S releasing properties of ACS14...
with concentration of 300 μM by HSip-1 (Figure 3). Given that ACS14 and HSip-1 might have own fluorescence emission, both were taken into test consideration. The data illustrated that ACS14 itself actually did not show any fluorescence intensity and would have no potential interference. In contrast, HSip-1 group presented an absorbance peak. But based on this, ACS14 with HSip-1 had shown a 1.5-fold higher fluorescent intensity than HSip alone. Basically, the results confirmed that there was H₂S generated in solution by ACS14, which then can serve as a gas donor for use.

**Cytocompatibility of H₂S-releasing ACS14**

With H₂S donor ACS14 obtained, cytocompatibility of six groups were investigated next: blank, DMSO, ASA, ACS14 1 μM, ACS14 5 μM, and ACS14 10 μM groups. Endothelial cells were co-culture with samples for 1 and 3 days. At the beginning, DMSO, ASA and ACS14 had no impact on cell adhesion on day 1; cells were in normal morphology and spread well without cytotoxicity observed in any groups (images not shown here). ECs numbers increased significantly in the presence of ACS14 10 μM (Figure 4B), having obvious growth difference with all other groups. Also, cells in ASA group increased in number compared to blank control, and kept similar viability with cells in ACS14 1, 5 μM groups. But at day 3 as fluorescent images in Figure 4A, big proliferation difference appeared in each group. In contrast to a lower viability of ASA group, cell viability gradually increased with ACS14 concentration, and 10 μM ACS14 facilitated ECs proliferation most, about 1.6 folds than ASA (Figure 4B). Having examined the cytocompatibility of samples, we applied WSP-1 to monitor the intracellular H₂S. In consistence with proliferation results, cells in ACS14 group showed obvious green H₂S fluorescence (Figure 5A) with little fluorescence detected in other groups, and a twofold increase in fluorescent intensity was observed in ACS14 5 μM and 10 μM groups than ACS14 1 μM group (Figure 5B). In brief, ACS14 is cytocompatible, and could support ECs proliferation through H₂S release into cells.

**Anti-oxidation ability of ACS14**

ECs were seeded with samples for 24 hours, and then re-cultured for extra 12 hours in the presence of 400 μM H₂O₂. To observe to what extent ECs were damaged, cells were respectively stained by acridine orange/propidium iodide and dihydroethidium, and subsequently were lively imaged. Cells in blank group only added with H₂O₂, were extremely sensitive to oxidation, resulting in a dramatic increase in apoptosis rate as well as cells in DMSO (Figure 6A), of which, as Figure 6B showed, the apoptosis rates approached to 60% with other groups below 10%. In contrast, ECs in both ASA and ACS14 groups maintained normal morphology and viability as cells in control group (no H₂O₂), with apoptosis rate in ACS14 reduced to 1.6% from 4.7% in ASA. Furthermore, we examined the ROS production of ECs after H₂O₂ treatment. The nuclei fluorescence in ACS14 groups was the lowest with ASA having a half fluorescence decrease in comparison with control.
Zhao et al. / Med Gas Res www.medgasres.com

Figure 4: Effects of ACS14 on ECs proliferation.
Note: (A, B) ECs were cultured for 3 days (d), examined by phalloidin (red), DAPI (blue) staining (A) and CCK-8 kit assay (B). Scale bars: 200 μm. All data are expressed as the mean ± SD (n = 4). ***P < 0.001 (one-way analysis of variance followed by two-tailed Student’s t-test). ACS14: 2-Acetyloxybenzoic acid 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl ester; EC: endothelial cell; DAPI: 4′,6-diamidino-2-phenylindole; CCK-8: cell counting kit-8; OD: optical density. ASA: aspirin; DMSO: dimethyl sulfoxide.

Figure 5: H2S detection in human umbilical vein endothelial cells.
Note: (A, B) Cells were cultured with each sample and examined by H2S probe WSP-1 (green) (A), followed with fluorescent intensity detection (B). Scale bars: 50 μm. All data are expressed as the mean ± SD (n = 4). *P < 0.05, ***P < 0.001 (one-way analysis of variance followed by two-tailed student’s t-test). H2S: Hydrogen sulphide; ACS14: 2-acetyloxybenzoic acid 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl ester; ASA: aspirin; DMSO: dimethyl sulfoxide.

Figure 6: Anti-oxidative ability of ACS14.
Note: (A, B) Endothelial cells were treated with H2O2, and examined by AOPI staining (A), followed with apoptosis rate calculation (B). Scale bars: 200 μm. All data are expressed as the mean ± SD (n = 4). *P < 0.05, ***P < 0.001 (one-way analysis of variance followed by two-tailed student’s t-test). ACS14: 2-Acetyloxybenzoic acid 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl ester; ASA: aspirin; DMSO: dimethyl sulfoxide; AOPI: acridine orange and propidium iodide.

These results demonstrated that in the event of H2O2 treatment, ECs viabilities in ACS14 and ASA groups were apparently higher than those in blank and DMSO groups. Being confirmed via intracellular ROS detection, ACS14 protective effects by releasing H2S could more effectively enhance the anti-oxidation lesions ability of ECs than ASA.
Anti-inflammation ability of ACS14

To investigate the anti-inflammation potential of ACS14, RAW264.7 macrophages were cultured in the presence of samples for 1–3 days. Compared to macrophages in the first 24 hours (Figure 8A) that blank group has more cells adhered on the surface, macrophages on the 3rd day showed a higher viability in the presence of ASA and ACS14 than blank and DMSO groups (Figure 8B). Essentially, macrophages proliferated in a similar way with ECs that big differences appeared at day 3 and ACS14&ASA groups had a 30% higher increased cell numbers than others (Figure 8C). To confirm inflammation condition further, we thereafter measured the inflammatory factor TNF-α in macrophages, followed with the detection of ROS generation which was correlated with the activation of pro-inflammatory signal pathway nuclear factor-kappaB. ACS14 and ASA groups having higher cell amounts showed 15% decreased TNF-α content compared with control with lower cell numbers (Figure 8D). Correspondingly, we tested ROS level which is related to macrophages activation and TNF-α release. In Figure 9A, cells themselves and DMSO did not show any effect on ROS reduction under oxidative context. However, it was obviously observed in the presence of ASA and ACS14. Macrophages cultured with ASA had a 20% lower intracellular ROS fluorescent intensity than counterparts in Blank and DMSO, with 1 μM ACS14 having the same effect. However, macrophages cultured with 5 μM and 10 μM ACS14 showed a 60% dramatic decrease of ROS generation (Figure 9B). That is to say, via reducing ROS production and TNF-α release, both ACS14 and ASA have the potential to protect macrophages against H_{2}O_{2} induced oxidative injury and enhance anti-inflammation ability of macrophage, and 5 μM and 10 μM ACS14 had a more outstanding performance.

Figure 7: ROS detection in human umbilical vein endothelial cells.

Note: (A, B) Cells were cultured with each sample and treated with H_{2}O_{2} for 12 hours, followed with ROS probe DHE (red) detection (A) and fluorescent intensity measurement (B). Scale bars: 50 μm. All data are expressed as the mean ± SD (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance followed by two-tailed Student's t-test). ACS14: 2-Acetyloxybenzoic acid 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl ester; ASA: aspirin; DMSO: dimethyl sulfoxide; ROS: reactive oxygen species; DHE: dihydroethidium.

Figure 8: Effects of ACS14 on anti-inflammation ability of macrophages.

Note: (A–D) After 1, 3 days (d) culture, macrophages was examined by fluorescence staining (A, B), CCK-8 assay test (C), and TNF-α measurement (D). Scale bars: 25 μm. All data are expressed as the mean ± SD (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance followed by two-tailed Student's t-test). ACS14: 2-Acetyloxybenzoic acid 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl ester; ASA: aspirin; DMSO: dimethyl sulfoxide; CCK-8: cell counting kit-8; TNF-α: tumor necrosis factor alpha.
In recent year, H₂S, produced in mammal cells via three H₂S producing enzymes, cystathionine β-synthase, cystathionine γ-lyase and 3-mercaptopryruvate sulfurtransferase, has been reported to possess versatile physiological benefits. Exogenous H₂S donors were continually explored to assist different endogenous progresses. One H₂S donor aspirin was manufactured on the basis of aspirin, aiming to maintain or enhance aspirin’s hemocompatibility as well as ameliorate the situation via H₂S that mother compound aspirin would cause gastrointestinal mucosal damage in spite of outstanding antithrombotic effects.²⁹ H₂S released from ACS14 can increase glutathione formation and heme oxyenase-1 promoter activity, resulting in a lower level of 8-isoprosrane, and concurrently the intracellular H₂S/glutathione formation could counteracts gastric damage-related redox imbalance.³⁰ By virtue of ACS14’s outstanding hemocompatibility quite expected in vascular microenvironment, we further focused on whether or not ACS14 has anti-oxidative lesion and anti-inflammation abilities, which are also critical in the progress of vascular intima repair for atherosclerosis.

Here, by anethol trithionum and o-acetylsalicyloyl chloride, we successfully prepared dimethylated anethol trithionum and thereafter synthesized H₂S-releasing product ACS14. The fact that the intensity fluorescence of probe-marked H₂S is 1.5 folds than probe itself confirmed the obtained ACS14 could serve as an exogenous H₂S provider. Also, compared to aspirin and blank groups, our results showed ACS14 had better cyto-compatibility and significantly increased HUVECs viability, in the presence of which, H₂S was obviously detected inside cells in ACS14 group, indicating that H₂S released by ACS14 could become assimilated and utilized by cells. Although almost no H₂S was detected in aspirin group, HUVECs cultured with aspirin still showed certain viability enhancement, attributing to aspirin’s capability to stimulate cyclic guanosine monophosphate level and increase nitric oxide bioavailability.³⁵

In order to investigate ACS14 potential for intima repair in the context of AS inflammatory microenvironment, we examined its cyto-protective property. Under the condition of H₂O₂ induced oxidative injuries, ACS14 and aspirin, compared to blank group, respectively reduced the apoptosis rate from 60% to 1.6% and 4.7%, with ACS14 showing best cyto-protective property. Our ROS detection results further deciphered this. Just in consistent with Osborne et al.’s research,³⁶ aspirin cultured with HUVECs could half reduce intracellular ROS level than blank group, with ACS14 decreasing ROS production most which is the same as Feng’s finding in H9C2 cells.³⁷ That ACS14 has better anti-oxidation protective property might be benefited from the combined effort, since both aspirin and H₂S were reported to have antioxidant effects. According to early studies, aspirin was able to prevent the increase of intracellular ROS formation by elevating hemeoxyenase-1 protein, ferritin and telomerase content levels,³⁵,³³,³⁸,³⁹ while released H₂S which can enter into cytoplasm as detected previously, can elevate the intracellular cysteine levels and form a free radical scavengers glutathione.⁴⁰ The cyto-protective effect of aspirin and ACS14 was also found on macrophages. As being reported to augment the anti-inflammatory effects by Li et al.,²¹ H₂S released from ACS14 could slightly lessen TNF-α release without cell numbers reduction, which was correlated with intracellular ROS generation and macrophages activation. Macrophages cultured with aspirin had 20% decrease of ROS production than blank group, with 5 μM and 10 μM ACS14 showing the lowest ROS signal. However, 1 μM ACS14 only showed the same ROS scavenging effect as aspirin. Although both aspirin and H₂S can impede the pro-inflammatory progression via inhibiting nuclear factor-kappaB expression under oxidation condition,⁴¹,⁴² ACS14 at low concentration still possessing aspirin’s anti-inflammation ability, would fail to further obviously enhance related effects on macrophages. This finding indicated that ACS14 alleviate inflammation based on the efforts of aspirin and H₂S by reducing ROS production. 

**Figure 9: Effects of ACS14 on ROS in macrophages.**

Note: (A, B) Macrophages were cultured with samples, followed with H₂O₂ treatment for 12 hours. Then ROS probe DHE (red) detection (A) and fluorescent intensity measurement (B) were performed. Scale bars: 50 μm. All data are expressed as the mean ± SD (n = 4). ***P < 0.001 (one-way analysis of variance followed by two-tailed Student’s t-test). ACS14: 2-Acetyloxybenzoic acid 4-(3-thioxo-3H-1,2-dithiol-5-yl)phenyl ester; ASA: aspirin; DMSO: dimethyl sulfoxide; ROS: reactive oxygen species; DHE: dihydroethidium.

**DISCUSSION**

In order to investigate ACS14 potential for intima repair in the context of AS inflammatory microenvironment, we examined its cyto-protective property. Under the condition of H₂O₂ induced oxidative injuries, ACS14 and aspirin, compared to blank group, respectively reduced the apoptosis rate from 60% to 1.6% and 4.7%, with ACS14 showing best cyto-protective property. Our ROS detection results further deciphered this. Just in consistent with Osborne et al.’s research, aspirin cultured with HUVECs could half reduce intracellular ROS level than blank group, with ACS14 decreasing ROS production most which is the same as Feng’s finding in H9C2 cells. That ACS14 has better anti-oxidation protective property might be benefited from the combined effort, since both aspirin and H₂S were reported to have antioxidant effects. According to early studies, aspirin was able to prevent the increase of intracellular ROS formation by elevating hemeoxyenase-1 protein, ferritin and telomerase content levels, while released H₂S which can enter into cytoplasm as detected previously, can elevate the intracellular cysteine levels and form a free radical scavengers glutathione. The cyto-protective effect of aspirin and ACS14 was also found on macrophages. As being reported to augment the anti-inflammatory effects by Li et al., H₂S released from ACS14 could slightly lessen TNF-α release without cell numbers reduction, which was correlated with intracellular ROS generation and macrophages activation. Macrophages cultured with aspirin had 20% decrease of ROS production than blank group, with 5 μM and 10 μM ACS14 showing the lowest ROS signal. However, 1 μM ACS14 only showed the same ROS scavenging effect as aspirin. Although both aspirin and H₂S can impede the pro-inflammatory progression via inhibiting nuclear factor-kappaB expression under oxidation condition, ACS14 at low concentration still possessing aspirin’s anti-inflammation ability, would fail to further obviously enhance related effects on macrophages. This finding indicated that ACS14 alleviate inflammation based on the efforts of aspirin and H₂S by reducing ROS production.
and TNF-α synthesis. These evidences suggest that ACS14 can promote HUVECs and macroparticles proliferation, mitigate HUVECs and macroparticles from oxidation lesions, mitigates inflammation. Taken together, besides the outstanding homocompatibility as researches reported, ACS14 further had cyto-protective and anti-inflammatory capabilities, which is quite promising to serve as a H₂S donor to be applied in the context of atherosclerosis for vascular intima repair.

Author contributions
Concepts, design, definition of intellectual content, data analysis, manuscript editing, manuscript review and guarantor: ASZ; literature research: ASZ, DZ, HHW, XH; experimental studies: DZ, HHW, XH; data acquisition: DZ, HHW, XH; statistical analysis: DZ, HHW, XH; manuscript preparation: ASZ, DZ. All authors approved the final version of manuscript for publication.

Conflicts of interest
No potential conflict of interest was reported by the authors.

Financial support
This research was financially supported by the National Natural Science Foundation of China, No. 81771988 (to ASZ) and the National Natural Science Foundation of China, No. 81401522 (to ASZ).

Copyright license agreement
The Copyright License Agreement has been signed by all authors before publication.

Data sharing statement
Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Plagiarism check
Checked by iThenticate.

Peer review
Externally peer reviewed.

Open access statement
This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-Non-Commercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

REFERENCES
1. Reifsteinstein RJ, Hulbert WC, Roth SH. Toxicology of hydrogen sulfide. Anna Rev Pharmacol Toxicol. 1992:32:109-134.
2. Wang R. Two’s company, three’s a crowd: can H₂S be the third gaseous transmitters? FASEB J. 2002;16:1792-1798.
3. Szabó C. Hydrogen sulphide and its therapeutic potential. J Pharmacol. 2015:27:205-213.
4. Meister A, Fraser PE, Tice SV. Enzymatic desulfuration of beta-mercaptopyruvate to pyruvate. J Biol Chem. 1954:206:561-575.
5. Verma A, Hirsch DJ, Glatt CE, Ronnet GV, Snyder SH. Carbon monoxide: a putative neural messenger. Science. 1993:259:381-384.
6. Brunstein AE, Goryachkova EV, Toluja EA, Willhardt HH, Yefremova LL. Specificity and some other properties of liver serine sulfhydrase: evidence for its identity with cystathionine -synthase. Biochim Biophys Acta. 1971:242:253-256.
7. Cavallini D, Mondovi B, De Marco C, Sciascia-Santoro A. The mechanism of desulphhydration of cysteine. Enzymology. 1962:242:253-266.
8. Powell CR, Dillon KM, Matson JB. A review of hydrogen sulfide (H2S) donors: Chemistry and potential therapeutic applications. Biochem Pharmacol. 2018:149:110-123.
9. Papapetropoulos A, Priyachou A, Alhany Z, et al. Hydrogen sulfide is an endogenous stimulator of angiogenesis. Proc Natl Acad Sci U S A. 2009:106:21972-21977.
10. Kohn C, Dubovska G, Huang Y, Gallach M. Hydrogen sulfide: potent regulator of vascular tone and stimulator of angiogenesis. Int J Biochem Cell Biol. 2012;44:81-86.
11. Geng B, Chang L, Pan C, et al. Endogenous hydrogen sulfide regulation of myocardial ischemia induced by isolated rat heart. Biochem Pharmacol Res Commun. 2004:318:756-763.
12. Whitteman M, Armstrong JS, Chu SH, et al. The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite scavenger? J Neurochem. 2004:96:765-768.
13. Webb GD, Lim LH, Oht VM, et al. Contractile and vasorelaxant effects of hydrogen sulfide and its biosynthesis in the human internal mammary artery. J Pharmacol Exp Ther. 2002:384:876-882.
14. Li D, Huang X, Meng XM, et al. Exogenous HŠS enhances mice gastric-mucosal muscle tension through S-sulfhydration of K 4.3, mediating the inhibition of the voltage-dependent potassium current. Neurogastroenterol Motil. 2012:24:1705-1716.
15. Zhao K, Ju Y, Li S, Alhany Z, Wang Y, Yang G. S-sulfhydration of MEK1 leads to PARP-1 activation and DNA damage repair. EMBO Rep. 2014:15:792-800.
16. Xie J, Feng H, Li S, et al. SIRT3 mediates the antioxidant effect of hydrogen sulfide in endothelial cells. Antioxid Redox Signal. 2016:24:329-343.
17. Iida T, Sawa T, Itaha H, et al. Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. Proc Natl Acad Sci U S A. 2014:111:7606-7611.
18. Webb GD, Lim LH, Oht VM, et al. Contractile and vasorelaxant effects of hydrogen sulfide and its biosynthesis in the human internal mammary artery. J Pharmacol Exp Ther. 2002:384:876-882.
19. Zhao K, Ju Y, Li S, Alhany Z, Wang Y, Yang G. S-sulfhydration of MEK1 leads to PARP-1 activation and DNA damage repair. EMBO Rep. 2014:15:792-800.
20. Xie J, Feng H, Li S, et al. SIRT3 mediates the antioxidant effect of hydrogen sulfide in endothelial cells. Antioxid Redox Signal. 2016:24:329-343.
21. Iida T, Sawa T, Itaha H, et al. Reactive cysteine persulfides and S-polythiolation regulate oxidative stress and redox signaling. Proc Natl Acad Sci U S A. 2014:111:7606-7611.
22. Web GD, Lim LH, Oht VM, et al. Contractile and vasorelaxant effects of hydrogen sulfide and its biosynthesis in the human internal mammary artery. J Pharmacol Exp Ther. 2002:384:876-882.