Experimental study on oral sulfhydryl as an adjuvant for improving nitrate ester tolerance in an animal model

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Abstract. – **OBJECTIVE:** As an initial step in exploring the feasibility of oral sulfhydryl as an adjuvant for improving nitrate ester tolerance, this study was designed to experimentally test the adjuvant therapy in a rabbit model of atherosclerosis (AS).

MATERIALS AND METHODS: New Zealand white rabbits with induced AS were randomly divided into four groups: AS group, AS + nitrate ester group, AS + nitrate ester tolerance group, and AS + drug combination group. Additionally, four equivalent groups with healthy New Zealand white rabbits without AS were also conformed. After feeding the animals for 5 days, the concentrations of superoxide anion ($\cdot O_2$-), superoxide dismutase (SOD), malondialdehyde (MDA), nitric oxide (NO), and endothelin-1 (ET-1) in blood and the relaxation response of the aortic ring were determined in each subject. The vascular plaques in different treatment groups were assessed by Hematoxylin and eosin (HE) staining to investigate the therapeutic value of sulfhydryl as coadjuvant for improving nitrate ester tolerance, and changes in blood vessels in different treatment groups were studied by immunohistochemical assays.

RESULTS: Our results showed no significant differences through time in the concentrations of $\cdot O_2$-, SOD, MDA, NO, ET-1 between the healthy control and the nitrate ester groups ($p > 0.05$). The levels of SOD and MDA in the nitrate ester tolerance group increased with time, however, the levels of $\cdot O_2$-, NO and ET-1 decreased gradually ($p < 0.05$). The NO, $\cdot O_2$- and ET-1 levels in both the AS and AS + nitrate ester tolerance groups were significantly decreased, but SOD and MDA were significantly increased ($p < 0.05$). SOD and MDA in the AS + nitrate ester group decreased gradually with time, but $\cdot O_2$-, NO- and ET-1 levels increased ($p < 0.05$). The levels of SOD and MDA in the AS + drug combination and the drug combination group decreased significantly with time, in contrast, those of $\cdot O_2$-, NO- and ET-1 increased ($p < 0.05$). The results of HE staining proved that the atherosclerosis model was established successfully.

CONCLUSIONS: We conclude the use of a sulfhydryl compound as an adjuvant significantly reduced nitrate ester tolerance, and this strategy was safe and looks promising for humans.

Key Words:
  Sulfhydryl compound replacement therapy, Nitrate ester tolerance, Clinical application.

Introduction

Nitrate esters are a traditional drug for the treatment of cardiovascular diseases[1]. In 1879, it was Doctor William Murrell who first used time nitrate esters in the treatment of angina pectoris in London[2]. Nitrate esters include isosorbide mononitrate, nitroglycerin, and isosorbidedinitrate, and others. These drugs play an important role in the treatment of mixed angina pectoris, acute angina pectoris, and persistent angina pectoris. They come in contact with the mitochondrial aldehyde dehydrogenase and then release nitric oxide (NO), a potent natural vasodilator. Atherosclerosis may be the cause of the ischemic heart disease, angina pectoris, which is manifested by chest pain caused by inadequate blood flow. At low doses, nitrate esters cause venodilation (widening of the venous blood vessels), nitroglycerin will dilate veins more than arteries, thereby reducing the preload; this is thought to be its primary mechanism of action. However, at higher doses, nitrate esters also dilate arteries, thereby reducing the afterload[3,4]. At the same time, nitrate esters help reduce arterial pressure acting as anti-atherosclerosis agents decreasing afterload. Dilating the veins decreases cardiac preload, and lowers the oxygen requirement of the heart whilst at the same

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time reducing ventricular transmural pressure and improving coronary blood flow[5,6]. Patients suffering from angina pectoris can anticipate episodes and take nitrate esters 5 to 10 min before performing certain physical activities. Nitrate esters are available in oral tablets and solution for intravenous use. The use of these drugs should be judicious as studies have shown that continuous exposure to nitrate esters can cause the body to stop responding normally to the medications (frequent dosing or continuous medication for 24-72 hours can cause tolerance). The use of diuretics, sulfhydryl donors, folic acid, or other drugs' can improve or prevent the nitrate esters tolerance, thus ensuring better long-term therapeutic effects.

A sulfhydryl is a functional group with a strong odor and weak acidity (ready to be oxidized). The redox signal transduction of sulfhydryl groups has been associated with the pathogenesis of diverse diseases[7]. The isosorbide mononitrate (ISMN) capsule is an oral sustained-release formulation commonly used in the clinic; each capsule contains about 100 extended-release pellets, 30% of the drug content gets released very quickly, while the rest exhibits a sustained-release pattern[8,9]. The bioavailability of oral administration is 100%. The rapidly released ingredients ensure the onset of action at 15 to 20 min after oral administration, and the elimination half-life at 4-5 hours. Due to the sustained-release components in ISMN, the clinical cardiovascular protection time is extended for up to 17 hours[10,11]. The most common method in the clinic for the prevention of nitrate resistance tolerance is to provide a discontinuous distribution of 24-hour plasma concentrations, i.e., by alternating administration of isosorbidedinitrate and ISMN tablets[12].

To explore an alternative solution, by which nitrate esters can effectively be used to dilate blood vessels and improve myocardial ischemia, while at the same time avoiding the development of drug tolerance, we designed a research with an animal model where we tested an approach using oral administration of the sustained-release capsule of ISMN. The effects on -SH, superoxide anion ($\bullet O_2^-$) and endothelin-1 (ET-1) in the tolerance mechanism of nitrate esters were investigated.

This study explores the possibility of improving the tolerance to nitrate esters by oral administration of sulfhydryl groups. Also, experiments were carried out to investigate the effectiveness of the adjuvant therapy, thereby providing a first step for clinical future clinical investigations.

Materials and Methods

Experimental Materials

1. Twenty-four healthy New Zealand white rabbits of either gender, weighing 2000 ± 200 g, were randomly divided into four groups: healthy control, nitrate ester, nitrate ester tolerance and drug combination groups, with 6 animals in each. At the same time, twenty-four more healthy New Zealand white rabbits were selected for the establishment of atherosclerosis (AS) model. Briefly, the abdominal aorta of each animal was catheterized, and the local endothelial layer was disrupted by a balloon-catheter endothelium-stripping method. Then, animals were fed with a high-fat diet (0.5% cholesterol + 5% lard + 15% egg yolk powder + 79.5% normal diet) for 3 weeks, following with a similar diet without 0.5% cholesterol (5% lard + 15% egg yolk powder + 79.5% normal diet) for another 5 weeks. Finally, the animals with AS were randomly divided into four groups: AS, AS + nitrate ester, AS + nitrate ester tolerance and AS + combination drugs groups, again with 6 animals in each. The rabbits in different groups were kept as follows:

1. Control and AS groups: animals were given no treatment, and general feeding was offered.
2. Nitrate ester group: animals were administered oral isosorbide mononitrate capsules (manufacturer: Yangtze River Pharmaceutical Group, Shanghai Haini Pharmaceutical Ltd., Shanghai, China approval number: Zhunzi H10970331) at the same time once a day for 5 days (25 mg/pellet), and offered general feeding.
3. Nitrate ester tolerance group: animals were administered oral isosorbide mononitrate capsules at the same times (25 mg/pellet), twice a day (12 hours intervals), for a total of 5 days, and offered general feeding.
4. Combination drugs group: animals were administered oral isosorbide mononitrate capsules + N-Acetylcysteine (NAC) granules, and isosorbide mononitrate (25 mg/pellet) + NAC 100 mg, twice daily every 12 hours for a total of 5 days. The animals were offered general feeding.
5. AS + nitrate ester group: animals were administered oral isosorbide mononitrate capsules at the same time every day (25 mg/pellet), for a total of 5 days.

6. AS + nitrate ester tolerance group: animals were administered oral isosorbide mononitrate capsules every 12 hours (25 mg/pellet) for a total of 5 days.
7. AS + combination drugs group: animals were administered oral isosorbide mononitrate capsules + NAC granules, and isosorbide mononitrate (25 mg/pellet) + NAC 100 mg, twice daily every 12 hours, for a total of 5 days.

2. After 5 days, all of the animals were injected with heparin sodium 1000 U via the ear vein and, then, were sacrificed by intravenous injection of 3% pentobarbital sodium. A thoracotomy was performed, immediately, the abdominal aorta was dissected, removed and placed in K-H solution. Vessels were cut into 5 mm long segments.

3. The aorta segments from all groups were collected, and the concentrations of $\cdot O_2$- and superoxide dismutase (SOD) in the supernatant of the homogenate of vessels were determined using a Luminol Chemiluminescence System. The concentration of malondialdehyde (MDA) in the supernatant was determined by TBA colorimetry, and the concentrations of NO and ET-1 in the tissue fluids were measured using an enzyme-linked immunosorbent assay (ELISA) kit for NO and ET-1. Finally, the relaxation responses of the blood vessels to different concentrations of NTG (N-Methyl-N -nitro-N-nitrosoguanidine) were measured with a bench-scale automatic balance recorder after stimulation with phenylephrine, and the dose-effect relationship was recorded. This study was approved by the Animal Ethics Committee of Shanghai Jiao Tong University Animal Center.

Determination of $\cdot O_2$- Concentration

5 ml of aorta blood from different subjects were extracted and, then, centrifuged at $1000 \times$ g for 10 min at 4°C. The plasma supernatant was collected, and the concentrations of $\cdot O_2$- were determined with a Luminol Luminescence Kit (Suzhou Alpha, Suzhou, China) according to the manufacturer's instructions[13].

Determination of SOD Concentration

5 ml of aorta blood were extracted from different subjects and, then, centrifuged at $1000 \times$ g for 10 min at 4°C. The plasma supernatant was collected, and the concentrations of SOD were determined with a Luminol Luminescence Kit (Thermo Fisher, Waltham, MA, USA) according to the manufacturer's instructions.

Determination of MDA Concentration

Samples of 5 ml of aortic blood were extracted and centrifuged at $1000 \times$ g, for 10 min at 4°C. The plasma supernatants were collected, and the concentrations of MDA were determined with Luminol Luminescence Kit (Suzhou Alpha, Suzhou, China) by measuring the absorbance at 495 nm in a Microplate Reader[13].

Determination of Nitric Oxide Concentration

Samples of 5 ml of aortic blood were extracted and, then, centrifuged at $1000 \times$ g, for 10 min at 4°C. The plasma supernatants were collected, and the concentrations of NO were determined using an ELISA Kit (Thermo Fisher, Waltham, MA, USA) by measuring the absorbance at 495 nm in a Microplate Reader[14].

Determination of Endothelin-1 Concentration

Samples of 5 ml of aortic blood from different subjects were extracted and, then, centrifuged at $1000 \times$ g, for 10 min at 4°C. The plasma supernatants were collected, and the concentrations of endothelin-1 were determined using an ELISA Kit (Thermo Fisher, Waltham, MA, USA) by measuring the absorbance at 495 nm with a Microplate Reader.

HE Staining

Hematoxylin and eosin (HE) staining was used to determine the morphological features in different samples; the experiments were conducted following the instructions in the staining kit (ABI, Foster City, CA, USA). Briefly, frozen sections were unfrozen at room temperature for 30 min. They were washed three times with phosphate-buffered solution (PBS) for 5 min each, immersed in hematoxylin solution for 3 min, and rinsed with running tap water for 30 s. Next, they were covered with 1% hydrochloric acid alcohol for 5 s, and finally, washed with running tap water for 7 min. A "bluing-up" of cell nuclei was observed. Then, sections were stained with eosin for 3 min and washed with running tap water for 30 s. A sequential submersion into different solutions followed: 70% ethanol for 5 s, 80% ethanol for 10 s, 90% ethanol for 1 min, 95% ethanol for 2 min, 100% ethanol I for 4 min, 100% ethanol II for 5 min, xylene I for 5 min, and xylene II for 5 min. The slides were then fixed with neutral mounting medium[14].

Statistical Analysis

All data were analyzed using the SAS 8.1 statistical software package (SAS Campus Drive Cary, NC, USA). Data were presented as $\bar{x} \pm s$, comparisons between two groups were assessed with double-tailed t-test, comparisons within a group were assessed with a one-way Analysis of Variance (ANOVA) followed by Least Significant Difference as the Post-hoc test. A p-value of less than 0.05 was considered statistically significant.

Results

Time-dependent Changes in the Levels of $\cdot O_2$-, SOD, MDA, NO, and ET-1 in the Control Group

The levels of $\cdot O_2$-, SOD, MDA, NO, ET-1 were determined in the control group at different time points. The results showed no significant differences between time points in the control group, see Figure 1.

Time-dependent Changes in the Levels of $\cdot O_2$-, SOD, MDA, NO, ET-1 in the AS Group

The levels of $\cdot O_2$-, SOD, MDA, NO, ET-1 were measured at different time points in the AS group. Results showed the levels of $\cdot O_2$-, SOD, MDA, NO, ET-1 exhibited different change trends through the different time points, as shown in Figure 2. The levels of SOD and MDA increased gradually in the AS group, by contrast, the levels of NO, $\cdot O_2$-, and
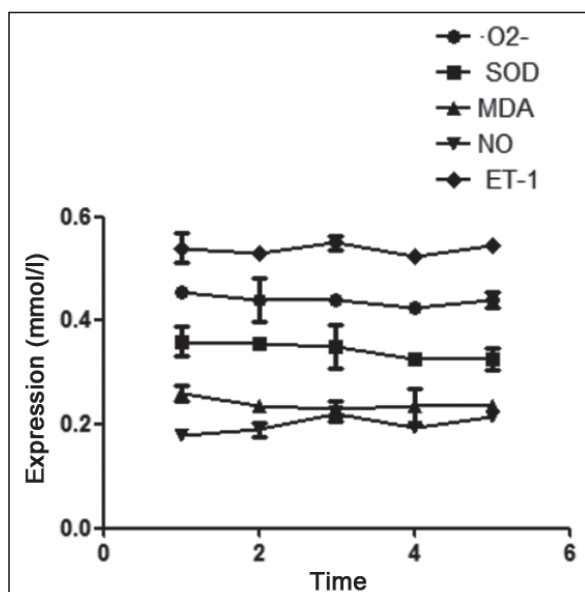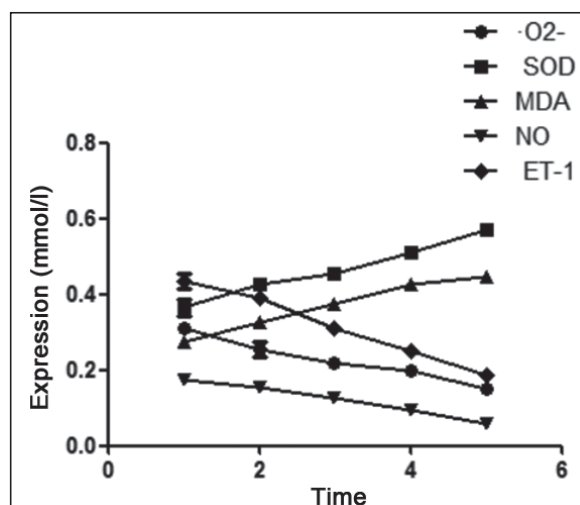

Figure 2. Time dependent changes in the levels of •O_2-, SOD, MDA, NO, and ET-1 in the AS group.

ET-1 decreased gradually, suggesting that the AS had an impact on the levels of the markers. When compared with the levels in the control group, the levels of NO, $\cdot O_2$-, and ET-1were significantly reduced in AS group, but those of SOD and MDA were significantly increased ($p < 0.05$).

Time-dependent Changes in the Levels of $\cdot O_2$-, SOD, MDA, NO, ET-1 in the Nitrate Ester Group

The results of Figure 3 suggest that the levels of $\cdot O_2$-, SOD, MDA, NO, and ET-1 in the nitrate ester group had different change trends, as com-
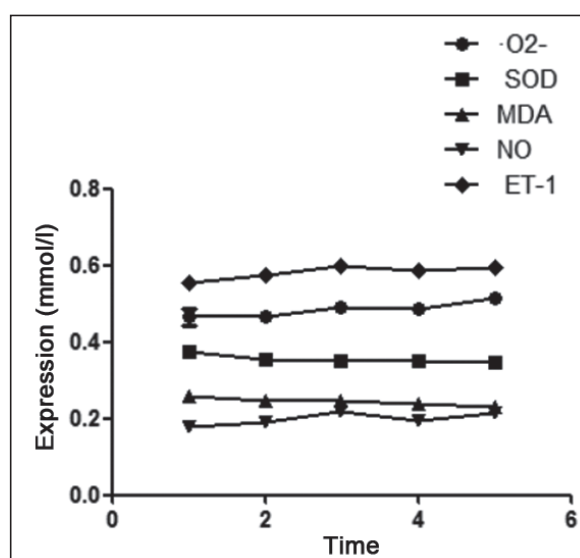

Figure 1. Time dependent changes in the levels of •O_2-, SOD, MDA, NO and ET-1 in the control group.

Figure 3. Time dependent changes in the levels of •O_2-, SOD, MDA, NO, and ET-1 in the nitrate ester group.

pared to the trends seen in the other groups. For instance, SOD and MDA showed a decreasing trend, whereas $\cdot O_2$-, NO, and ET-1 increased gradually, however, the changes were not enough to show statistical significance ($p > 0.05$).

Time-dependent Changes in the Levels of $\cdot O_2$-, SOD, MDA, NO, and ET-1 in the AS + Nitrate Ester Group
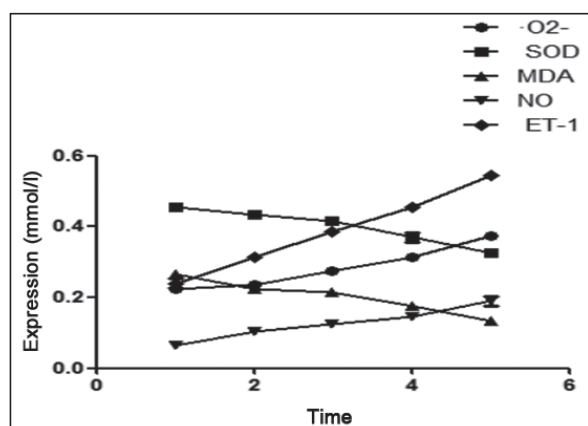
The results shown in Figure 4 showed that $\cdot O_2$-, SOD, MDA, NO, and ET-1 levels varied with time. The levels of SOD and MDA were high in the AS + nitrate ester group, but they decreased gradually with time ($p < 0.05$). In contrast, the levels of $\cdot O_2$-, NO, and ET-1 were low in the AS + nitrate ester group, but they increased gradually with time ($p < 0.05$). This suggests that isosorbide mononitrate capsules can significantly increase the levels of $\cdot O_2$-, NO and ET-1, therefore improving the body's resistance to AS.

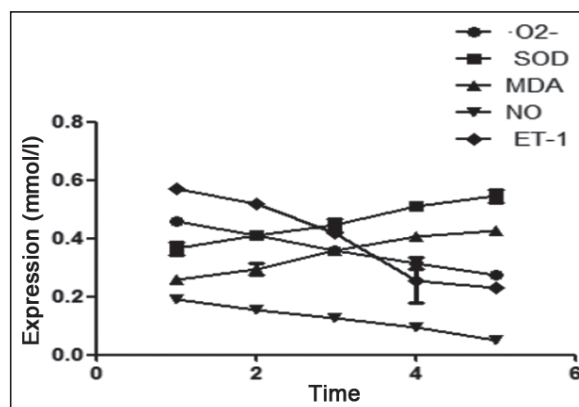### Time-dependent Changes in the Levels of $\cdot O_2$-, SOD, MDA, NO, and ET-1 in the Nitrate Ester Tolerance Group

The results of Figure 5 show the levels of $\cdot O_2$-, SOD, MDA, NO, and ET-1 exhibited different change trends with time. For instance, the levels of SOD and MDA enhanced gradually with time, but those of $\cdot O_2$-, NO, and ET-1 diminished gradually in the nitrate ester tolerance group ($p < 0.05$).

Time-dependent Changes in the Levels of $\cdot O_2$-, SOD, MDA, NO and ET-1 the AS + Nitrate Ester Tolerance Group

As seen in Figure 6, the change trends in the levels of $\cdot O_2$-, SOD, MDA, NO, and ET-1

Figure 5. Time dependent changes in the levels of $\bullet O_2$-, SOD, MDA, NO and ET-1 in the nitrate ester tolerance group.

were different. SOD and MDA increased, but the levels of $\cdot O_2$-, NO, and ET-1 decreased with time in AS + nitrate ester tolerance group. The differences showed a statistical significance ($p < 0.05$).

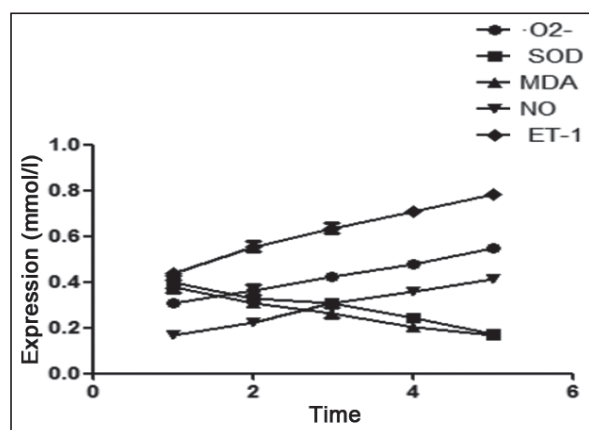### Time-dependent Changes in the Levels of $\cdot O_2$-, SOD, MDA, NO, and ET-1 in the Drug Combination Group

As seen in Figure 7, that the levels of SOD and MDA reduced gradually with time, in contrast, those of $\cdot O_2$-, NO and ET-1 increased instead ($p < 0.05$).

Time-dependent Changes in the Levels of $\cdot O_2$-, SOD, MDA, NO, and ET-1 in the AS + Drug Combination Group

The results showed the levels of $\cdot O_2$-, SOD, MDA, NO, and ET-1 had different change trends,

Figure 4. Time dependent changes in the levels of $\bullet O_2$-, SOD, MDA, NO and and ET-1 in the AS + nitrate ester group.
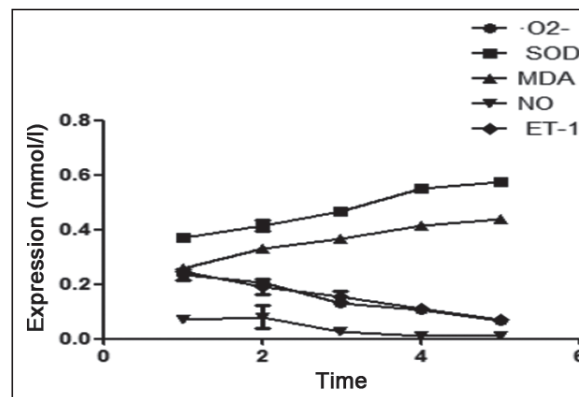
Figure 6. Time dependent changes in the levels of $\bullet O_2$-, SOD, MDA, NO and ET-1, in the AS + nitrate ester tolerance group.

1473

Figure 7. Time dependent changes in the levels of $\cdot O_2-$, SOD, MDA, NO and ET-1 in the drug combination group.

see Figure 8. In the case of SOD and MDA, their levels decreased with time, but the levels of $\cdot O_2-$, NO and ET-1 increased significantly instead ($p < 0.05$).

Comparison of Vascular Plaques in Different Groups by HE Staining

The plaques in the blood vessels of different groups were observed by HE staining (Figure 9). The results show the area of vascular plaques in the AS group was significantly larger than that in the control group. Also, the area of vascular plaques in the AS + nitrate ester tolerance group was significantly larger than that in the nitrate ester tolerance group ($p < 0.05$). However, there were no significant differences regarding the area of plaques between the AS + drug combination group and the non-AS drug combination group (p
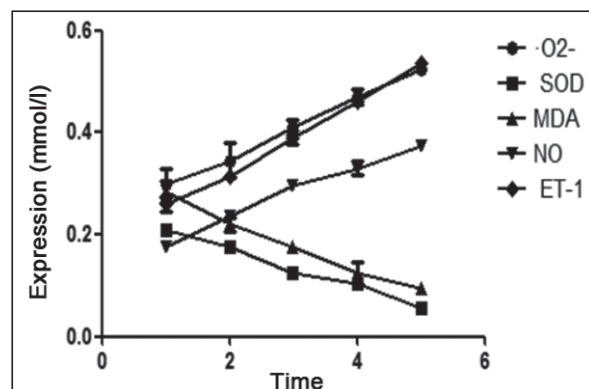

Figure 8. Time dependent changes in the levels of $\cdot O_2-$, SOD, MDA, NO and ET-1 in the AS + drug combination group.

> 0.05), suggesting that combined drugs significantly reduced the area of vascular plaques, thus improving the condition of atherosclerosis.

Discussion

In 1879, 26-year-old British doctor William Murrell opened a new chapter in the clinical application of nitrate esters by first reporting that nitroglycerin was effective in the treatment of angina pectoris. However, frequent dosing or continuous administration for 24-72 hours may induce drug tolerance, manifested by rapid decline or complete disappearance of the effects against myocardial ischemia and the effects on hemodynamics, rendering the treatment ineffective. Besides, there is cross-tolerance between the different classes of nitrate esters; thus, tolerance has become a major obstacle for its clinical application[15]. Different theories have been put forth as possible underlying mechanisms of tolerance including neurohormonal activation by the drug, oxidative stress induction, biochemical reactions resulting in thiol depletion, NO transduction obstruction, and mitochondrial dysfunction. Clinical approaches tried to prevent the occurrence of drug tolerance, including the use of statins and sulfhydryl group donor drugs.

In this study, we used white rabbits to establish an AS model and conformed groups with those and healthy controls in order to examine different manifestations of nitrate ester tolerance in the different groups. Our results showed that the levels of SOD and MDA (markers of oxidative stress) in AS rabbits were significantly higher, but $\cdot O_2-$, NO, and ET-1 were significantly lower when compared with healthy control group rabbits. Also, the levels of SOD and MDA were significantly higher ($p < 0.05$), but the levels of $\cdot O_2-$, NO, and ET-1 were decreased in the AS + nitrate ester tolerance group compared to the same levels in the AS + nitrate ester rabbits. In addition, the levels of $\cdot O_2-$, NO, and ET-1 were significantly increased in the AS + drug combination compared with those in the AS + nitrate ester tolerance group ($p < 0.05$). Nitrate esters act as non-endothelium-dependent exogenous nitric oxide (NO) donors[16], they can bind thiol groups in the sarcolemma forming nitrosothiol (S-nitrosothiols) by way of the glutathione S-transferase of smooth muscle cells of blood vessels. NO binds to Fe^{2+} contained in the active site of the heme of soluble guanylyl
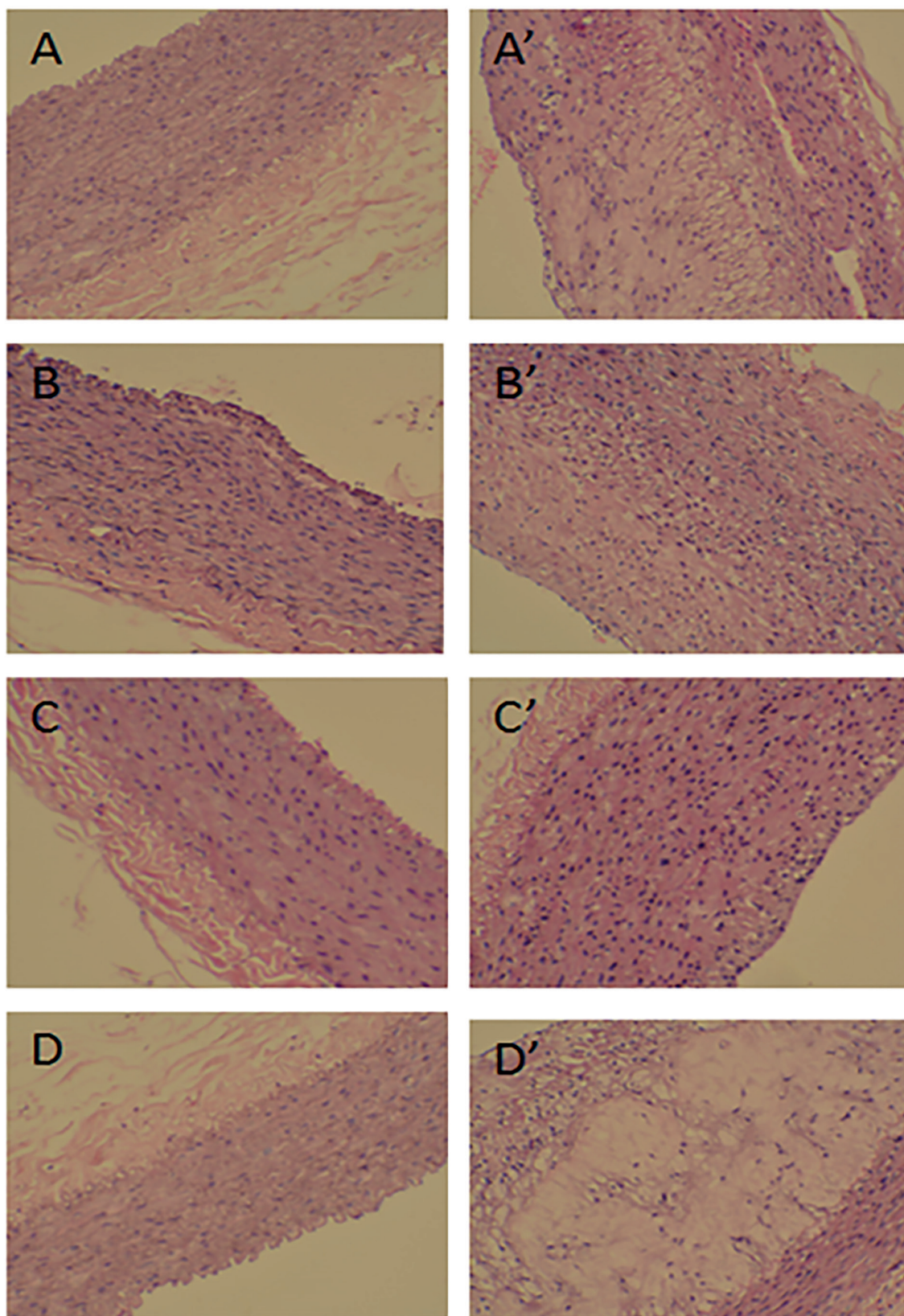
Figure 9. Observation of the area of vascular plaques by HE staining. **A,** control group; **A′,** AS group; **B,** nitrate ester tolerance group; **B′,** AS + nitrate ester group; **C,** nitrate ester tolerance group; **C′,** AS + nitrate ester tolerance group; **D,** drug combination group; **D′,** AS + drug combination group. The blue color shows the lipid (Magnification * 100).

cyclase (sGC) forming a complex, thereby activating sGC[17]. Guanylate cyclase produces cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP), cGMP, in turn, activates cGMP-dependent protein kinase C (PKC), which plays a role in decreasing intracellular calcium levels, for instance, inhibiting extracellular Ca^{2+} influx, and accelerating the sarcoplasmic reticulum's intracellular Ca^{2+} uptake, leading to relaxation of the muscle cells and thus to dilation of blood vessels[18]. N-Acetylcysteine (NAC) is a carrier of an active -SH, a precursor of active intracellular glutathione (GSH), and an oxygen free radical scavenger *in vivo*. The oral agent can be absorbed well, reaching the peak of plasma concentration after 2-3 hours of ingestion, and it displays an average terminal elimination half-life of 5.6 hours[19]. At present, the commonly used agents in the clinic include oral medicine (effervescent tablets and granules) and injections; they have been widely used as expectorants, for liver protection, and for detoxification. The roles of NACs as -SH donors and oxygen free radical scavengers can effectively counter the "thiol depletion" and "superoxide anion" actions, which are closely related to the mechanism of nitrate esters tolerance, providing a theoretical basis for antagonizing the tolerance of nitrate esters20.

Conclusions

Oral administration of a sulfhydryl group adjuvant with the nitrate therapy can significantly improve nitrate esters' tolerance, and this strategy is safe and promising for clinical trials.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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