

# *Salvia miltiorrhiza* Inhibit Vascular Smooth Muscle Cells Proliferation with Antioxidative Effect



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## Abstract

Vascular smooth muscle cells (VSMCs) proliferation plays a key role in the pathogenesis of atherosclerosis which is the main cause of cardiovascular or cerebrovascular deaths worldwide. This study aims to explore whether SM has antioxidant effects to inhibit VSMCs proliferation on the cultured rat aortic smooth muscle A10 cells. Cell viability assay, intracellular reactive oxygen species (ROS) measurement and thiobarbituric acid-reactive substance assay were applied. One way analysis of variance and Scheffe test were used for the statistical analysis. The results demonstrated that homocysteine could induce more cell viability, intracellular ROS and lipid peroxidation of VSMCs, but reduced by *Salvia miltiorrhiza* (SM) or N-acetyl cysteine (NAC) treatment. Both SM and NAC can significantly decrease cell viability, intracellular ROS and lipid peroxidation on the rest or homocysteine-induced VSMCs. SM can inhibit VSMCs proliferation with suppressing intracellular ROS and lipid peroxidation.

## Introduction

Atherosclerosis is the global burden [1,2] and the leading causes of morbidity and mortality of ischemic heart disease, stroke, and peripheral vascular diseases all over the world [3,4]. They resulted in 247.9 deaths per 100,000 persons, accounting for 84.5% of cardiovascular or cerebrovascular deaths and 28.2% of all-cause mortality in 2013 [5]. VSMCs proliferation would play a key role in the pathogenesis of atherosclerosis [6,7]. Their phenotype would change from a contractile phenotype in quiescent state to a active synthetic phenotype in the pathologic process of atherosclerosis [8]. They would change from oval to spindle shape then.

*Salvia miltiorrhiza* (SM) known as "Danshen" in traditional Chinese medicine was commonly used to treat the blood stasis syndrome [9,10] cancers [11,12] and atherosclerosis related vascular diseases [13,14] for thousands of years in Asia. Previous population-based studies also found that SM was the most common Chinese herb used to treat heart disease [15,16] and stroke [17,18].

The purpose of this study is to find out whether SM has antioxidant effects to inhibit VSMCs proliferation on the cultured rat aortic smooth muscle A10 cells. In addition to the general control group, both negative control with homocysteine [19,20] and positive control with antioxidant NAC [21,22] were used for SM research on the cultured A10 cells.

## Materials and Methods

### Preparation of herbal extract *Salvia miltiorrhiza*

The roots of cultivated *Salvia miltiorrhiza* Bunge were purchased from a Traditional Chinese Medicine dispensary in Taiwan. The 400g of this *Salvia miltiorrhiza* root was cut into 0.5cm long pieces and boiled in 500mL of freshly double distilled and deionized water under reflux condition through 1h. After that, the mixture was filtered through a Type JH membrane filter (0.45m, 47mm Id, Millipore, Bedford, MA, USA) and the filtrate was carefully collected. Next, further 500mL water was added to the residue and boiled for 1h. The filtrate was collected again and combined with the previous filtrate sample and cooled to ambient temperature. Final powdered form of the aqueous extract of *Salvia miltiorrhiza* root was obtained by freeze-drying at -40 °C to a light brownish residue with an approximate yield of 76g (ca. 19.0%) using Alpha 2-4LSC apparatus (Martin Christ, Osterode, Germany). The crude sterile SM extract was stored at -20 °C until further use in all subsequent experiments. The concentration used in each experiment was calculated upon the dry weight of the *Salvia miltiorrhiza* extract (mg/mL) which was resuspended in freshly prepared double distilled and deionized water as purified by Milli-Q filtration system (Millipore). SM 0.015mg/ml would be used in this study.

### Rat aortic smooth muscle cells culture

The rat A10 cells line, a vascular smooth muscle cells (VSMCs) line isolated from rat thoracic embryonic aorta [23], was purchased from the Food Industry Research and Development Institute in Taiwan. Cells were routinely cultured in Dulbecco's modified Eagle medium (DMEM, Gibco Life Technologies, Sao Paulo, Brazil) containing 10.0% fetal bovine serum, penicillin (100units/mL), streptomycin (100g/mL) and NaHCO<sub>3</sub> (3.7g/L) and grown in humidified atmosphere of 5.0% CO<sub>2</sub> in air at 37 °C. Cells were characterized as being vascular smooth muscle cells by immunofluorescence detection of alpha-smooth muscle actin (Sigma). Cells between passages 7 and 15 were used for experiments.

### MTT cell viability assay

This assay is based on the capacity of mitochondrial enzymes (succinate dehydrogenase) in the cell to reduce thiazolyl blue tetrazolium bromide (MTT) to form insoluble product formazan [24]. The amount of  $1.0 \times 10^5$  cells were seeded in 24-well tissue culture plates and replaced with or without 100M of homocysteine. Different groups including control, *Salvia miltiorrhiza* and antioxidant n-acetyl cysteine (NAC) were compared. For the growth-rate determination, after 3 days of culture, the isopropanol solution mixed with freshlymade MTT salt was added to wells in triplicate. Next, the optical density of dissolved material was measured spectrophotometrically at 560nm with subtract background at 670 nm using a 96-well plate reader (Elx 800, Bio-Tek Instrument, Winooski, VT, USA).

### Measurement of reactive oxygen species(ROS)

To assess the effect of SM on the cellular redox status, the intracellular ROS was measured by the fluorimetric assay with 2, 7 - dichlorofluorescin (DCFH) as described by Wang and

Joseph [25]. Briefly, 5M of the ROS prober dye in the form of 2, 7 -dichlorofluorescin diacetate (DCFH-DA) was added and incubated for 1h at 37 °C. Next, the rat A10 cells were washed with PBS, and fluorescence of DCFH was monitored in the Perkin-Elmer LS 5B fluorometer (Perkin-Elmer, Norwalk, CT, USA) with excitation at 485 nm and emission at 530 nm, respectively.

### Thiobarbituric acid-reactive substance assay

Thiobarbituric acid-reactive substances (TBARS) were determined according to the assay described by Janero [26]. The antioxidant effect on lipid peroxidation was evaluated by determining malondialdehyde (MDA) level in the cultured VSMC medium via the thiobarbituric acid reacting substances method (Sigma). The cultured VSMC medium mixed with trichloroacetic acid and thiobarbituric acid was boiled. 2ml butanol was then added to the each tube and results were obtained at 535nm after centrifugation at 2500rpm for 10min.

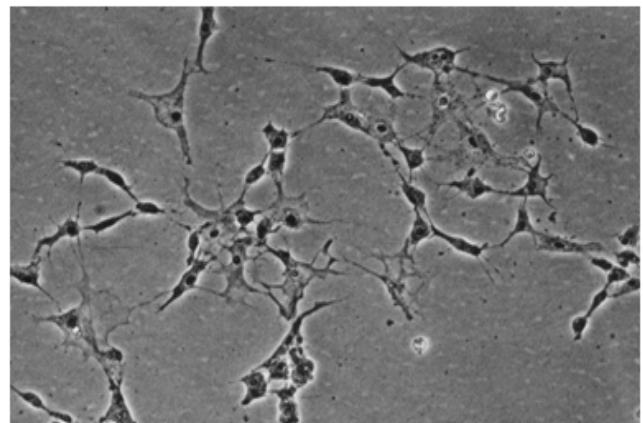
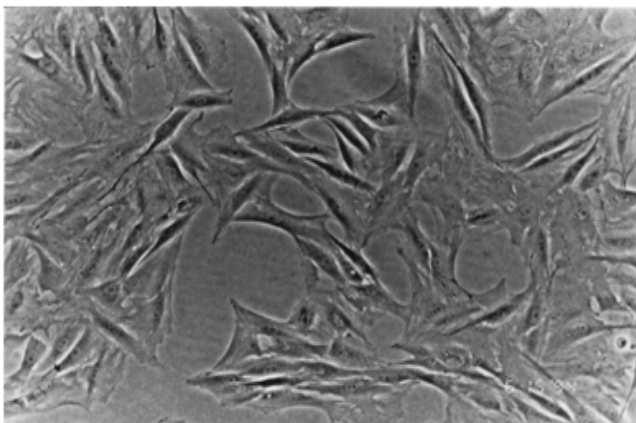
### Statistical analysis

All experiments were repeated 5 times and the results were expressed as means  $\pm$  standard error mean. One way analysis of variance and Scheffe test using SPSS software (SPSS Inc, Chicago, IL, USA) were applied for the statistical analysis. A value of  $p < 0.05$  was considered to be statistically significant

### Results

#### Morphology change of A10 cells with *Salvia miltiorrhiza*

A10 cells were authenticated as being VSMCs by immunofluorescence detection initially. After 3 days of A10 cells cultured , morphology of spindle synthetic phenotype of VSMCs without *Salvia miltiorrhiza* treatment were changed to irregular shape with *Salvia miltiorrhiza* treatment (Figure 1).



**Figure 1:** Morphology differences between rest cells and SM induced A10 cells. It shows spindle shape on the rest cells and irregular shape on the SM induced VSMCs.

### Inhibitory A10 cells viability with *Salvia miltiorrhiza*

MTT results (Figure 2) showed the mean cell viability of control group was  $47.45 \pm 1.49\%$  on the rest cultured VSMC , but significantly decreased to  $34.91 \pm 1.26\%$  in the SM group or

$33.62 \pm 1.28\%$  in the NAC group ( $P < 0.001$ ). Scheffe test (Table 1) revealed that the mean of difference  $12.54 \pm 0.85\%$  between control group and SM group was statistically significant (95% CI: 10.167~14.910%). The other mean of difference  $13.83 \pm 0.85\%$

between control group and NAC group was also statistically significant (95% CI: 11.459~16.202%). However, there was no significant difference 1.29±0.85% (95% CI: 1.080~3.664%) between SM and NAC group. Homocysteine (100µM) can significantly stimulate A10 cells growth (P<0.001), but would be inhibited by SM or NAC treatment. The mean cell viability of control group was 100±0.00% on the homocysteine induced VSMC culture, but significantly decreased to 48.17±1.21% in the SM group or 48.01±0.80% in the NAC group (P<0.001). Scheffe test (Table 2) revealed that the mean of difference 51.83±0.53% between control group and SM group was statistically significant (95% CI: 50.356~53.304%). The other mean of difference 51.99±0.53% between control group and NAC group was also statistically significant (95% CI: 50.518~53.466%). However, there was no significant difference 0.16±0.53% (95% CI: 1.080~3.664%) between SM and NAC group. The results demonstrated that SM 0.015mg/ml and NAC 16ng/ml did inhibit the proliferation of A10 cells (Table 3). The inhibition phenomenon was more pronounced in the homocysteine induced VSMC growth.

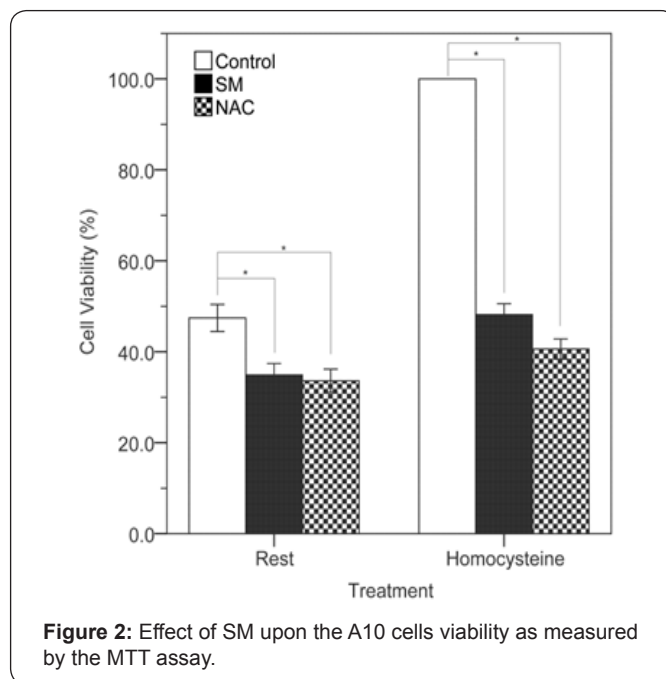


Figure 2: Effect of SM upon the A10 cells viability as measured by the MTT assay.

Table 1: Cell viability, ROS and lipid peroxidation differences compared with Scheffe method on Rest A10 cells.

Homocysteine A10	Group comparison	Mean <sub>diff</sub> ±SD	P value	95% CI
Cell Viability (%)	Control-SM	51.83±0.53	0.000	(50.356,53.304)
	Control-NAC	51.99±0.53	0.000	(50.518,53.466)
	SM-NAC	0.16±0.53	0.954	(-1.312,1.636)
ROS (%)	Control-SM	23.71±0.60	0.000	(22.031,25.389)
	Control-NAC	25.14±0.60	0.000	(23.463,26.821)
	SM-NAC	1.43±0.60	0.099	(-0.247,3.111)
Lipid peroxidation	Control-SM	0.19±0.01	0.000	(0.174,0.213)
	Control-NAC	0.21±0.01	0.000	(0.191,0.230)
	SM-NAC	0.02±0.01	0.111	(-0.003,0.036)

Table 2: Cell viability, ROS and lipid peroxidation differences compared with Scheffe method on homocysteine induced A10 cells.

Rest A10	Group comparison	Mean <sub>diff</sub> ±SD	P value	95% CI
Cell Viability (%)	Control-SM	12.54±0.85	0.000	(10.167,14.910)
	Control-NAC	13.83±0.85	0.000	(11.459,16.202)
	SM-NAC	1.29±0.85	0.348	(-1.080,3.664)
ROS (%)	Control-SM	3.66±0.54	0.000	(2.138,5.174)
	Control-NAC	4.65±0.54	0.000	(3.132,6.168)
	SM-NAC	0.99±0.54	0.230	(-0.524,2.512)
Lipid peroxidation	Control-SM	0.09±0.01	0.000	(0.080,0.108)
	Control-NAC	0.10±0.01	0.000	(0.083,0.111)
	SM-NAC	0.00±0.01	0.806	(-0.011,0.017)

Table 3: Cell viability, ROS and lipid peroxidation differences comparison.

VSMC	Control	SM	NAC	F value	P value
<b>Rest</b>					
Cell Viability (%)	47.45±1.49	34.91±1.26	33.62±1.28	161.265	0
ROS (%)	19.49±0.98	15.83±0.81	14.84±0.78	40.427	0
Lipid peroxidation	0.38±0.01	0.29±0.01	0.28±0.01	237.454	0
<b>Homocysteine</b>					

Cell Viability (%)	100±0.00	48.17±1.21	48.01±0.80	6428.153	0
ROS (%)	42.93±1.25	18.22±0.84	17.79±0.68	1099.746	0
Lipid peroxidation	0.59±0.02	0.39±0.01	0.38±0.01	544.751	0

### Inhibitory reactive oxygen species of A10 cells with *Salvia miltiorrhiza*

The results of intracellular ROS measurement (Figure 3) revealed the mean value of control group was 19.49±0.98% on the rest cultured VSMC, but significantly decreased to 15.83±0.81% in the SM group or 14.84±0.78% in the NAC group (P<0.001). Scheffe test (Table 1) noted that the mean of difference 3.66±0.54% between control group and SM group was statistically significant (95% CI: 2.138~5.174%). The other mean of difference 4.65±0.54% between control group and NAC group was also statistically significant (95% CI: 3.132~6.168%). However, there was no significant difference 0.99±0.54% (95% CI: 0.524~2.512%) between SM and NAC group. Homocysteine (100µM) can significantly increase A10 cells intracellular ROS level (P<0.001), but would be reduced by SM or NAC treatment. The mean value of control group was 42.93±1.25% on the homocysteine induced VSMC culture, but significantly decreased to 18.22±0.84% in the SM group or 17.79±0.68% in the NAC group (P<0.001). Scheffe test (Table 2) revealed that the mean of difference 23.71±0.60% between control group and SM group was statistically significant (95% CI: 22.031~25.389%). The other mean of difference 25.14±0.60% between control group and NAC group was also statistically significant (95% CI: 23.463~26.821%). However, there was no significant difference 1.43±0.60% (95% CI: 0.247~3.111%) between SM and NAC group. The results demonstrated that SM 0.015mg/ml and NAC 16ng/ml did reduce intracellular ROS of A10 cells on with or without homocysteine induced VSMC culture (Table 3).

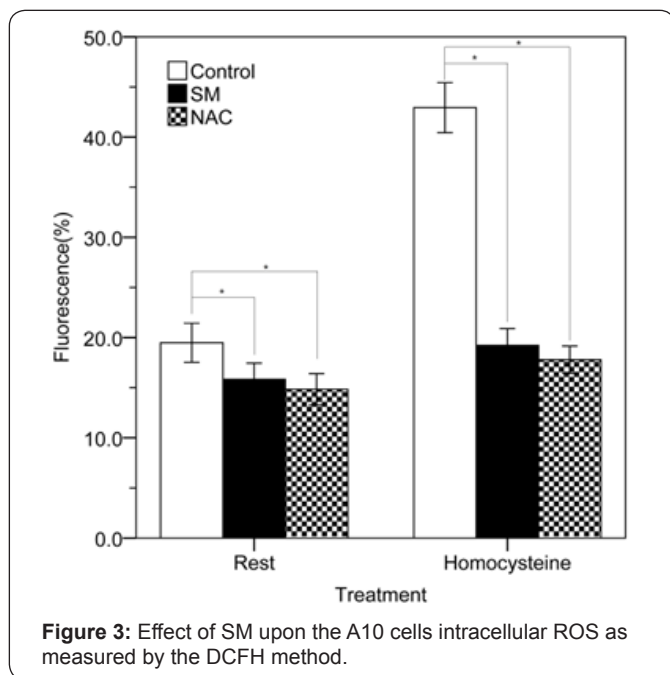


Figure 3: Effect of SM upon the A10 cells intracellular ROS as measured by the DCFH method.

### Inhibitory lipid peroxidation of A10 cells with *Salvia miltiorrhiza*

The results of lipid peroxidation measurement (Figure 4) showed the mean value of control group was 0.38±0.01 on the rest cultured VSMC, but significantly decreased to 0.29±0.01 in the SM group or 0.28±0.01 in the NAC group (P<0.001). Scheffe test (Table 1) revealed that the mean of difference 0.09±0.01 between control group and SM group was statistically significant (95% CI: 0.080~0.108). The other mean of difference 0.10±0.01 between control group and NAC group was also statistically significant (95% CI: 0.083~0.111). However, there was no significant difference 0.00±0.01 (95% CI: 0.011~0.017) between SM and NAC group. Homocysteine (100µM) can significantly increase A10 cells lipid peroxidation level (P<0.001), but would be reduced by SM or NAC treatment. The mean value of control group was 0.59±0.02 on the homocysteine induced VSMC culture, but significantly decreased to 0.39±0.01 in the SM group or 0.38±0.01 in the NAC group (P<0.001). Scheffe test (Table 2) revealed that the mean of difference 0.19±0.01 between control group and SM group was statistically significant (95% CI: 0.174~0.213). The other mean of difference 0.21±0.01 between control group and NAC group was also statistically significant (95% CI: 0.191~0.230). However, there was no significant difference 0.02±0.01 (95% CI: 0.003~0.036) between SM and NAC group. These results demonstrated that SM 0.015mg/ml and NAC 16ng/ml did improve lipid peroxidation of A10 cells on the with or without homocysteine induced VSMC culture (Table 3).

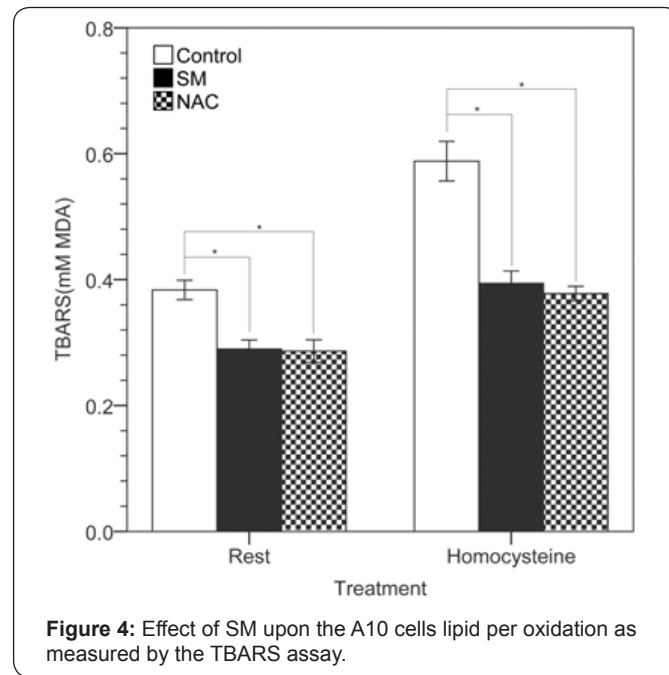


Figure 4: Effect of SM upon the A10 cells lipid per oxidation as measured by the TBARS assay.

## Discussion and Conclusion

Through activating circulation and dispersing blood stasis, interpretation of Traditional Chinese Medicine, SM could be used to treat blood stasis syndrome before. Until now, SM is still the most commonly used Chinese herb in cardiovascular [15,27] and cerebrovascular diseases [17,18]. It mainly contains the hydrophilic phenolic acids and the lipophilic tanshinones [28] which may have some pharmacological effects including cardioprotection, neuroprotection, anticancer, anti-inflammation and antifibrosis [29,30].

The morphology of spindle shape was changed by SM treatment. It would represent the condition that the VSMCs treated with SM were not in the active synthetic phenotype. SM may reduce the growth of VSMCs (Figure 1) to inhibit the pathological process of atherosclerosis.

In addition to the general control group, homocysteine was used as a negative control and NAC was used as a positive control for SM research on the cultured A10 cells. Homocysteine could be the high risk factor of atherosclerosis and induce proliferation and migration of VSMCs [19,20]. Another NAC is a strong antioxidant [21,22]. This study noted that homocysteine could induce more cell viability, intracellular ROS, and lipid peroxidation of VSMCs, but reduced by SM or NAC treatment. Both SM and NAC can significantly decrease VSMCs cell viability, intracellular ROS, and lipid peroxidation on the rest or homocysteine-induced VSMCs (Table 3).

Excessive ROS and oxidative stress can cause endothelium dysfunction, lipid peroxidation, and vascular inflammation [31]. They would regulate differentiation and contractility of VSMCs and lead to vascular remodeling in the initiation and progression of atherosclerosis [32,33]. Another lipid oxidation products and oxidized low-density lipoproteins could impair platelet-derived growth factor receptor activity and induce smooth muscle cell migration and proliferation in the vessel [34]. This study demonstrated that SM may be similar to NAC with antioxidant capacity and could inhibit VSMCs proliferation with suppressing intracellular ROS and lipid peroxidation. SM may reduce ROS production through downregulating oxidizes, superoxide, oxidative modification of LDLs, mitochondrial oxidative stress, but up regulating catalase, MnSOD, glutathione peroxidase and coupled eNOS [14].

No advanced molecular research about antioxidative mechanism of SM was the limitation of this study. Another limitation of this study was not carried out on the hydrophilic phenolic acids and the lipophilic tanshinones. However, the conclusion of this pilot study is SM could inhibit VSMCs proliferation with suppressing intracellular ROS and lipid peroxidation, which still can be used as a reference for further molecular medicine or clinical research.

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