Original Paper

Suppression of diet-induced hypercholesterolemia by scutellarin in rats

Qing Li$^{1, 2}$, Jian-Hong Wu$^2$, De-Jian Guo$^2$, Huan-Le Cheng$^2$, Shi-Lin Chen$^{1, 2}$, Shun-Wan Chan$^{2, 3,*}$

Affiliation

$^1$ Institute of Medicinal Plant Development, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, PR of China

$^2$ State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen, PR of China

$^3$ Open Laboratory of Chirotechnology, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR, PR of China

Correspondence

Dr. Shun-Wan Chan, Open Laboratory of Chirotechnology, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR, PR of China. E-mail address: bcswhchan@polyu.edu.hk; Phone: +852-34008718; Fax: +852-23649932
Abstract

Hypercholesterolemia is a major risk factor for the development and progression of cardiovascular diseases including atherosclerosis. A major active ingredient scutellarin, from the plant *Erigeron breviscapus* was investigated for its hypocholesterolemic and atheroscleroprotective effects (30 and 100 mg/kg/day, p.o.). The serum lipid profile (total cholesterol, triglycerides, high density lipoprotein cholesterol and low density lipoprotein cholesterol) was monitored and aortic functions in Sprague-Dawley rats fed with normal diet, atherogenic diet or atherogenic diet plus oral administration of either scutellarin or simvastatin (a positive control) were tested. It was found that scutellarin markedly attenuated the increased serum total cholesterol induced by atherogenic diet. It caused a significant reduction in the atherogenic index. In addition, scutellarin administration could significantly enhance acetylcholine-induced nitrate/nitrite production, increase the gene expression of endothelial nitric oxide synthase and improve acetylcholine-induced endothelium-dependent vasorelaxation in rat isolated aortas. These data revealed that scutellarin could reduce the atherogenic properties of dietary cholesterol in rats. However, whether scutellarin’s atheroscleroprotective potential targets endothelial function directly or indirectly on its antioxidative activity remains to be determined.

Key words
Scutellarin; Atherosclerosis; Hypercholesterolemia; Vasorelaxation; Nitric Oxide; Cholesterol

Abbreviations: 7α-hydroxylase (CYP7A1); endothelial nitric oxide synthase (eNOS); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); high cholesterol diet group (HCD); high density lipoprotein cholesterol (HDL); low density lipoprotein cholesterol (LDL); nitric oxide (NO); nitric oxide synthase (NOS); simvastatin (10 mg/kg/day, p.o.) treated group (SIM); low dose scutellarin (30 mg/kg/day, p.o.) treated group (SL) and high dose scutellarin (100 mg/kg/day, p.o.) treated group (SH).
Introduction

Atherosclerosis is the leading cause of death in developed countries and the incidence rate is increasing in other parts of the world [1,2]. Hypercholesterolemia (high blood total cholesterol) is a dominant risk factor for the development and progression of atherosclerosis and other related cardiovascular diseases which have emerged as a major health problem in many countries [2,3]. A high cholesterol diet is a major contributor to an unbalanced lipoprotein metabolism and associates with an increased prevalence of atherosclerosis [3].

In hypercholesterolemia and atherosclerosis, the physiological activity of nitric oxide (NO) is reduced and this resulted in impairment of the endothelium-dependent vasodilation, platelet aggregation enhancement and increased endothelial adhesiveness for monocytes [4]. Endothelial dysfunction is recognized as the basic mechanism for initiation and maintenance of atherosclerosis [5]. Therefore, the protection of endothelial integrity by elimination of certain risk factors via lipid lowering agents has been proven to be effective in restoring endothelial function and in slowing the progress of the disease [6].

Scutellarin, 7-(β-D-glucopyranuronosyloxy)-5,6-dihydroxy-2-(4-hydroxyphenyl)-4-H-1-benzopyran-4-one (C_{21}H_{18}O_{12}) (Fig. 1), is one of the active components isolated from a perennial wild plant distributed in southwest China, *Erigeron breviscapus* (Vant.) Hand.-Mazz. (Dengzhanxixin), which belongs to the family of *Compositae* and has been
used in clinical settings to treat cerebrovascular accident patients for many years [7]. In recent years, it was reported that scutellarin possessed anticoagulatory [8], antioxidative [9], anti-inflammatory [10], neuroprotective [11], vasorelaxation [12] as well as cardiovascular and cerebrovascular ischemia protective effects [13,14]. Despite the broad therapeutic uses of *Erigeron breviscapus*, there have been no reports on its effects on the reduction of lipid levels and atheroscleroprotection.

Therefore, an investigation was studied on scutellarin for any effect on high cholesterol diet-induced hypercholesterolemic rats, in particular the serum lipid profile [total cholesterol, triglycerides, high density lipoprotein cholesterol (HDL) and low density lipoprotein cholesterol (LDL)]. To investigate the protective role of scutellarin treatment under hypercholesterolemic condition, further investigations were carried out on the endothelium-dependent vasorelaxation, acetylcholine-induced nitrate/nitrite production [presumably NO production] and endothelial nitric oxide synthase (eNOS) mRNA expression of isolated aortas from rats with or without treatment with scutellarin. The effect of simvastatin, a known hypocholesterolemic and hypolipidemic drug, was also used for comparison.
Materials and Methods

**Chemicals**

Scutellarin was purchased as a yellow powder (>98% purity, formula weight 464.4) from Xi’an Guanyu Bio-Technique Co. Ltd (Xi’an, China) while simvastatin 20 mg tablets (10% purity, the content was confirmed by HPLC) were purchased from Hangzhou MSD Pharmaceutical Co. Ltd. (Hangzhou, China). Phenylephrine, acetylcholine, Nω-nitro-L-arginine methyl ester (L-NAME), indomethacin and neostigmine were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). NO kit was from Nanjing Keygen Biotech. Co. Ltd. (Nanjing, China). All the other chemicals used were of analytical grade.

**Animals and experimental treatment**

Male Sprague-Dawley rats (170 ± 10 g), supplied by Guangdong Provincial Medical Laboratory Animal Center (Guangzhou, PR of China), were housed separately (4 animals/cage) under a temperature controlled (25 ± 2 °C) room with a regular 12 hr light: 12 hr dark cycle. After one week, the rats were randomly assigned to one of five experimental groups (n = 8) for an additional 38 days. These groups were control group (Control), high cholesterol diet group (HCD), simvastatin (10 mg/kg/day, p.o.) treated group (SIM), low dose scutellarin (30 mg/kg/day, p.o.) treated group (SL) and high dose scutellarin (100 mg/kg/day, p.o.) treated group (SH). The control group was fed with standard normal rat chow with protein (~14%), fat (~10%) and carbohydrate (~76%) while the other groups were fed with HCD, which is a standard rat chow supplemented
with 1% cholic acid, 2% pure cholesterol and 5.5% oil. The rats were administered with distilled water or their corresponding drugs by oral gavage (20 mL/kg) once every morning for 38 days. At the end of the experimental period, the rats were fasted overnight and killed by cervical dislocation. Blood samples and aortas were then collected for further analysis. All experiments were approved by the Ethical Committee of the Hong Kong Polytechnic University.

**Analysis of lipid levels in blood samples**

Immediately after cervical dislocation, blood was collected in chilled centrifuge tubes by cardiac puncture. The blood was then centrifuged (4500 rpm) at 4 °C for 15 min and serum was collected. Total serum cholesterol, triglycerides, LDL and HDL were measured by using the Keygen’s reagents.

**Isolation of thoracic aorta**

At the end of the treatment period, the rats were sacrificed and their thoracic aortas were immediately placed in 4 °C Tyrode's solution of the following composition: NaCl 118 mM, KCl 4.7 mM, KH2PO4 1.2 mM, NaHCO3 25 mM, glucose 11 mM, CaCl2 2.5 mM, and MgSO4 1.2 mM. The isolated aorta from each animal was cut into three ring segments with any fat and connective tissue removed. One ring from each aortic preparation was used for isometric tension measurement (aortic ring ~3 mm), *in vitro* nitrate/nitrite production (aortic ring ~15 mm) and real-time PCR analysis (aortic ring ~15 mm).
Measurement of the isometric tension of the isolated thoracic aorta

One of the ring segments was mounted in 5 ml organ baths filled with Tyrode's solution (37 °C, gassed with 95% O₂ and 5% CO₂ mixture), under passive tension of 1.2 g for 60 min. After 60 min of equilibration, the aortic rings were challenged with 60 mM KCl twice to ensure a suitable contractile set up of the preparation. The contractile response (isometric tension, in g) was measured. To investigate the relaxant effects of acetylcholine on isolated aorta, the preparations were pre-contracted with 1 μM phenylephrine in the presence of 1 μM indomethacin (a nonselective cyclo-oxygenase inhibitor) and 1 μM neostigmine (an anticholinesterase). After a steady-state contraction was established, cumulative concentrations (10 nM – 10 μM) of acetylcholine were added to the organ bath.

In vitro nitrate/nitrite production in the isolated thoracic aorta

To evaluate the endothelial damage in blood vessels caused by high cholesterol diet, in vitro nitrate/nitrite production in the aortic ring was tested to estimate NO production. The isolated aortas were washed twice with Tyrode's solution, and then cut into 15mm segments (weight = 0.03 – 0.04 g). The segments were incubated in a 24-well plate (containing 2 mL Tyrode's solution per well) with 1 μM acetylcholine and 1 μM neostigmine. After incubation (37 °C) for 2 hr, each segment was blotted dry and weighed and the incubated culture solution of each well was collected in a separate microcentrifuge tube. The solution of each tube was subsequently dried by vacuum freeze-drying and the resulting pellets were re-dissolved with 300 μL of distilled water. Nitrate/nitrite levels were measured using the Keygen’s NO kit. The absorbance was
determined using the spectrophotometer at 540 nm. The concentrations of nitrate/nitrite were calculated following the instruction of the kit.

**Real-time polymerase chain reaction analysis**

Isolated thoracic aortas were homogenized and total RNA was extracted using Trizol reagent (Life Technologies Inc., Rockville, MD, USA) for the determination of gene expression levels in various groups. 3.5 µg of total RNA was reverse transcribed into cDNA using RevertAid™ first strand cDNA synthesis kit (Fermentas). Real-time PCR was performed with an ABI PRISM® 7000 Sequence Detection System with SYBR® GreenER™ qPCR SuperMix for ABI PRISM® (Invitrogen) in 20 µL of total reaction mixture. The primers for eNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Shanghai GeneCore Bio Technologies Co. Ltd. (China) (Table 1). Expression levels of the cDNA of interest were related to an internal standard: housekeeping gene (GAPDH), to correct for differences in quantity and quality between different RNA samples.

**Statistical analysis**

Data was expressed as means ± S.E.M. and n denotes the number of replications for each data point. After validation of each parameter for homogeneity of variance, the differences between groups were assessed by one-way analysis of variance using SPSS (Version 12) software package for Windows (Chicago, IL, USA). Post hoc testing was performed for intergroup comparisons using the least significance difference test; with P values of < 0.001, < 0.01 and < 0.05 are indicated in the figures or tables.
Results

The lipid profiles: serum total cholesterol, triglycerides, HDL and LDL from various rat groups are summarized in Table 2. Elevated serum total cholesterol and LDL were observed in the HCD. Treatment with simvastatin (10 mg/kg/day) and scutellarin (both 30 mg/kg/day and 100 mg/kg/day) altered the lipid profiles to normal levels and a notable increase in serum HDL/LDL ratio was observed. The serum total cholesterol was significantly reduced in the SIM (3.75 ± 0.40 mmol/L, P < 0.001), SL (4.86 ± 0.32 mmol/L, P < 0.05) and SH (4.34 ± 0.21 mmol/L, P < 0.01), as compared with that in the HCD (5.73 ± 0.36 mmol/L). However, total cholesterol in serum did not return to normal level (2.25 ± 0.05 mmol/L) in all treatment groups. The notable increase in the ratio of HDL/LDL among treatment groups was brought about by a markedly increase of HDL. The levels of HDL in all treatment groups were comparable to the level in the control group (P > 0.05). A high cholesterol diet significantly up-regulated LDL content but not the level of triglycerides in serum. Both simvastatin and scutellarin treatments have no significant effect on the levels of LDL and triglycerides (P > 0.05).

Further analysis of the impact of serum lipid profiles on the progression of atherosclerosis, the atherogenic index [(total serum cholesterol – HDL) / HDL] which measures coronary heart disease risk was calculated. It was found that elevated atherogenic index was observed in the HCD. There was a significant decrease in the atherogenic indexes in both the SIM and SH (Fig. 2) which suggested the atheroscleroprotective potential of simvastatin or scutellarin in the current experimental
setting. The atherogenic indexes were 2.69 ± 0.32 in the SIM and 2.58 ± 0.13 in the SH as compared to the HCD with 3.63 ± 0.26. The indexes for both the SIM and SH were close to the level of the control group (2.26 ± 0.32).

To evaluate the protective effect of drug administration on vascular endothelial activity, the thoracic aorta (with intact endothelium) was isolated for various analyses. When phenylephrine (1 μM)-induced contraction reached a steady condition, acetylcholine was added cumulatively to the aortic preparation. Acetylcholine elicited a concentration (10 nM – 10 μM)-dependent aortic relaxation of various groups of rats with ~55 – 80% maximum relaxation at 10 μM (n = 6 – 8) (Fig. 3). However, a significant smaller magnitude of relaxation caused by acetylcholine was observed from HCD rats compared to those observed in other groups. Scutellarin (30 mg/kg/day & 100 mg/kg/day) dose dependent improved the acetylcholine-induced relaxation in rats with high cholesterol diet. There was amelioration in the acetylcholine-induced relaxation in animals treated with scutellarin (100 mg/kg/day) and simvastatin (10 mg/kg/day).

The effect of drug treatments on high cholesterol diet-induced damage on aortic endothelial cells was also investigated. The isolated aortic rings from various groups were tested with regard to their effect on nitrate/nitrite production. Without acetylcholine (1 μM), aortic rings released undetectable levels of nitrate/nitrite after 2 hr incubation (data not shown). When acetylcholine (1 μM) was added to the incubation medium, nitrate/nitrite production in control group dramatically increased to 0.86 ± 0.21 nmol/mg of aortic tissue for the 2 hr incubation period. The effect of acetylcholine on nitrate/nitrite
production could be abolished by a NOS inhibitor, L-NAME (20 μM) (data not shown). It was found that high cholesterol diet markedly attenuated nitrate/nitrite production (0.25 ± 0.05 nmol/mg of tissue, P < 0.05). As shown in Fig. 4, all treatment groups significantly potentiated nitrate/nitrite production. The increase in nitrate/nitrite production for scutellarin was in a dose-dependent manner.

Change in the gene expression of eNOS was also examined (Fig. 5). The gene expression level of eNOS in the aortas from HCD group was markedly suppressed (P < 0.001); whereas treatment of simvastatin (10 mg/kg/day) and scutellarin (100 mg/kg/day) could significantly increase eNOS expression level back to the level of control group (1.81 ± 0.324) (P < 0.05). However, animals treated with scutellarin (30 mg/kg/day) showed no significant enhancement in the gene expression of eNOS.
Discussion

This study described for the first time that scutellarin has hypocholesterolimic effect and is orally active. In the present study, we have illustrated that oral scutellarin administration significantly reduced serum total cholesterol and increased serum HDL/LDL ratio. In addition, the elevated atherogenic index induced by atherogenic diet was reversed thus suggesting atheroscleroprotection by scutellarin. Although the testing dosage for scutellarin was up to 100 mg/kg/day, it can be considered as non-toxic for the fact that intraperitoneal LD$_{50}$ values in mice is 2402 mg/kg [13]. To evaluate the applicability of the current animal model, simvastatin (positive control) was used. It is a potent hypocholesterolemic and hypolipidemic drug of statin series and is commonly for the treatment of coronary heart disease, hypercholesterolemia and hyperlipidemia [15]. In this study, simvastatin has been shown to improve serum lipid profile and atherogenic index similar to the effects seen with scutellarin. However, the effects of simvastatin in the present study were associated with an increase in HDL level but no change in LDL level in animals fed with atherogenic diet. This is different from the function of statins in lowering LDL. This warrants further study in the current experimental setting.

In the current study, the HCD had abnormal serum lipid levels with significantly increased total cholesterol and LDL but no significant changes in triglyceride and HDL levels. This is consistent with previous studies with similar experimental settings [3,16]. In fact, elevated plasma total cholesterol and LDL levels are significant enough to demonstrate the atherogenic effect of the model since both parameters play a significant
role in atherosclerosis development and subsequent coronary heart disease [17].

Hypercholesterolemia and atherosclerosis have a close relationship with vascular dysfunction [5]. The present findings showed endothelial dysfunction in aortic rings from hypercholesterolemic rats. Vascular relaxation in response to acetylcholine was clearly blunted in aortas from the HCD. This provides supporting evidence to other studies where hypercholesterolemia and early stages of atherosclerosis in experimental animals and humans, the most common vascular functional abnormality is a reduction of endothelium-dependent relaxation [18]. Scutellarin could prevent the high cholesterol diet-induced reduction of eNOS gene expression. This in turn, increased the acetylcholine-induced nitrate/nitrite production and acetylcholine-induced vasorelaxation in aortic rings dose dependently.

NO responds to various pathophysiological stimuli in order to protect the integrity of the vasculature [19]. It is rapidly oxidized to nitrite/nitrate. The measurement of plasma nitrite/nitrate after an overnight fast was used as an index of NO synthase (NOS) activity [20]. Indeed, either activation or inhibition of NOS activity was associated with corresponding increases or decreases in circulation nitrite concentrations [21]. However, it should be noted that approximately 70% of plasma nitrite has been shown to be derived from NOS activity in the endothelium [21]. To enhance the accuracy of NOS activity estimation, we adopted a method to measure acetylcholine-induced nitrate/nitrite production in vitro instead of monitoring nitrite concentration in the circulation.
Atherogenic lipoprotein, LDL, was shown to increase eNOS generation of superoxide anion [22]. An increase in reactive oxygen species (ROS) resulted in oxidative stress and cellular oxidative damage [23]. In a hypercholesterolemic condition, increased ROS in the endothelium increases NO breakdown [24]. Scutellarin possesses a potent antioxidative activity and protected cells (including endothelium) from oxidative damage [25]. It was demonstrated as a good ROS scavenger [8,26]. Therefore, scutellarin could protect the vasculature through its antioxidative activity.

In the present study, scutellarin treatment dose dependently improved acetylcholine-induced relaxation on aortic rings. This can be explained by the up-regulation of eNOS RNA expression and/or increase in acetylcholine-induced nitrate/nitrite production, which represented an increase in NO availability. Scutellarin was shown to increase eNOS expression in a rat model of cerebral ischemia/reperfusion [14]. However, whether the vasoprotective effect is due to the up-regulation of eNOS gene, the antioxidative activity of scutellarin or both is presently unclear and awaits further investigation. In the HCD group, eNOS RNA expression was down-regulated in isolated aorta. However, several studies have shown that cardiovascular risk factors, including hypercholesterolemia are associated with an increase rather than a decrease in eNOS expression [27,28]. The reason for a decrease in NO availability is more likely related to eNOS dysfunction (eNOS uncoupling), which in many cases is resulted from a reduced availability of tetrahydrobiopterin, an essential co-factor for NOS [28]. eNOS uncoupling will lead to superoxide and H$_2$O$_2$ production [29]. The discrepancy in eNOS RNA expression warrants further study.
In conclusion, the current results indicate for the first time that scutellarin possess prominent hypocholesterolemic and atheroscleroprotective effects. The effects may be brought about by scutellarin on the gene expression of eNOS in endothelial cells, antioxidative activity or both. Confirmatory studies in other models would provide more evidence of scutellarin's effects.

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References


15 **McCord EL, Goenka S.** Development of thyroid follicular adenoma on simvastatin therapy. Tenn Med 2000; 93: 210-212.


17 **Kannel WB.** Range of serum cholesterol values in the population developing coronary artery disease. Am J Cardiol 1995; 76: 69C-77C.

18 **Sorensen KE, Celermajer DS, Georgakopoulos D, Hatcher G, Betteridge DJ,**


Figure legends

Fig. 1 The chemical structure of scutellarin. *Chiral center.

Fig. 2 The antherogenic indexes of animals from the Control, HCD, SIM, SL and SH. Data are expressed as means ± SEM, n = 5 - 8. #P < 0.05 represents significant differences when compared with the Control group. *P < 0.05 and **P < 0.01 represent significant differences when compared with the HCD.

Fig. 3 The concentration-response curves to acetylcholine are expressed as decrease in (percentage) steady state tension obtained with 1 μM phenylephrine precontracted thoracic aortic rings from the Control, HCD, SIM, SL and SH. Data are expressed as means ± SEM, n = 6 - 8. *P < 0.05 and **P < 0.01 represent significant differences when compared with the Control group.

Fig. 4 In vitro nitrate/nitrite production from isolated aortas under challenging with acetylcholine (1 μM). Data are expressed as means ± SEM, n = 6 - 8. #P < 0.05 represents significant differences when compared with the Control group. *P < 0.05 represents significant differences when compared with the HCD.

Fig. 5 Real time RT-PCR analysis of the gene expressions of eNOS in isolated aortas from the Control, HCD, SIM, SL and SH. The expression level of each gene was normalized to that of the GAPDH gene. Data are expressed as means ± SEM, n = 3 - 8.
# P < 0.05 represents significant differences when compared with the Control group. *P < 0.05 represents significant differences when compared with the HCD.
### Table 1 The primer sets used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product size (bp)</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>5’GGATCTGCAAGACCGATT3’</td>
<td>5’GGTGAGGACTGTCCAAACACT3’</td>
<td>159</td>
<td>U18336</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’TGCACCACCAACTGTTAG3’</td>
<td>5’AGTGGATGCAGGGATGATGT3’</td>
<td>180</td>
<td>NM_017008</td>
</tr>
</tbody>
</table>
Table 2 Serum lipid levels of rats in Control, HCD, SIM, SL and SH groups

<table>
<thead>
<tr>
<th>Animals</th>
<th>Control</th>
<th>HCD</th>
<th>SIM</th>
<th>SL</th>
<th>SH</th>
</tr>
</thead>
<tbody>
<tr>
<td>groups</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>2.25 ± 0.05</td>
<td>5.73 ± 0.36***</td>
<td>3.75 ± 0.40***</td>
<td>4.86 ± 0.32***</td>
<td>4.34 ± 0.21***</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>0.71 ± 0.03</td>
<td>0.73 ± 0.04</td>
<td>0.73 ± 0.06</td>
<td>0.83 ± 0.04</td>
<td>0.70 ± 0.06</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>0.29 ± 0.05</td>
<td>0.90 ± 0.07***</td>
<td>0.92 ± 0.12***</td>
<td>0.95 ± 0.11***</td>
<td>0.83 ± 0.04***</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.15 ± 0.11</td>
<td>0.62 ± 0.08</td>
<td>1.38 ± 0.09***</td>
<td>1.29 ± 0.07***</td>
<td>1.31 ± 0.06***</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM, n = 3 – 8.

### p < 0.001 represents significant differences when compared with the Control group.

* p < 0.05 represents significant differences when compared with the HCD group.

** p < 0.01 represents significant differences when compared with the HCD group.

*** p < 0.001 represents significant differences when compared with the HCD group.
Fig. 1.
Fig. 2.
Fig. 3.

- Log [Acetylcholine] (M)
- Percentage relaxation (%)
- 0 20 40 60 80 100
- Control
- HCD
- SIM
- SH
- SL
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Fig. 4.

Nitrate/nitrite content (nmol/mg of aorta)
Fig. 5.

![Graph showing eNOS/GAPDH mRNA expression level across different groups: Control, HCD, SIM, SL, SH. The graph indicates significant differences (*) and a trend noted by #.]