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The effect of ecdysterone on cerebral vasospasm following experimental subarachnoid hemorrhage *in vitro* and *in vivo*

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Abstract

Objectives—Cerebral vasospasm has been the dreaded complication of ruptured intracranial aneurysms. Worldwide effort has led to many promising experimental treatments but none was confirmed to be effective in clinical trials. Ecdysterone is an insect steroid hormone. Our previous study showed that ecdysterone might prevent cerebral vasospasm *in vitro*. Even after all these works, rare attempts have been made to test the effect of ecdysterone on vascular adventitial fibroblast (VAF) proliferation, a process known to play an important role in various pathogenic vascular conditions. Thus, we tested the hypothesis that ecdysterone could affect VAF characteristics and have an effect on SAH induced cerebral vasospasm.

Methods—OxyHb of 100 μ M was used in the *in vitro* study to mimic the clinical situation. The effect of OxyHb on the cell proliferation and migration of cultured aortic smooth muscle cells was investigated. In the *in vivo* study, 20 rabbits were equally divided into four groups: control group, SAH group, SAH/nimodipine group and SAH/ecdyserone group. Changes in neurological function and cerebral angiograms were observed after SAH.

Results—OxyHb increased the proliferation of vascular adventitial fibroblasts at 24 hours. Ecdysterone co-treatment was apparently similar to the suppression of proliferation. Cell cycle analysis indicated that ecdysterone inhibited the progression of vascular adventitial fibroblasts from G1 to S. The results of the migration assay showed that 100 μ M OxyHb obviously prompted vascular adventitial fibroblast migration and that ecdysterone would attenuate this effect. In the SAH/nimodipine and SAH/ecdyserone groups, neurological deficit, cerebral vasospasm and structural changes in basilar artery were alleviated with nimodipine or ecdysterone treatment.

Conclusion—Ecdysterone could affect vascular adventitial fibroblast characteristics and attenuate vasospasm after SAH.

Keywords

Cerebral vasospasm; ecdysterone; oxyhemoglobin; rabbit animal model; vascular adventitial fibroblast

INTRODUCTION

Despite years of intensive clinical and experimental investigation, delayed cerebral vasospasm remains the dreaded complication of ruptured intracranial aneurysms. Worldwide effort has

led to many promising experimental treatments that reverse or prevent cerebral vasospasm, but none was confirmed to be effective in clinical trials¹.

Ecdysterone, an analog of insect steroid hormone ecdysteroid isolated from rhizomes, roots and the stem bulk of many plants, has long been known for its role in insect development and metamorphosis. In the last few decades, a substantial body of evidence showed that ecdysterone may have significantly positive pharmacologic properties^{2,3}. This is consistent with the use of several ecdysteroid-containing plant species in Chinese herbs such as *Achyranthes bidentata* which has been well known for its ability to activate blood and eliminate stasis in the treatment of trauma and thrombosis in traditional Chinese medicine. Ecdysterone was considered the main active monomer component of *Achyranthes bidentata*. Recent research has found that ecdysterone also has beneficial effects on cerebral infarction, cerebral ischemia and cerebral trauma^{4,5}. Our previous study showed that ecdysterone might attenuate the NO secretion disorder of endothelial cells and the corresponding conditioned medium had a lower proliferative activity on vascular smooth muscle cells *in vitro*^{6,7}.

Even after all these works, rare attempts have been made to test the effect of ecdysterone on vascular adventitial fibroblast proliferation, a process known to play an important role in various pathogenic vascular conditions. Thus, we tested the hypothesis that ecdysterone could affect vascular adventitial fibroblast (VAF) characteristics and have an effect on subarachnoid hemorrhage (SAH) induced cerebral vasospasm. The present study is, to our knowledge, the first report that shows direct evidence of the protective effects of ecdysterone in experimental subarachnoid hemorrhage both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell culture and identification

Vascular adventitial fibroblasts were obtained from the descending thoracic aorta of female 200 g Sprague–Dawley rats. All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals. Animals were killed with intravenously administered pentobarbital (150 mg/kg). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 200 mg/ml L-glutamine, as described previously⁸. Over 90% of cells were in accordance with their special cell markers: VAFs stained positively to collagen III and vimentin but negatively to smooth muscle α-actin (Figure 1A,B). The cell culture exhibited typical morphology. Cells were passaged with trypsin (0.2 g/l)–EDTA (0.5 g/l) at ~95% confluence and were used in passages without changes in growth characteristics (VAFs for passages 3–7). When cells reached 100% confluence, they were growth arrested by maintenance in serum-free medium (DMEM containing 0.1% BSA and 200 mg/ml L-glutamine) for 24 hours before the experiment. All cells were set in three groups: control group, 100 µM OxyHb group and 100 µM OxyHb and EDS (200 mg/L) co-treated groups.

Proliferation assay: MTT assay and cell cycle assay

Methyl thiazolyl tetrazolium (MTT) was used for assaying cell proliferation. Cells (1×10^4 cells/well) were plated in 96-well plates and treated for 24 hours. Then the cells were treated with MTT (0.5 mg/ml) for 4 hours at 37°C. The culture medium was removed from 96-well plates, and DMSO (150 µl/well) was added to dissolve the formazan in the cells. The metabolized MTT was measured in an enzyme-linked immunosorbent assay reader at 490 nm. Cell viability of the VAFs was expressed with corresponding OD value. Changes in the cell cycle were assayed by flow cytometry (Facstar, USA). Cells were harvested into tubes and fixed with 70% ethanol, then washed with PBS, and assayed by flow cytometer.

Migration assay: wound healing assay and transwell assay

To detect the effect of OxyHb on cell migration *in vitro*, a modified monolayer-wounding cell migration assay was used. The cells were seeded at a density of 2×10^5 cells/well in six-well plates containing sterile glass coverslips. After the culture reached confluence, cells were scraped with a sterile 200 μ l pipet tip and treated according to the group. The distance of cell migration was measured after 12 hours, 24 hours, 2 days, 4 days and 7 days. Data are expressed as the percentage of the residual scraped area relative to the original scraped area. Cell migration was also assessed using a modified Boyden's chamber method⁹. The cells were harvested after treatment in accordance with the groups. After the cell suspension was cultured with DMEM containing 20% FBS for 1 hour, the cells were seeded at a density of 1×10^5 cells/well in a 24-well plate. Medium containing 20% FBS was used to induce the migration. Cells were allowed to migrate for 4 hours (12), then stained with hematoxylin dye and counted under a light microscopy ($\times 100$). The number of cells was recorded from at least five fields per well.

Rabbit model of SAH

A total of 20 male Japanese white rabbits weighing 2.8–3.4 kg were used in this study. All of them were treated in advance by right common carotid artery (CCA) ligation. After 7 days, they were randomly divided into the following four groups. Animals of Group 1 served as controls and had saline instead of blood injected into the cisterna magna ($n=5$), Group 2 received experimental SAH without additional treatment (SAH only; $n=5$). In Group 3, animals were administered nimodipine injections at 0.05 mg/kg/h for 4 hours per day by intravenous infusion through a cannulated marginal ear vein after SAH (SAH/nimodipine; $n=5$). Animals of Group 4 received injections of 10 mg/kg/d ecdysterone immediately after SAH (SAH/ecdyserone; $n=5$). All surgical and angiographic procedures were performed under anesthesia with 50 mg/kg i.m. ketamine hydrochloride and 10 mg/kg i.m. xylazine. The animals were placed in supine position.

The right CCA was approached in a sterile manner through a 1.5 cm median incision in the cervical region. The right CCA was ligated and a catheter (16-gauge polyethylene) was inserted in it to prepare for the angiograph. The skin wound was closed. After the ligation, all animals were observed for 7 days and no significant neurological symptoms were found in this study.

The central ear artery was cannulated to obtain 3 ml arterial blood. A 23-gauge butterfly needle was inserted percutaneously into the cisterna magna, and the autologous non-heparinized arterial blood was injected during a 10 second period. The animal was then positioned head down for 20 minutes to facilitate the settling of blood in the basal cistern. This procedure was repeated 48 hours later. The animal was allowed free access to food and water during the next 72 hours and observed closely for adequate food intake and for any possible neurological deficits.

Cerebral angiograph and transcranial Doppler sonography

Two angiographs were performed 1 day before and 3 days after SAH. After the animals were anesthetized, a peripheral vein catheter was placed. Body temperature was monitored and maintained with a water-heated blanket. End tidal and arterial carbon dioxide pressures, arterial oxygen pressure, oxygen saturation, blood pressure and heart rate were monitored and maintained within normal physiologic limits. Angiograms were obtained with retrograde injection of 5 ml 60% iohalamate meglumine within the catheter that was inserted in the ligated right CCA. Identical exposure factors and magnifications were used for all angiograms. After angiograms were obtained, mannitol (0.5 g/kg) was administered intravenously and the arterial carbon dioxide pressure was decreased to 30 mmHg. Transcranial Doppler sonography (TCD) was also performed with Medasonic CDS, a 2 MHz probe equipment (USA). The basilar artery

was explored through the pillow window. Peaksystolic velocity and mean velocity were measured.

Behavior and morphologic assessment

Rabbits were maintained on standard pellet feed and tap water in day/night regulated quarters at 23°C. Each rabbit underwent neurological examination and assessment of food intake before SAH and daily thereafter for 3 days. For the neurological evaluation, the animals were observed on a flat floor. Manual muscle testing was also carried out. Combining the assessment results, the neurological symptoms and food intake were categorized into four grades as Endo *et al.* described¹⁰.

Rabbits were killed 3 days after SAH. After the abdominal aorta was blocked, routine transcardial perfusion was preformed. Specimens of the superior half of the brainstem with the basilar artery were subjected to routine light microscope examination using standard H&E stain and the remaining half of the basilar artery was examined with routine transmission electron microscopy.

Drugs

OxyHb was prepared as described previously¹¹. The concentration of OxyHb was determined spectrophotometrically. Ecdysterone was obtained from the Kunming Institute of Botany (Kunming, China) and was dissolved in 0.05% DMSO and immediately diluted giving a final concentration of less than 0.1% DMSO. Nimodipine was obtained from Nimotop (Bayer Inc., Germany). Other chemicals were commercial products of the highest available grade from Sigma Inc. (USA).

Statistical analysis

All results are expressed as mean $\pm$ SEM. The data were analysed using one-way analysis of variance and Newman–Keuls–Student's *t*-test. $p < 0.05$ was considered significant.

RESULTS

In vitro model

The results showed the effect of ecdysterone and OxyHb on VAFs (Table 1). In a morphologic examination, VAFs stimulated by OxyHb had a more decurtated shape compared to those of the control group (Figure 1C). OxyHb of 100 μ M increased the proliferation of VAFs after 24 hours. In 100 μ M groups, the curve of the OD–time diagram turned out to be bidirectional, increasing acutely at first and then dropping down. Although co-treatment with ecdysterone failed to inhibit cellular proliferation with statistical significance when OxyHb was used to induce proliferation ($p > 0.05$), the trend was apparently similar to a suppression of proliferation (Figure 1D). The effects of OxyHb on cell cycle progression were determined by flow cytometry. Quiescent VAF was induced to enter the S phase by 24 hour stimulation with 100 μ M OxyHb (Figure 2B). The population of G₀/G₁ cells decreased (84.1 and 73.6%, $p < 0.05$), with a concomitant rise significantly in S phase cells (7.6 and 23.5%, $p < 0.05$). It seemed that ecdysterone inhibited the progression of VAFs from G₁ to S, as shown by the increase in G₀/G₁ cells (94.7%) accompanied by a concurrent decrease in S phase cells (3.04%) ($p < 0.05$) (Figure 2C). Results of the wounding cell migration assay showed that 100 μ M OxyHb prompted VAF migration ($p < 0.05$) and ecdysterone would attenuate this effect ($p < 0.05$) (Figure 2D). We also evaluated the effect of OxyHb on cellular migration using a modified Boyden's chamber method. The results are consistent with the data shown by the wounding cell migration assay ($p < 0.05$).

Rabbit model of SAH

Behavior assessment—The neurological findings are summarized in Figure 1. Rabbits in the three groups showed various neurological deficits after SAH. Two rabbits in Group 2 were hemiplegic, and no recovery was seen 3 days after SAH. In Groups 3 and 4, no rabbits were hemiplegic. The reduction in neurological deficits was not obvious in all groups 3 days after SAH. A significant decrease in food intake was seen in Group 2. In Group 3, the food intake of rabbits decreased, possibly because of the use of narcotics for 4 hours each day (Figure 3).

Angiography and TCD sonography—The initial mean diameter of the basilar arteries was 0.65 mm in Group 2 (SAH group), 0.67 mm in Group 3 and 0.64 mm in Group 4. Three days after SAH, the mean basilar artery diameters in the three groups were 0.50, 0.53 and 0.55 mm, and the average decreases were 23, 20 and 14%, respectively. The post-SAH basilar artery diameters in the three groups were significantly smaller compared to the relative initial levels and those of the SAH group was narrower than those of the other two groups. However, there was no difference in these last two groups (Figure 4). TCD revealed vasospasm of the basilar artery after the second blood injection. The SAH group showed the most typical change in the TCD index (Table 2).

Morphologic changes—Histopathologic examination revealed a thick subarachnoid clot around the basilar artery in all animals with SAH. There were no significant morphologic changes in the control group. Endothelial cell injury, endothelial desquamation, corrugation of the internal elastic lamina (IEL), necrotic changes, phenotype modulation of smooth muscle cells (SMC) and inflammatory cell infiltration were significant in Group 2 (SAH group). In Group 3, there was cytoplasmic blebbing of the partial endothelial cells, vacuolate, slight corrugation of the internal elastic lamina, and an absence of visible necrotic changes of the smooth muscle cells. Similar changes were found in Group 4. Moreover, inflammatory cell infiltration was not observed in Groups 3 and 4 (Figure 5).

DISCUSSION

We have observed that: (1) OxyHb could induce VAF proliferation and migration *in vitro*, and this effect could be inhibited by ecdysterone; (2) ecdysterone could alleviate neurological function deficits, attenuate vasospasm and protect the vessel structure in the rabbit SAH model. These effects are comparable with the effects of nimodipine.

Achyranthes bidentata has been used in the treatment of trauma, thrombosis and arthritis in Chinese traditional medicine for more than 1800 years. However, recent works indicate that it also has various pharmacologic effects on brain trauma. Works have shown that its effective element ecdysterone exerts protective effects on cerebral infarction, cerebral ischemia and cerebral trauma in cell models or animal models⁵. Our previous works have also shown that ecdysterone could alleviate the morphologic changes in the rabbit brain microvessel endothelial cells caused by bloody cerebrospinal fluid. It also decreases the expression of ICAM-1, attenuates the NO secretion disorder of endothelial cells after blood stimulation and inhibits the proliferative activity of endothelial cells in the corresponding conditioned medium^{6,7}.

The adventitia surrounding the blood vessels has long been exclusively considered as a supporting tissue, the main function of which is to provide adequate nourishment to the muscle layers of the tunica media. In fact, fibroblasts have the ability to rapidly respond to injury and to modulate their function to adapt rapidly to local vascular needs. Fibroblasts appear to be uniquely equipped to proliferate, transdifferentiate and migrate under pathologic conditions. The role of the adventitia in the vascular response to injury and vascular remodeling has recently received considerable attention. The activation of adventitial fibroblasts induces the expression of α -actin and phenotypic modulation to smooth muscle-like cells, the

myofibroblasts. The expression of contractile proteins in myofibroblasts may contribute to vascular remodeling by constricting vessels and contributing to late lumen loss. Furthermore, myofibroblasts are involved in tissue repair by the deposition of extracellular collagen, which also contributes to vascular remodeling^{12,13}.

Unlike the involvement of medial smooth muscle cells and intimal endothelial cells, the activation of the adventitia has not previously been intensively investigated during vascular response to injury following SAH. Smith *et al.*¹⁴ found that myofibroblasts and type V collagen within the medial layer were abundant in a vasospastic cerebral artery after SAH. In several animal SAH models, a similar result was reported following SAH¹⁵. Transmission electron microscopy of normal arteries revealed Virchow–Robin (intra-adventitial) spaces lined by simple planar epithelium-like cells. But in SAH arteries, these spaces were filled almost entirely with strands of connective tissue and fibroblasts¹⁶. Yamamoto *et al.*¹⁷ reported that fibroblast populated collagen lattice contraction was significantly accelerated by cerebrospinal fluid taken from patients with symptomatic vasospasm. Works by other investigators used a similar fibroblast populated collagen lattice model to evaluate the role of fibroblasts in collagen compaction induced by the bloody cerebrospinal fluid or hemolysate of vasospasm patients, and the possible mechanisms involved, such as ET-1, tyrosine kinase, PKC, protein kinase A and G, and myosin light-chain kinase^{18,19}. Some works also found that there is adventitia-dependent relaxation in the cerebral arteries expressing the recombinant eNOS gene. This also suggests that adventitial fibroblasts in human cerebral arteries may play a part in vasodilatation²⁰. So the cerebral vascular disorders, such as cerebral vasospasm after SAH, may correlate with the functional modulation of the adventitial fibroblasts. After SAH, a complicated series of cellular and molecular events is elicited by the presence of blood in the subarachnoid space, culminating in a vigorous inflammatory response. Some of the cytokines that have been characterized and found to be up-regulated in experimental and/or clinical cerebral vasospasm after SAH include PDGF, TNF- α , IL-1 α , IL-1 β , IL-6, and IL-8^{21,22}. All of these cytokines and other vasogenic substances may have an effect on the VAFs. Proliferating cells are evident in the adventitia on the day of vascular injury^{12,13}. After vascular injury, growth factors and cytokines are released from platelets and cell debris, most of which control cell proliferation. These works demonstrate that OxyHb could modulate the proliferational state of the VAFs. In this study, OxyHb could induce VAF proliferation in the early phase (0–24 hours). Although ecdysterone co-treatment failed to inhibit cellular proliferation with statistical significance when OxyHb was used to induce proliferation, the trend was similar to the suppression of proliferation (Figure 1D). Furthermore, ecdysterone's anti-proliferative effect was also noted in vascular smooth muscle cells, another major cell type of vascular system (data not shown here).

In systemic arterial injury, there is some evidence to support the migration of fibroblasts from the adventitia to the intimal layer and the development of the neointimal lesion. The cells that are purported to be migrating display α -SM actin positivity (i.e. the myofibroblast phenotype). After balloon induced severe endoluminal coronary injury, translocation of bromodeoxyuridine labeled cells suggests that proliferating adventitial cells migrate to the intimal layer, and their phenotypic modulation to myofibroblasts. This finding was confirmed by Scott *et al.*²³, who, using almost the same method, found that adventitial myofibroblasts possibly migrate into the neointima. An especially interesting observation was that the time course of the increased synthesis of α -smooth muscle actin in the adventitial cells after arterial injury was consistent with the time course of SAH. Furthermore, primary adventitial fibroblasts, stably transfected with LacZ retrovirus and introduced in the injured carotid artery, were found all along the wall from the adventitia to the neointima²⁴.

In SAH, there was no direct evidence for adventitial fibroblast migration. But in some SAH models, myofibroblasts were found abundantly in a vasospastic cerebral artery after SAH^{14–}

¹⁶. Our results showed that 100 μ M OxyHb obviously prompted VAF migration. When VAFs were co-treated with ecdysterone, OxyHb induced cellular migration was significantly suppressed (Figure 2D). Two specific markers for migration support this conclusion: monolayer-wounding cell migration assay and Boyden's chamber migration assay, which both showed similar results. The mechanism by which adventitial fibroblasts migrate to the neointima remains unclear. Migration is independent of the proliferation rate and is controlled by specific levels of metalloproteinases and their tissue inhibitors. Matrix metalloproteinase-9 is necessary for the migration of cells into the intimal layer after vascular injury. The expression of matrix metalloproteinases in the adventitia is increased after vascular injury and may favor the migration of fibroblasts¹³. In relation to these anti-proliferative and anti-migratory effects of ecdysterone, its effect on the cell cycle was also evaluated. Cell cycle analysis indicated that ecdysterone decreased the number of cells entering S phase (Figure 2A–C).

There is currently no ideal *in vivo* model mimicking SAH-induced cerebral vasospasm in humans. The model of Chan *et al.*²⁵ is the popular rabbit model which is used for the study of vasospasm. However, it might seldom elicit cerebral ischemia, which is the main complication of CVS following SAH. Endo *et al.*¹⁰ developed a symptomatic rabbit model of vasospasm. In that model, CCA is bilaterally tied to establish the blood supply route mainly from the vertebrobasilar arterial system into the entire brain and increase the frequency of neurological symptom of rabbits. However, after bilateral CCA ligation, the rabbits were more likely to show definite symptoms or died acutely, so it is not fit for the investigation of anti-vasospasm drugs. This new rabbit model of SAH which was used in our study is simple, replicable and has low mortality. No experimental rabbit died after CCA ligation, compared with the six of 21 rabbits that died in Endo's model. The neurological deficit of experimental rabbits was also notable, and it mainly occurred in 1–2 days after the second injection of blood into the cisterna magna. In this new model, hippocampal ischemia and infarct were found on histologic study, and angiographic study also proved that a relatively vasospasm had occurred. Based on the description of Schwartz and Masago²⁶, we could figure out that the induction of SAH and cerebral vasospasm of this new model was simple, efficient, reproducible, inexpensive and associated with an acceptably low mortality rate when compared with Endo's model.

This study further proved that ecdysterone could improve the outcome of experimental animals, attenuate cerebral vasospasm and prevent morphologic changes of basilar artery following SAH, and some of its protective effects may compare with nimodipine. According to this study, it was notable that the morphologic changes of the arterial wall were alleviated in the ecdysterone group compared to the SAH group. Moreover, in the ecdysterone group, inflammatory cell infiltration was seldom seen in the arterial wall. Our previous works also prove that ICAM-1 expression in microvessel endothelial cells and the adhesion of polymorphonuclear cells to microvessel endothelial cells following bloody cerebrospinal fluid stimulation could be reduced by ecdysterone. Other works also show that ecdysterone has an anti-inflammatory effect⁷. All of these suggest that ecdysterone may have a potent protective effect on the components of the arterial wall, and attenuate the inflammatory response associated with cerebral vasospasm after SAH. However, the mechanism of ecdysterone remains incompletely understood.

In the present study, we report that ecdysterone inhibits OxyHb induced VAF proliferation and migration *in vitro* and improves the outcome in a rabbit SAH model. Despite these positive end results, we did not find *in vivo* evidence of VAF proliferation and migration. Although it was supported in other *in vivo* works mentioned above, the *in vitro* results could be reconsidered to some extent. In this study, we use OxyHb to mimic the clinical situation. OxyHb, generated by hemoglobin in erythrocytes, is the most likely pathogenic agent for vasospasm, although the specific mechanism is uncertain²². Considering that the OxyHb level definitely is not fully equal to that in blood, the difference of *in vitro* and *in vivo* results may be reasonable. The

reported levels of hemoglobin range from 500 μM in a subarachnoid hematoma to 30 μM in the cerebrospinal fluid, and OxyHb could be released continuously from subarachnoid clots in patients and an animal SAH model. In a study using microdialysis, corresponding levels of oxyhemoglobin were shown (50 μM)²⁷. So we use this concentration as the base to set our concentration, but the real concentration is still unknown. Considering that we only did *in vitro* testing using OxyHb in the early period after the stimulation, if we evaluate the *in vitro* changes at a time point later than the one we used in this study, we might be able to observe different results. On the other hand, or another possibility, OxyHb induced VAF proliferation and migration may be just a temporary pathologic event and a cell event of VAF activation, but not the final result. Our laboratory also found that the proliferative activity of VAFs conditioned medium on cultured VSMCs was enhanced by OxyHb, and this effect could be suppressed by ecdysterone. This may be evidence of VAF activation, or VAFs could modulate VSMC in SAH, and ecdysterone may take effect in SAH (data not shown).

At present, no consistently efficacious and ubiquitously applied preventive and therapeutic measures are available in clinical practice¹. According to this study, nimodipine and ecdysterone had similar protective effects on vascular following SAH, in line with the recent finding that ecdysterone acts as a protective agent on cerebral ischemia, which suggests the possibility of ecdysterone being a potent agent for controlling cerebral vasospasm after SAH and may be a valid medical therapy.

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References

1. Feigin VL, Rinkel GJ, Lawes CM, et al. Risk factors for subarachnoid hemorrhage: An updated systematic review of epidemiological studies. *Stroke* 2005;36:2773–2780. [PubMed: 16282541]
2. Simon AF, Shih C, Mack A, et al. Steroid control of longevity in *Drosophila melanogaster*. *Science* 2003;299:1407–1410. [PubMed: 12610309]
3. Kononova NP, Mitrokhin I, Volkova LM. Ecdysterone modulates antitumor activity of cytostatics and biosynthesis of macromolecules in tumor-bearing animals. *Izv Akad Nauk Ser Biol* 2002;6:650–658.
4. Wu X, Jiang YG, Fan SZ, et al. Effect of ecdysterone on cultured endothelial cell injured by hypoxia. *Acta Acad Med Milit Tert* 1998;20:358–360.
5. Xu NJ, Guo YY, Rui WX, et al. Protective effect of ecdysterone on cerebral ischemia-induced impairment. *J Shenyang Pharm Univ* 1999;16:118–121.
6. Chen Z, Zhu G, Tang WH, et al. Effects of ecdysterone on injury of endothelial cells following experimental subarachnoid hemorrhage. *Chin J Clin Pharmacol Ther* 2004;9:540–543.
7. Chen Z, Feng H, Tang WH, et al. The effect of ecdysterone on adherence of polymorphonuclear cells to endothelial cells following experimental SAH. *Chin J Geriatr Cardiovasc Cerebrovasc* 2004;6:270–272.
8. Patel S, Shi Y, Niculescu R, et al. Characteristics of coronary smooth muscle cells and adventitial fibroblasts. *Circulation* 2000;101:524–532. [PubMed: 10662750]
9. Li G, Chen YF, Greene GL, et al. Estrogen inhibits vascular smooth muscle cell-dependent adventitial fibroblast migration *in vitro*. *Circulation* 1999;100:1639–1645. [PubMed: 10517736]
10. Endo S, Branson PJ, Alksne JF, et al. Experimental model of symptomatic vasospasm in rabbits. *Stroke* 1988;19:1420–1425. [PubMed: 3188126]
11. Martin W, Villani GM, Jothianandan D, et al. Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta. *J Pharmacol Exp Ther* 1985;232:708–716. [PubMed: 2983068]

12. Sartore S, Chiavegato A, Faggin E, et al. Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: From innocent bystander to active participant. *Circ Res* 2001;89:1111–1121. [PubMed: 11739275]
13. Strauss BH, Rabinovitch M. Adventitial fibroblasts: Defining a role in vessel wall remodeling. *Am J Respir Cell Mol Biol* 2000;22:1–3. [PubMed: 10615057]
14. Smith RR, Clower BR, Grotendorst GM, et al. Arterial wall changes in early human vasospasm. *Neurosurgery* 1985;16:171–176. [PubMed: 3883222]
15. Smith RR, Clower BR, Cruse JM, et al. Constrictive structural elements in human cerebral arteries following aneurysmal subarachnoid haemorrhage. *Neurol Res* 1987;9:188–192. [PubMed: 2891067]
16. Espinosa F, Weir B, Shnitka T. Electron microscopy of simian cerebral arteries after subarachnoid hemorrhage and after the injection of horseradish peroxidase. *Neurosurgery* 1986;19:935–945. [PubMed: 3808241]
17. Yamamoto Y, Bernanke DH, Smith RR. Accelerated non-muscle contraction after subarachnoid hemorrhage: Cerebrospinal fluid testing in a culture model. *Neurosurgery* 1990;27:921–928. [PubMed: 2274134]
18. Ogihara K, Barnanke DH, Zubkov AY, et al. Effect of endothelin receptor antagonists on non-muscle matrix compaction in a cell culture vasospasm model. *Neurol Res* 2000;22:209–214. [PubMed: 10763512]
19. Patlolla A, Ogihara K, Zubkov A, et al. Role of tyrosine kinase in fibroblast compaction and cerebral vasospasm. *Acta Neurochir Suppl* 2000;76:227–230. [PubMed: 11450013]
20. Tsutsui M, Onoue H, Iida Y, et al. Adventitia-dependent relaxations of canine basilar arteries transduced with recombinant eNOS gene. *Am J Physiol* 1999;276:H1846–H1852. [PubMed: 10362662]
21. Dumont AS, Dumont RJ, Chow MM, et al. Cerebral vasospasm after subarachnoid hemorrhage: Putative role of inflammation. *Neurosurgery* 2003;53:123–133. [PubMed: 12823881]
22. Mayberg MR. Cerebral vasospasm. *Neurosurg Clin N Am* 1998;9:615–627. [PubMed: 9668192]
23. Scott NA, Cipolla GD, Ross CE, et al. Identification of a potential role for the adventitia in vascular lesion formation after balloon overstretch injury of porcine coronary arteries. *Circulation* 1996;93:2178–2187. [PubMed: 8925587]
24. Li G, Chen SJ, Oparil S, et al. Direct *in vivo* evidence demonstrating neointimal migration of adventitial fibroblasts after balloon injury of rat carotid arteries. *Circulation* 2000;101:1362–1365. [PubMed: 10736277]
25. Chan RC, Durity FA, Thompson GB, et al. The role of the prostacyclin-thromboxane system in cerebral vasospasm following induced subarachnoid hemorrhage in the rabbit. *J Neurosurg* 1984;61:1120–1128.
26. Schwartz AY, Masago A. Experimental models of subarachnoid hemorrhage in the rat: A refinement of the endovascular filament model. *J Neurosci Methods* 2000;96:161–167. [PubMed: 10720681]
27. Pluta RM, Afshar JK, Boock RJ, et al. Temporal changes in perivascular concentrations of oxyhemoglobin, deoxyhemoglobin, and methemoglobin after subarachnoid hemorrhage. *J Neurosurg* 1998;88:557–561. [PubMed: 9488312]

Figure 1.

(A) VAF stain positive to vimentin. (B) VAF stain positive to collagen III. (C) VAFs stimulated by OxyHb had a more decurtated shape compared to control group. (D) The effect of OxyHb on VAF proliferation. Each study was repeated six times

Figure 2.

(A) Control group. (B) VAF incubated with 100 μ M OxyHb for 24 hours. (C) VAF incubated with 100 μ M OxyHb and EDS for 24 hours. (D) The effect of OxyHb on VAF cell migration. Each study was repeated three times. The percentage that residual scraped area relative to the original scraped area was measured after 12 hours, 24 hours, 2 days, 4 days and 7 days

Figure 3. Number of rabbits in each neurological deficit grade and food intake class before (day 0) and during 3 days after SAH

Figure 4.

Angiograms of rabbit basilar artery in Groups 2–4. (A) Notable angiographic spasm of basilar artery was seen in Group 2 (SAH group). (B and C) Unobvious angiographic spasm of basilar artery were seen in Groups 3 and 4 (nimodipine and ecdysterone groups). (D) Graph shows comparisons of day 0 versus day 3 (after SAH) basilar artery midpoint diameters (mm) in three groups. Significant diameter reductions were observed between days 0 and 5 angiograms in control (* $p < 0.05$), nimodipine (** $p < 0.05$) and ecdysterone (*** $p < 0.05$) groups. No significant differences in day 0 (baseline) diameters between groups (all $p > 0.05$)

Figure 5.

The BA in all groups. (A) Internal elastic lamina (IEL) lies smoothly under the monolayer endothelial cells, and SMCs are well-arranged in Group 1 (control) (28.8 μ m). (B) BAs of rabbit in Group 2 (SAH) are contracted with IEL corrugation. The smooth muscle cells are shortened, rounded up and dystrophic with a lot of vacuoles (arrow) and condensation of chromatin (triangle) (38.4 μ m). (C) Morphology of BA in Group 3 (nimodipine) is similar to that in control group (28.8 μ m). (D) IEL corrugates, and SMC are shortened slightly and not well-arranged in Group 4 (ecdysterone) (28.8 μ m)

Table 1

The effect of OxyHb or OxyHb and EDS co-treated versus control group

	100 μ M OxyHb	100 μ M OxyHb + EDS
VAF proliferation	+++++ *	++++*
VAF migration	++*	---* [†]

⁺: increase; ⁻: decrease.

* $p < 0.05$ versus control group

[†] $p < 0.05$ versus OxyHb group.

Table 2The index of TCD after the secondary blood injection (mean $\pm$ SD)

Group	Index		
	0 day	1 day	3 days
SAH	57.66 $\pm$ 5.11	78.00 $\pm$ 8.97**	80.54 $\pm$ 6.17**
Ni	62.00 $\pm$ 7.37	47.32 $\pm$ 6.01**	56.74 $\pm$ 6.79
EDS	63.80 $\pm$ 5.65	92.94 $\pm$ 4.33**	81.83 $\pm$ 8.67**
SAH	47.28 $\pm$ 6.79	62.74 $\pm$ 12.76*	64.08 $\pm$ 9.26*
Ni	50.18 $\pm$ 6.01	37.06 $\pm$ 5.59**	40.18 $\pm$ 6.64*
EDS	50.2 $\pm$ 4.33	68.94 $\pm$ 8.63*	59.28 $\pm$ 2.95*