4′-O-β-D-glucosyl-5-O-methylvisamminol, an active ingredient of *Saposnikovia divaricata*, attenuates high-mobility group box 1 and subarachnoid hemorrhage-induced vasospasm in a rat model

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Abstract

**Background:** High-mobility group box 1 (HMGB1) was observed to be an important extracellular mediator involved in vascular inflammation associated with subarachnoid hemorrhage (SAH). This study is of interest to examine the efficacy of 4′-O-β-D-glucosyl-5-O-methylvisamminol (4OGOMV), C₂₂H₂₈O₁₀, in the alternation of cytokines and HMGB1 in an animal model.

**Methods:** A rodent double hemorrhage SAH model was employed. Administration with 4OGOMV was initiated 1 h after animals were subjected to SAH. Basilar arteries (BAs) were harvested and cortexes examined for HMGB1 mRNA, protein expression (Western blot) and monocyte chemoattractant protein-1 (MCP-1) immunostaining. Cerebrospinal fluid samples were collected to examine IL-1β, IL-6, IL-8 and MCP-1 (rt-PCR).

**Results:** Morphological findings revealed endothelial cell deformity, intravascular elastic lamina torture, and smooth muscle necrosis in the vessels of SAH groups. Correspondingly, IL-1β, IL-6 and MCP-1 in the SAH-only and SAH-plus vehicle groups was also elevated. 4OGOMV dose-dependently reduced HMGB1 protein expression when compared with the SAH groups. (p < 0.01) Likewise, 400 μg/kg 4OGOMV reduced IL-1β, MCP-1 and HMGB1 mRNA levels as well as MCP-1(+) monocytes when compared with the SAH groups.

**Conclusion:** 4OGOMV exerts its neuro-protective effect partly through the dual effect of inhibiting IL-6 and MCP-1 activation and also reduced HMGB1 protein, mRNA and MCP-1(+) leukocytes translocation. This study lends credence to validating 4OGOMV as able to attenuate pro-inflammatory cytokine mRNA, late-onset inflammasome, and cellular basis in SAH-induced vasospasm.

**Keywords:** 4′-O-β-D-glucosyl-5-O-methylvisamminol, High-mobility group box 1, Tumor necrotic factor-α, Subarachnoid hemorrhage, Vasospasm
and molecular events in the subarachnoid space and further result in a robust inflammatory response [2, 3]. Even if the systemic inflammation has been recognized in the pathogenesis of cerebral vasospasm, the putative importance of intracranial sterile inflammatory activity has not been fully emphasized. Till now, various inflammosomes, including adhesion molecules, cytokines, leukocytes, immunoglobulins, and complements, were observed in the pathogenesis of SAH-induced cerebral infarct and vasospasm [1, 7, 8, 17–20].

The levels of pro-inflammatory cytokines and adhesion molecule have been found increased in cerebrospinal fluid (CSF) after SAH [10, 15]. However, the benefits of inflammation associated with SAH remain unclear. High-mobility group box 1 (HMGB1), a ubiquitous nuclear protein, is expressed in activated monocytes, macrophages, circulating neutrophils, and platelets [13, 17, 21]. In Wolfson et al’s study, HMGB1 is proved to be able to mediate vascular monocyte chemotaxis, neuron dendrite outgrowth, and the proinflammatory reaction of endothelial cells [16]. The released HMGB1 is late-onset inflammatory cytokines and adhesion molecule that have been known increased in cerebrospinal fluid (CSF) after SAH [10, 15]. However, the benefits of inflammation associated with SAH remain unclear. HMGB1 is known as a proinflammatory cytokine that is involved in the pathogenesis of various inflammatory diseases including acute myeloid leukemia and focal cerebral ischemia [17].

Given the promising result of 4OGOMV on the block of cellular mitosis and its various effects of pro-inflammatory cytokines stimulation on endothelial dysfunction, the rat SAH model was used to test the hypothesis that 4OGOMV, a natural histone deacetylase inhibitor, attenuates SAH-related vasospasm and HMGB1-associated neuro-inflammation.

### Methods

#### Materials

4′-O-β-d-glucosyl-5-O-methylvisamninol (4OGOMV) has been characterized as a naturally occurring and potent histone H3 phosphorylation inhibitor, and was bought from Baoji Plant Bio-Engineering Co., Ltd., Shaanxi 710000-710090 PRC. Monoclonal anti-rat IL1-β, IL-6, IL-8 and MCP-1 antibody were obtained from Abcam (Cambridge, MA 02139, USA), BD Transduction Lab (BD Biosciences, San Jose, CA 95060, USA), Upstate Biotech (Lake Placid, NY 12946, USA), and Santa Cruz Biotechnology, Inc. Santa Cruz, CA 95060, USA). Rabbit anti-rat HMGB1 antibody was purchased from Biochieldom international Co., Ltd, Taipei 11659, Taiwan distributing Abcam biochemicals, Cambridge, MA 02139, USA CNM protein extraction kits were from Biochain (Hayward, CA 94545, USA). 4OGOMV was prepared by Ms. Wu SC (Kaohsiung Medical University Hospital, Kaohsiung 807. Taiwan, ROC). Dimethyl sulfoxide (DMSO) at a concentration of 10 mM was used as a solvent as well as a vehicle.

#### Induction of double hemorrhage SAH

Fifty-four male Sprague–Dawley rats (260–320 g; bought from BioLasco Taiwan Co., Ltd., Taipei 115, Taiwan. authorized by Charles River Lab), were enclosed in this study. All the protocols were approved and supervised by the University of Kaohsiung Medicine Animal Research Committee and were compliant with the Declaration of Helsinki (1964). The rats received anesthesia by an intraperitoneal injection of 7 mg/kg Zoletil 50 (VIRBAC, L.I.D., Carros 06516, France). 0.3 ml fresh arterial blood was withdrawn and injected into the cisterna magna via a stereotactic apparatus (Stoelting, Wood Dale, IL 60191, USA). 4OGOMV or a vehicle was injected intraperitoneally. 3.0 ml Cerebrospinal fluid (CSF) was drawn by No30 Terumo needle to make sure the tip of the needle had been into subarachnoid space. Animals were placed in ventral recumbent position for 20 min to allow clot formation. After monitoring for respiratory distress and giving artificial ventilation if needed, the animals were returned to the vivarium when fully awake. A habitat with a 12 h light–dark cycle was offered with access to food and water ad libitum. 48 h after 1st SAH, the animals received secondary injection of SAH to maintain...
the tendency of vasoconstriction. Thereafter, the animals received perfusion–fixation 72 h after 2nd SAH.

**General design of experimental groups**

The animals were randomly subdivided into the following groups (N = 9 rats): (1) sham operated (no SAH); (2) SAH-only; (3) SAH-plus vehicle; (4) 4OGOMV (100 μg/kg/day) treatment in SAH rats; (5) SAH rats received 200 μg/kg/day 4OGOMV treatment; and (6) treatment with 400 μg/kg 4OGOMV in SAH rats. The dosage was adjusted to be devoid of hepatic-renal toxicity based on the pilot rabbit artery ring study. The administration was initialized at 1 h after induction of SAH by using an osmotic mini-pump (Alzet corp, Palo Alto, CA 94301, US) to offer a constant serum level. Cortical tissue samples were obtained by inserting a 24-gauge needle 5 mm into the skull bone (N = 5) through a burr hole craniectomy (2 mm apart from the bregma) under a negative pressure.

**Perfusion–fixation**

By 72 h after 2nd SAH, the animals were re-anesthetized by administration of 7 mg/kg Zoletil 50. Perfusion–fixation was performed to open the chest, catheterize with a NO16 needle into the left ventricle as well as the clamped descending aorta, and incise the right atrium. 100 ml of 0.01 M phosphate buffer (pH 7.4) at a pressure of 80 mmHg was dripped, followed by 120 ml 2% paraformaldehyde in the PBS solution at 36 °C. The brain was extracted and immersed in a fixative at 4 °C overnight. Visual inspection observed blood clots formed over the basilar artery (BA) in all SAH animals.

**Hemodynamic measurements**

Heart rate, blood pressure, and rectal temperature were monitored in the duration of the administration of 4OGOMV as well as 3 intervals of 12 h by a tail-cuff method (SC1000 Single Channel System, Hatteras Instruments, NC 27518, US) and rectal thermometer (BIO-REX-2-ISO, FL 33409, US). The femoral artery was catheterized to obtain blood samples to determine arterial blood gases, Na, K, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), blood urea nitrogen (BUN) and creatinine levels.

**Neurological assessment**

Two neurological tests were enclosed in this study. A modified limb-placing test (MLPT) [35] was performed before and at a 48 h interval after the animals were subjected to SAH. The behavioral assessment was composed of forelimb and hindlimb ambulation and placing/stepping reflex examinations performed before and after animals were subjected to SAH. A motor deficit index (MDI) was calculated for each rat at an interval of 24 h. The final index was the sum of the scores (walking with lower extremities and placing/stepping reflex). Animals with MDI score more than three were considered as paraplegia, whereas MDI score less than three was considered as neurological deficit. Another modified Voetsch neuro-score was performed, which is composed of the evaluation of head movement, confrontation, reflex stimulation by ear pinch, proprioception and four limb movement. The summation of neuroscore ranged from 3 to 0, which stood for normal performance to comatose status and paraplegia [36]. The neuroscore was represented as the percentage difference of the mean performance from sham-operated groups.

**Basilar artery morphological studies**

Five BA cross-sections of each animal (the middle third) were analyzed by two investigators blinded to the experiment set. The basilar arteries were frozen and cut into 25-μm-thick sections with the assistance of ultramicrotome (Reica EM UC7, Union Optical and Instruments and Nanotechnology. New Taipei City, 22101 Taiwan.) The cross-sectional area of BA was automatically evaluated via a computer-assisted morphometer (Image-1/Metamorph Imaging System; Universal Imaging Corp. Sunnyvale, CA 94089, USA). The mean average of five cross-sections of a given animal was collected for every animal. Group data were expressed as the mean ± standard deviation.

**Quantification of mRNA expression of IL-1β, IL-6, IL-8, and MCP-1**

The CSF cytokines mRNA was examined via the ABI PRISM® 7900 System (Applied Biosystems, Foster City, CA 94404, USA). According to the manufacturer’s instructions, the mRNAs for IL-1β, IL-6, IL-8, and MCP-1 expression were determined, while 18S was used as a standard control based on its stability. Primer sequences were employed for IL-1β: (242 bp: forward 5′-GCTCATCTGGGATCCTTCC-3′ and reverse 5′-CCTGCTGAGAGCTTGTGG-3′); IL-6: (91 bp: forward 5′-GACAACCTTGGCATGTTG-3′ and reverse 5′-ATGCAGGGATGATGTTCTG-3′); IL-8: (229 bp: forward 5′-TCAGGATGATGTTG-3′ and reverse 5′-ACTTCTCCACAACCTCCTGC-3′) and MCP-1: (457 bp: forward 5′-CTTCTCCACCATACAGTC-3′ and reverse 5′-CTTGCTAATCAGTGGATC-3′). Each sample was launched into a TaqMan® Human Cytokine Card that enclosed probes and primers for specific targets. This procedure was performed via an affixed filling reservoir and a vacuum-loading process via the ABI PRISM® Card Filling Station. Final data were expressed as a relative fold from...
the baseline. Comparative mRNA expression was set by the Livak and Schmittgen \( \Delta \Delta CT \) method [38]. The results were analyzed if a fivefold increase in the mRNA levels compared with the baseline to allow for data consistency.

**HMGB1 protein analyzed by western blotting**

The cortical homogenates (20 \( \mu \)g) were stirred with LDS sample buffer (contains 40 % glycerol, 4 % lithium dodecyl sulfate (LDS), 0.8 M triethanolamine-CI pH 7.6, 4 % Ficoll®-400, 0.025 % phenol red, 0.025 % Coomassie G250, 2 mM EDTA disodium, NuPAGE®-LDS Sample Buffer (4×) NP0007; Invitrogen, Carlsbad, CA 92008, USA). Samples were loaded for 8 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and then separated after centrifuging at 12,000 rpm for 10 min twice. The specimen was then mounted onto a polyvinylidene difluoride membrane and incubated in blocking buffer (5 % non-fat dry milk in Tris-buffered saline with 0.2 % Tween 20) at room temperature. Rabbit anti-rat HMGB1 monoclonal antibody (1:50,000; Biocheifdom International Co., Ltd Wenshen District, Taipei 11659, Taiwan. distributing Abcam biochemicals, Cambridge, MA 02139, USA) was used, while \( \beta \)-Actin (monoclonal anti-\( \beta \)-actin, dilution 1:40,000; Sigma-Aldrich, Taipei 116, Taiwan) was used as a loading control. A secondary antibody was conjugated with horseradish peroxidase (HRP) in TBS-t at room temperature for 1 h. The immune blots were developed via a GS-700 digital scan and Molecular Analyst® (a GS-700 digital densitometer, Bio-Rad, Ramsey, MN 55303, USA). Relative optical densities were determined by comparison between the measured values and the mean values from the vehicle plus SA groups.

**Detection of HMGB1 mRNA by rt-PCR**

The activated HMGB1 mRNA levels in the cortical homogenates were determined by TriPure RT-PCR Reagent (Roche Diagnostics Corp. IN 46256, USA). According to the supplier’s instructions, the primer sequences were designed according to the cleaved caspase-3, -9a and GAPDH gene sequences as stated in GenBank. Cleaved caspase-3 primer is as: 393 bp; forward: 5′-CTGGGTACCCCTGATTTGTA-3′; reverse: 5′-GGTATTGAAGGTGATGCTT-3′; caspase-9a primer: 889 bp; forward: 5′-GGAGCCTTGAAGGAATTCTGCAC-3′; reverse: 5′-CCAGTGACGCAGCGGTTTCATC-3′. After incubation with Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) (Promega, WI 53711, USA), the cleaved caspase -3, -9a and GAPDH cDNA fragments were amplified and detected via agarose gel electrophoresis mixed with 1 \( \mu \)l of ethidium bromide. The intensity of the genes was measured via a comet assay method. GAPDH mRNA was used as a standard gene. The tissue samples of every five animals were used for rt-PCR and three measurements for each animal specimen performed by an investigator blind to the experiment set. Cleaved caspase-9a represented the majority of catalyzed pro-caspase-9.

**Immuno-staining with polyclonal anti-rat NeuN, GFAP, BrdU and MCP-1 antibody**

MCP-1 recruits monocytes, memory T cells, and dendritic cells were detected using a video-assisted microscope (\( \times 400 \)). Briefly, isolated rat basilar arteries were perfused and fixed with 4 % paraformaldehyde. Coronal sections of the basilar arteries were stored overnight on slides at –80 °C. Rabbit monoclonal anti-rat MCP-1 antibody was used at a dilution of 1:40, and immunostaining was performed for 40 min at 25 °C followed by drying overnight as described in the mouse monoclonal alkaline phosphatase anti-alkaline phosphatase (APAAP) technique [37]. The cerebral slice was frozen and cut into 30 \( \mu \)m-thick sections with the assistance of ultra-microtome E (Leica EM UC7, Union Optical and Instruments and Nanotechnology. New Taipei City 22101, Taiwan) Two temporal gyrus cross-sections of each animal were analyzed by two investigators blinded to the experiment set. A rabbit polyclonal BrdU (1:400), NeuN(1:100) and GFAP(1:500) antibody recruited neuronal-specific RNA splicing was performed for 40 min at 25 °C followed by drying overnight and detected by using a video-assisted microscope (\( \times 200 \)).
Statistical analysis
Data were expressed as the mean ± standard deviation. For comparison among groups, all statistical analyses were performed using SPSS 19.0 software (SPSS Inc., Chicago, IL 60614, USA). Difference was considered significant at a probability value less than 0.01.

Results
General observation
Throughout the study, there was no significant difference observed in the recorded physiological parameters, including GOT, GPT, BUN, creatinine, pH, blood pressure and arterial blood gas analysis among the experimental groups. This authenticated that 4OGOMV administration in the selected dosage was devoid of hepatic and renal toxicity.

Tissue morphometry and MCP-1 immunostaining
The internal elastic lamina (IEL) in the BAs of SAH and SAH+vehicle groups showed substantial corru-gation when compared with that obtained from the controls (Fig. 1, upper panel). IEL disruption was less prominent in the 200 and 400 μg/kg/day 4OGOMV treatment SAH groups. The cross-sectional areas of BAs in the SAH and SAH+vehicle groups were significantly reduced when compared with the control group (0.138 ± 0.028, and 0.135 ± 0.046). 400 μg/kg 4OGOMV attenuated SAH-induced vasoconstriction (Fig. 1, lower panel, P < 0.01). Peri-vascular MCP-1(+) cell level was significantly reduced in the 400 μg/kg/day 4OGOMV treatment and the healthy controls when compared with the SAH groups (P < 0.01, Fig. 2). Besides, the smaller branch of basilar artery was also dilated under the 400 μg/kg 4OGOMV treatment (Fig. 1, upper panel).

Neurological deficit
MLPT score was obtained to examine the motor-sen-sory incorporation of the forelimb and hindlimb and placing/stepping index as a reflective response to tactile and proprioceptive stimuli. The sum from these two tests is considered as motor deficit index (MDI). The mean MDI in the SAH and SAH+vehicle groups were 2.65 ± 0.38 and 2.42 ± 0.48, compared with...
the healthy controls. Treatment with 4OGOMV (at 400 μg/kg) significantly improved the MDI in the SAH groups (Table 1). Likewise, MDI ≥ 3 was substantially decreased in the 4OGOMV treatment SAH groups when compared with the SAH animals. The percentage difference of modified Voetsch neuro-scores was significantly induced in the 400 μg/kg/day 4OGOMV+SAH group and the healthy controls, when compared with the SAH groups (Table 2).

Table 1 Modified limb-placing test (MLPT)

| Group treatment | Ambulation | Placing/stepping reflex | MDI        |
|-----------------|------------|-------------------------|------------|
| Sham-operated   | 0          | 0                       | 0          |
| SAH             | 1.28 ± 0.17| 1.50 ± 0.13             | 2.78 ± 0.30|
| SAH+vehicle     | 1.28 ± 0.11| 1.58 ± 0.21             | 2.86 ± 0.32|
| SAH+4OGOMV 100 | 0.91 ± 0.60 | 1.54 ± 0.25             | 2.45 ± 0.85|
| μg/kg/day       | 200 μg/kg/day | 0.82 ± 0.25             | 1.65 ± 0.68|
|                 | 400 μg/kg/day | 0.56 ± 0.22*            | 1.21 ± 0.42*|

Results are expressed as the mean ± SD, n = 9; *p < 0.01 vs. SAH condition by Mann–Whitney U test

HMGB1 protein expression

HMGB1 were demonstrated to play a critical role in the onset of delayed and systemic inflammation. The expression of HMGB1 protein was not significantly different among the experimental groups at 48 h after the induction of SAH (Fig. 4, left column, p > 0.01). In this study, 4OGOMV (at 200 and 400 μg/kg/day) reduced
the expression of HMGB1 protein at 72 h after 2nd SAH, when compared with the SAH group (Fig. 4, right column, p < 0.01).

The activation of HMGB1 mRNA
To observe the neuronal inflammation subsequent to SAH, HMGB1 mRNA was examined. The activated HMGB1 mRNA was increased in the SAH groups when compared with the sham-operated groups (p < 0.01).

Results of cleaved caspase-3 and -9a mRNA expression
The cortical level of cleaved caspase-3 and -9a was significantly increased in the SAH group, compared with the 4OGOMV treatment+SAH groups and the healthy controls. (Fig. 6, P < 0.01) Treatment with 400 μg/kg/day 4OGOMV reduced activated caspase-3 and caspase-9a to a significant level when compared with that in the SAH rats, (Fig. 6) which corresponds to the result of neurobehavior examination.

NeuN, BrdU, GFAP immunostaining
NeuN(+) neurons were detected associated with vacuolated nuclear in the SAH in the SAH groups, which was absent in the 4OGOMV treatment and sham-operated groups. (Fig. 7, upper panel) Likewise, the GFAP(+) glia was observed increased in the SAH groups. 4OGOMV significantly reduced cerebral activated GFAP glia, when compared with SAH group. (Figure 7, lower panel). The

Table 2 Modified Voetsch neuroscores

| Group treatment | Head movement | Motor-sensory function | 4-limb movement | Sum          |
|----------------|---------------|------------------------|-----------------|--------------|
| Sham-operated  | 12 ± 0        | 12 ± 0                 | 6 ± 0           | 30 ± 0       |
| SAH            | 5.2 ± 2.2     | 4.6 ± 1.8              | 3.1 ± 0.5       | 12.9 ± 4.5   |
| SAH+vehicle    | 6.2 ± 2.6     | 5.5 ± 2.1              | 2.7 ± 0.8       | 14.4 ± 7.5   |
| SAH+4OGOMV 100 | 6.9 ± 1.6     | 6.5 ± 2.5              | 3.7 ± 0.5       | 17.1 ± 4.6   |
| SAH+4OGOMV 200 | 8.2 ± 2.4     | 9.6 ± 1.3              | 4.0 ± 1.0       | 21.7 ± 4.7   |
| SAH+4OGOMV 400 | 9.6 ± 2.5     | 10.2 ± 3.2*            | 4.3 ± 1.5       | 24.1 ± 7.2*  |

Results are expressed as the mean ± SD, n = 9; *p < 0.01 vs. SAH by Mann–Whitney U test

400 μg/kg/day 4OGOMV tended to decrease HMGB1 mRNA in the 48 h SAH rats (Fig. 5, left column, p > 0.05). By 72 h post 2nd SAH, 4OGOMV dose-dependently reduced HMGB1 mRNA when compared with the SAH groups (Fig. 5, right column, p < 0.01).

Fig. 3 Bar graph depicting 4OGOMV on the time-course change of pro-inflammatory cytokines after the induction of double shot SAH. Data are depicted for IL-1β, IL-6, IL-8, MCP-1 at 48 h after 1st SAH and 72 h post 2nd SAH. Data in the figure are presented as mean ± SD (n = 9). *, **P < 0.01, and #, ##, ###P > 0.01 when compared with the SAH group.
middle panel revealed the BrdU staining in the Sham-operated, SAH and 400 μg/kg/day 4OGOMV+SAH group. Even the contour of cerebrum was not deformed, the lethal effect of SAH related brain injury was observed, which corresponds to the observation of cleaved caspases mRNA related apoptosis (Fig. 6).

Discussion
In the present study, 4OGOMV, a natural compound, has been shown to attenuate SAH-induced IL-1β and MCP-1 expression in the initial stage of SAH. However, how to maintain the intensity of neuro-inflammation in delayed vasoconstriction remains unknown. HMGB1, a late-produced immunity mediator, showed a clue to investigate the mechanism of SAH-induced delayed cerebral ischemia. In Li et al's study, 4OGOMV is demonstrated to modulate the vasoconstriction on rabbit isolated basilar artery rings evoked by KCl and 5-HT [25]. In Wang et al's group, 4OGOMV was proven to be able to inhibit the proliferation of smooth muscle cells stimulated by TNF-α in a smooth muscle cell culture [27]. In the study of human colon cancer HT-29 cells, 4OGOMV is shown to inhibit the phosphorylation of histone H3 at Ser10, which is responsible for two important biological functions: serving as a feasible epigenetic strategy to inhibit the mitotic cell cycle progression and transcriptional activation of immediate pro-inflammatory genes during the interphase [11, 24]. Likewise, in the observation of NeuN, GFAP and BrdU polyclonal immunostating, our result supports 4OGOMV, in a selected dose, is able to reduce pro-inflammatory cytokines in the early stage of SAH-induced vascular deformity and also attenuate SAH induced cerebral apoptosis.

Elevated CSF IL-1β, IL-6, IL-8, TNF-α and adhesion molecule levels have been observed mediated by activated glia and imposed in the pathogenesis of aneurismal SAH-induced vasospasm [10, 39, 40]. In Bowman et al's study, a polyclonal antibody targeted against IL-6 was able to alleviate SAH-induced vasoconstriction in a femoral artery SAH model [41]. A cumulative result indicated that the upregulation of pro-inflammatory cytokines is antecedent to radiographic vasospasm (peak at 4th to 14th days after aneurysmal SAH in human subjects) [1, 40, 42], and attenuation of cytokines tends to minimize
vascular constriction and reduced cerebral infarct in animals. In this study, IL-1β, and MCP-1 (at 48 h after 1st SAH) levels were suppressed by the administration of 4OGOMV (at 200 and 400 μg/kg), while IL-6 level was reduced at 400 μg/kg 4OGOMV treatment group at 72 h after 2nd SAH.

Monocyte chemoattractant protein-1 (MCP-1), also known as chemokine ligand 2 (CCL2), is one of the major chemokines that regulate monocytes/macrophages migration and infiltration [11, 12, 43, 44]. Both CCL2 and its receptor CCR2 have been demonstrated to be induced and involved in various diseases such as idiopathic inflammatory myopathy, cytomegalovirus encephalitis, rheumatoid arthritis, Alzheimer’s disease, traumatic brain injury and rheumatic arthropathy [15, 22, 23]. Monocyte migration from the blood flow to the vascular endothelium is needed for routine surveillance of tissues in response to inflammation. Besides, CCL2 is reported to induce amylin expression through ERK1/ERK2/INK-AP1 and NF-κB-related signaling pathways. Amylin upregulation by CCL2 contributes to the elevation of the plasma amylin and insulin resistance in obesity [3]. In this study, MCP-1 positive cells were observed reduced in the media and adventitia of basilar artery in the 200 and 400 μg/kg 4OGOMV treatment SAH group, when compared with the SAH groups.

High-mobility group box 1 (HMGB1), a trigger of inflammation, tissue repair, with a characteristic of attracting inflammatory cells, recruiting stem cells and promoting their proliferation, is released by activated monocytes, macrophages, neutrophils, platelets and microglia [8, 17]. It is demonstrated to mediate the immediate and delayed inflammatory responses of vascular endothelial cells. As a late mediator, HMGB1 participated in the pathogenesis of chronic and preformed inflammation after the early immunity response has resolved [13]. Through bond to transmembrane receptors, like advanced glycation end products (RAGE), toll-like receptor (TLR)-2, TLR-4, activated nuclear factor-κB (NF-κB), extracellular regulated kinase (ERK) 1 and ERK 2 [16]. Moreover, HMGB1 also induced the expression of adhesion molecules from the activated platelets and microglia to up-regulate the recruitment of inflammation. Qiu et al. stated inhibition of HMGB1 expression is able to reduce focal cerebral ischemia insult by inhibiting the HMGB1/RAGE axis.
In this study, 4OGOMV (at 200 and 400 μg/kg) is able to restrict HMGB1 mRNA transcription and protein expression-induced delayed neuro-inflammation by 72 h after the induction of 2nd SAH, which corresponds to the observation of reduced IL-1β, IL-6 and MCP-1 level in the 4OGOMV treatment groups. Moreover, the peri-vascular MCP(+) cells were significantly decreased dose-dependently in the 4OGOMV treatment SAH rats.

In summary, the results of this study show that administration of 4OGOMV, at a therapeutic dosage, is efficacious in the prevention of experimental vasostenosis and improvement of neuro-behavior function. There are few reports about the bio-function of 4OGOMV, such as a novel epigenetic suppressor of histone H3 phosphorylation in a colon cancer cell lines, vasodilation effect on a rabbit isolated basilar artery rings, and suppression of smooth muscle cell hyperplasia induced by TNF-α [24, 26]. This is the first study indwelled in the immunosuppressive effect of 4OGOMV on SAH-induced vasospasm. Besides, 4OGOMV, by reducing HMGB1 mRNA activation, exerts a dual effect on microglia-related T cell transmigration and IL-6-related delayed inflammatory cascade.

Likewise, when considering the neuroprotective effect of 4OGOMV on the SAH rats, activated caspases-3 and -9a, which correspond to mitochondria related cellular apoptosis, were significantly reduced in the 400 μg/kg/day 4OGOMV treatment groups, when compared with the SAH rats. In mammals, there are three major pathways observed in the mitochondria related apoptosis: (1) the extrinsic receptor-related pathway; (2) the intrinsic pathway and (3) the cytotoxic lymphocyte-initiated granzyme B pathway [46, 48]. Shi et al has identified 14 caspases and subdivided them into one initiator group, such as caspase-8 and -9, one downstream effecter group, like caspase-3, -6, and -7, and another pro-inflammatory group, enrolled caspases-1, -4, -5, -11, -12, and -13 [47]. Caspase-3 is believed to be the major effecter caspase in neuronal apoptosis. Caspase-3 played a critical role in determining profound cerebral hyperplasia and deployment disorganization was observed in a study of caspase-3 knockout mice [46]. By now, the distinct roles of each caspase are still undefined. Besides, caspases act on upstream intrinsic apoptosis and downstream of cytochrome c release [47]. In this study, 4OGOMV was observed able to reduce the initiator caspase-9a (at 400 μg/kg/day) and decrease effecter Ccaspase-3 (at

![Fig. 6](image_url)
400 μg/kg/day). It is believed 4OGOMV, at a selected dose, is devoid of hepatic and systemic toxicity and has a neuroprotective effect by blocking both initiator caspase-9a and effector caspase-3 activation as other HDAI does [49].

Conclusions
The acuminated results arouse interest to consider the pathogenesis of SAH-induced acute and delayed neuro-inflammation and its effect dictates on the patient’s outcome. The breakout of T-cell immigration accompanying SAH is a critical and complicated pathway underlying the development and maintenance of delayed neurologic deficits. Our study shows that administration of 4OGOMV may diminish SAH-induced early pro-inflammation and subsequent HMGB1 stimulation in a rodent model of SAH. This study suggests that 4OGOMV, a natural occurring potent immunosuppressant in a selected dose, could prove clinically useful in preventing and treating SAH-induced vasospasm and early brain injury.

Fig. 7 The effect of 4OGOMV on the polyclonal NeuN, BrdU and GFAP immunostaining among the SAH groups. Upper panel: NeuN (+) neurons (red arrow head) with vaculated neucli and groups were depicted as: a Sham-operated; b SAH group and c 400 μg/kg/day 4OGOMV treatment SAH group. (n = 9/group) Middle and lower panels shows the BrdU (+) (black arrow head) and GFAP (+) cells (yellow arrow head) respectively. (X200) Standard bar = 100 μm

Abbreviations
BA: basilar artery; BrdU: 5-bromo-2′-deoxyuridine; CSF: cerebrospinal fluid; DIND: delayed ischemic neurological deficit; ET: endothelin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFAP: glial fibrillary acidic protein; HMGB-1: high-mobility group box 1; HRP: horseradish peroxidase; IEL: internal elastic lamina; IL-1β: interleukin-1β; IL-6: interleukin-6; IL-8: interleukin-8; Lactate dehydrogenase; N-methyl-d-aspartate; PBS: phosphate-buffered saline; MAPK: mitogen-activated protein kinase; MCP-1: monocyte chemoattractant protein-1; LCA: leukocyte common antigen; 4OGOMV: 4′-O-β-d-glucosyl-5-O-methylvisamminol; PDGF: platelet derived growth factor; PBS: phosphate-buffered saline; TGF-β1: transforming growth factor β1; TLRs: Toll-like receptors; TNF-α: tumor necrotic factor α; VEGF: vascular endothelial growth factor.

Authors' contributions
CZC planned the experiment and drafted the manuscript. SCW carried out the western blotting and rt-PCR. CLL offered statistic consultation and ALK helped the data analysis and gland support. All authors read and approved the final manuscript.

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