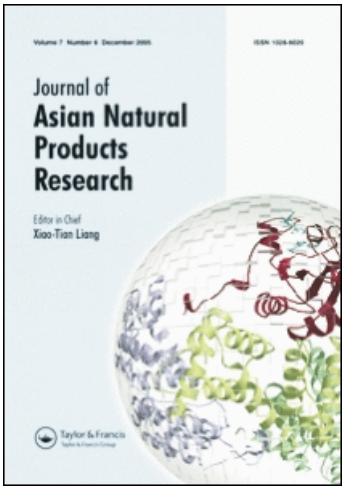

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Acute neurovascular unit protective action of pinocembrin against permanent cerebral ischemia in rats

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Acute vascular- and neuroprotective effects of pinocembrin (**1**) were evaluated in a rat model of focal cerebral ischemia. Focal cerebral ischemia was induced by the middle cerebral artery occlusion (MCAO) for 24 h. 5,7-Dihydroxyflavanone (compound **1**; at 3, 10, or 30 mg/kg), intravenously injected at 0, 8, and 16 h after MCAO, reduced the cerebral infarct volumes by 47, 39, and 37%, respectively, as visualized by 2,3,5-triphenyltetrazolium chloride staining ($P < 0.01$). Treatment with **1** also reduced brain swelling and improved behavioral deficits significantly ($P < 0.01$ and 0.05, respectively). To evaluate the effect of **1** on blood–brain barrier (BBB) disruption, mixture of Evans Blue (EB) and sodium fluorescein (NF) was intravenously injected immediately after MCAO. Global NF/EB uptake and fluorescence imaging of local BBB disruption were measured. Treatment with compound **1** reduced the leakage of both dyes, manifesting a preventive action in BBB integrity. This is the first time to demonstrate that **1** has acute neurovascular protective action against permanent focal cerebral ischemia. The mechanism of neurovascular protective action of **1** is under investigation.

Keywords: pinocembrin; neurovascular unit; cerebral ischemia; blood–brain barrier

1. Introduction

The strategies for comprehensive stroke treatment are now focusing on treating the complete neurovascular unit that consists of the blood–brain barrier (BBB) [endothelial tight junctions (TJ), basal lamina, pericytes, and astrocytic endfeet] and the brain tissue (neurons, glia, their processes, and the extracellular or interstitial space).[1,24] The BBB is a physical and metabolic barrier between the central nervous system and the systemic circulation. Disruption of the BBB plays an important role in the pathogenesis in many acute neuronal damages and chronic neurodegenerative diseases.[2–5] In particular, cerebral ischemia elicits biphasic elevations in the BBB permeability, occurring at 3–6 and 24–48 h, after the onset of ischemia.[6–8] This time-dependent increase in the BBB permeability is correlated with reversible and irreversible damage to the neurovascular unit, and may subsequently result in brain edema, inflammation, and, further, hemorrhagic transformation after stroke.[3,7,9,10] Neuroprotectants that act by reducing neuronal injury alone, therefore, would be expected to be less successful.[4,11] An effective therapy for ischemic stroke may need to offer wide cytoprotection to the neurovascular unit, which is composed of neuron and glial cell elements that are in physical proximity to the endothelium, and, thus, to prohibit progression of brain edema, BBB disruptions, and the maturation of final infarct after stroke.

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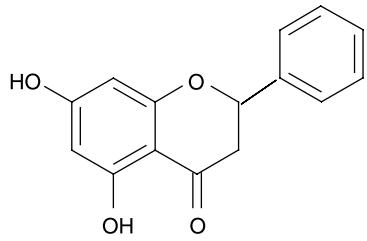

Figure 1. Chemical structure of **1**.

Compound **1** (pinocembrin) is the most abundant flavonoids in propolis.[12] The compound was reported to have multiple actions including anti-inflammatory, anti-oxidant, anti-thrombotic, anti-microbial, anti-allergic, hepatoprotective, anti-viral, cancer chemopreventive, and anti-asthmatic activities.[13–16] Our previous study also found that **1** induced relaxation of rat aortic rings through an endothelium-dependent and an endothelium-independent pathway,[17] and that **1** could improve rat cognitive impairments induced by chronic cerebral hypoperfusion, which contributed to its improvement of regional cerebral blood flow (rCBF) and mitochondrial structure and function.[18] In the present study, we further examined the acute neurovascular unit protective action of **1** against permanent cerebral ischemia at the initiation of occlusion in rats.

2. Results and discussion

2.1 Effects of 1 on regional cortical blood perfusion (rCBF) and saturation of blood oxygen (StO_2)

As shown in Table 1, 10 min after the ischemia, rCBF reduced to a level of about 10–20% of baseline values. At 90 min post-middle cerebral artery occlusion (MCAO), rats treated with **1** at 10 and 30 mg/kg had significantly higher rCBF ($P < 0.01$ and 0.05, respectively) than the controls. At 24 h of ischemia, all rats in the three groups of compound **1** treatment had remarkably elevated rCBF compared with vehicle group. The positive control drug edaravone treatment showed no action on reduced rCBF.

As to the saturation of blood oxygen, there was no significant difference among the groups.

2.2 Effects of 1 on neurological deficit scores and infarct volume

Neurological deficit scores (Figure 2) and infarct volume (Figures 3 and 4) of MCAO

Table 1. Regional cortical blood perfusion (rCBF) and saturation of blood oxygen (StO_2) data obtained prior to (pre-occlusion), at 10, 90 min, and 24 h of the permanent middle cerebral artery occlusion in rats.

			Percentage of pre-occlusion		
	n	Pre-occlusion	10 min of ischemia	90 min of ischemia	24 h of ischemia
rCBF (%)					
Vehicle	10	100	14.8 ± 0.5	27.1 ± 0.7	30.2 ± 1.9
pinocembrin (3 mg/kg)	12	100	14.1 ± 0.4	28.7 ± 0.4	39.6 ± 2.4*
pinocembrin (10 mg/kg)	12	100	13.2 ± 0.5	42.1 ± 1.2**	48.6 ± 1.7**
pinocembrin (30 mg/kg)	12	100	15.6 ± 2.1	35.9 ± 2.6*	40.1 ± 2.8*
Edaravone (3 mg/kg)	11	100	17.1 ± 0.7	29.7 ± 3.3	31.2 ± 1.2
StO_2 (%)					
Vehicle	10	58 ± 4	55 ± 2	60 ± 2	59 ± 1
pinocembrin (3 mg/kg)	12	60 ± 6	58 ± 4	61 ± 5	59 ± 1
pinocembrin (10 mg/kg)	12	63 ± 4	62 ± 1	63 ± 3	62 ± 2
pinocembrin (30 mg/kg)	12	59 ± 5	58 ± 4	58 ± 3	60 ± 4
Edaravone (3 mg/kg)	11	60 ± 2	58 ± 3	60 ± 5	60 ± 4

Data are represented as the means ± SEM. rCBF is expressed as a percentage of baseline values. *$P < 0.05$ and **$P < 0.01$ were considered significant compared with vehicle group.

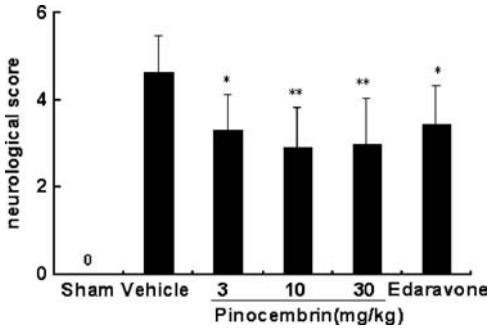

Figure 2. Effects of **1** on neurological deficits induced by MCAO operation. Data are expressed as means ± SEM and analyzed by one-way ANOVA. $*P < 0.05$ and $**P < 0.01$ were considered significant compared with vehicle group.

rats were significantly higher than that of sham-operated group 24 h after ischemia. Treatment with **1** (3, 10, and 30 mg/kg i.v.) significantly reduced the percentage of infarction in the ipsilateral hemisphere by 47, 39, and 37%, respectively, and decreased the neurological deficit scores as well. Treatment with **1** at 10 and 30 mg/kg showed better potency than the treatment with 3 mg/kg of edaravone. There is no significant difference between the groups treated with 10 and 30 mg/kg, respectively, of compound **1**.

2.3 Effects of 1 on brain swelling and EB/NF leakage

The contralateral (non-ischemic) hemispheric wet weight was used as a control for the calculation of ischemia-induced brain edema in the ipsilateral (ischemic) hemisphere. A net difference between the two hemispheric weights was obtained by subtracting the weight of the intact hemisphere from that of the infarcted hemisphere. Brain swelling was expressed as a percentage of the net difference between the ischemic hemispheric weight and the contralateral hemispheric weight. As shown in Table 2, the average

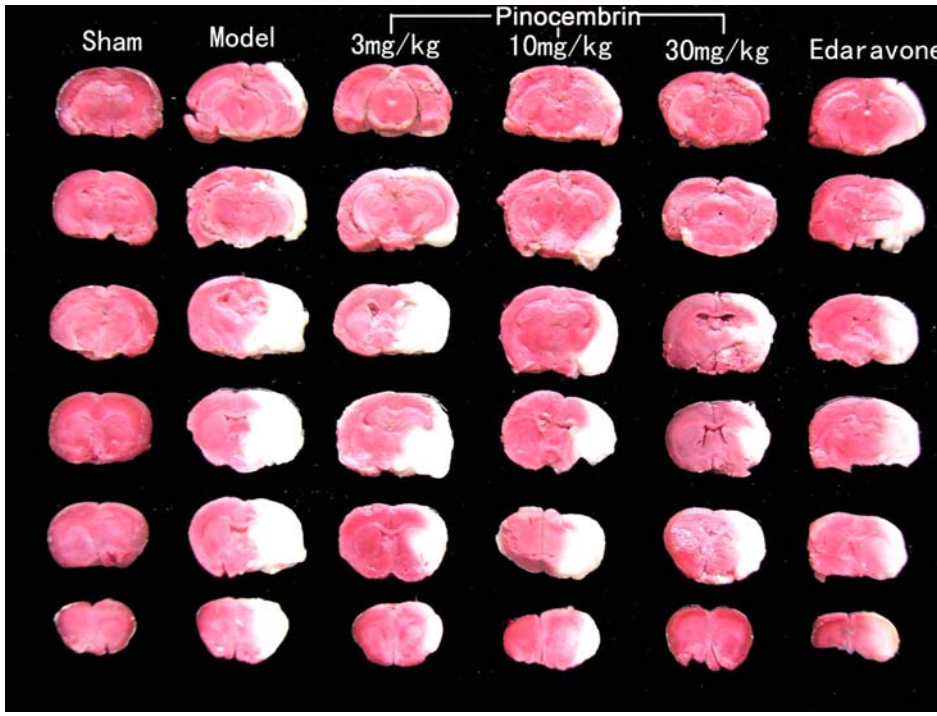

Figure 3. Illustrative coronal sections (2-mm thick) showing infarct area in the ischemic cerebral hemisphere as distinct pale-stained area in rats subjected to 24-h MCAO (model) and attenuation of infarct area by intravenously injection of **1** (3, 10, or 30 mg/kg) and edaravone (3 mg/kg) at 24-h ischemia.

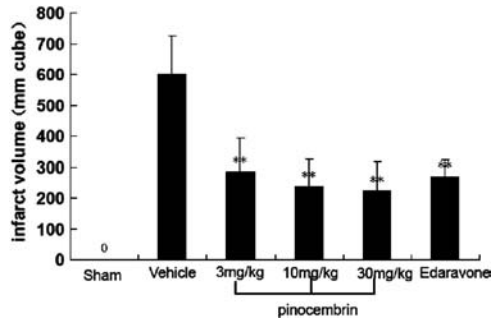

Figure 4. Effect of **1** on infarct volume of rats subjected to 24 h of permanent MCAO. Data expressed as means ± SEM were analyzed by one-way ANOVA. $^{**}P < 0.01$ *versus* vehicle group.

edema in vehicle group was 8.3 ± 1.9%, which decreased to 5.5 ± 1.7, 3.2 ± 2.1, or 5.1 ± 1.5%, respectively, after treatment with 3, 10 or 30 mg/kg of **1**.

The methods used for quantitative and qualitative BBB disruption were with some modifications.[22] The global NF/EB uptake and fluorescence imaging of local BBB disruption were evaluated. Treatment with 3, 10, or 30 mg/kg of **1** at the initiation time of occlusion reduced the NF/EB leakage significantly, which is consistent with the reduction effect on brain swelling. Treatment with 10 mg/kg of **1** manifested a superior action (Table 2).

2.4 Microscopic comparison of NF/EB uptake

Visualization of brain slices by fluorescence microscopy displayed minimal staining for NF and EB in most areas of the brain in sham-operated animals (Figure 5A/a). Brains from animals treated with compound **1** were distinguished by distinct areas of EB/NF staining, as well as by vascular or perivascular staining (Figure 5C/c,D/d). Much heavier and widespread staining for EB/NF was observed in the brains from vehicle group animals (Figure 5B/b). Figure 5E/e,F/f showed that brains from vehicle group rats were typically stained with EB/NF throughout the ischemic tissue; in contrast, brains from animals treated with compound **1** showed a discrete area of staining for EB/NF, typically in the area of the ventral midline (Figure 5G/g,H/h).

The findings of present paper are the first to show that treatment with **1** (3, 10, and 30 mg/kg) at the acute phase improved rCBF and reduced the postischemic damage to the neurovascular unit following permanent focal cerebral ischemia in rats. Additionally, treatment with **1** at the early phase after ischemic stroke is effective in preserving anatomical, biochemical, and functional integrity of the BBB permeability.

Each main cerebral vessel provides crucial blood supply to a distinct territory of the brain tissues. Occlusion of the MCA, the most common cause of stroke, affects both the gray and the white matter and the

Table 2. Effect of **1** on brain swelling and EB/NF leakage induced by 24-h occlusion of MCA in rats.

Group	n	Edema (%)	EB leakage (μg/g tissue)	NF leakage (μg/g tissue)
Vehicle	6	8.3 ± 1.9	8.6 ± 2.01	2.33 ± 0.30
pinocembrin (3 mg/kg)	6	5.5 ± 1.7*	6.16 ± 0.43*	1.39 ± 0.20*
pinocembrin (10 mg/kg)	6	3.2 ± 2.1**	4.39 ± 0.42**	1.30 ± 0.15**
pinocembrin (30 mg/kg)	6	5.1 ± 1.5*	6.03 ± 0.81*	1.48 ± 0.08*

Results are presented as the means ± SEM from six rats in each group, and are analyzed by one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* vehicle group.

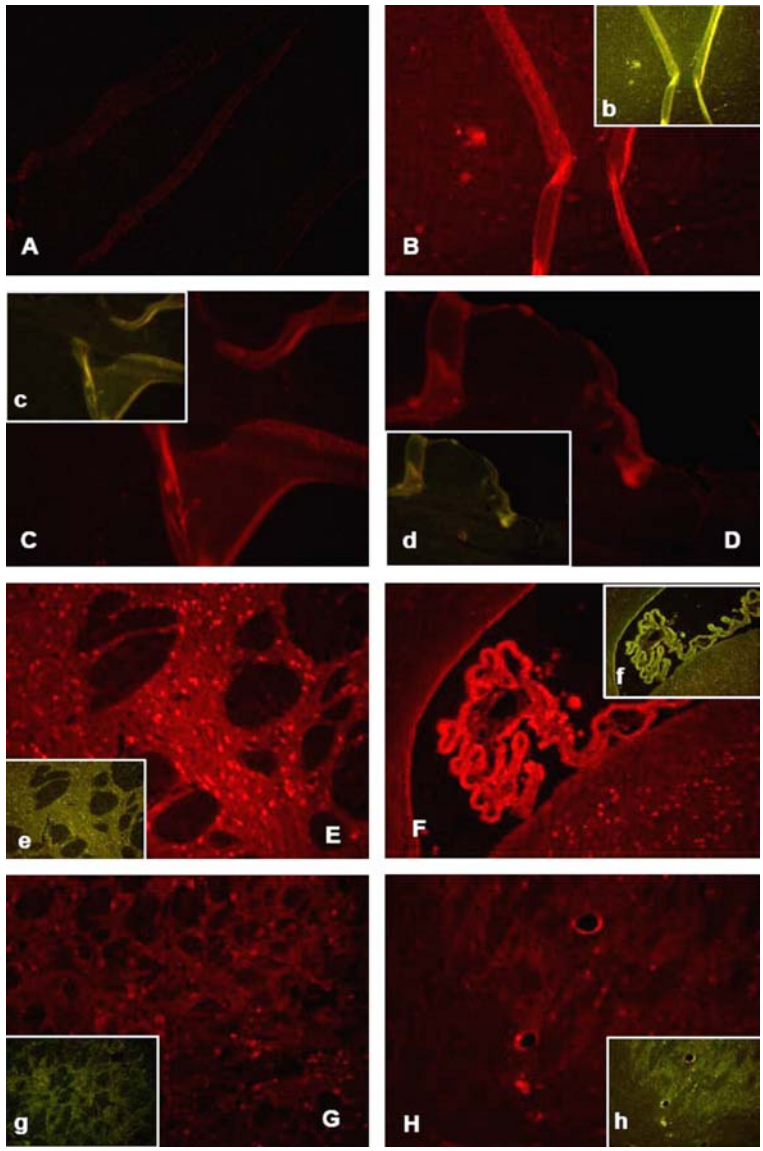

Figure 5. Microscopic comparison of NF/EB uptake. (A) Representative image from the parenchyma of a sham-operated rat. No staining is visible. (B), (E), and (F) Images from the lateral ventricle and the affected area of a vehicle group rat. Heavy NF/EB staining is observed throughout the parenchyma and the ischemic area. (C) and (G) Representative images from the lateral ventricle and the affected area respectively, in the animals treated with 3mg/kg of compound 1. (D) and (H) Representative images from the lateral ventricle and the affected area respectively, in animals treated with 10 mg/kg of 1. Every two pictures arranged together are from the identical area, displaying EB and NF staining, respectively, which shows these two dyes have almost the consistent distribution. All images are 400 × . Green, NF; red, EB; yellow, merge.

neurovascular interface as well.[10,11,23] These changes are a consequence of complex pathophysiological processes that eventually interfere with functional circuitries of the brain tissues. However, the assessment of ischemic pathology in experimental stroke models is dominated by histological staining of neuronal perikarya. In the development of

neuroprotective drugs for clinical use in stroke, increasing evidence has suggested that optimal functional recovery after an ischemic brain insult may require a therapy aimed at unique steps in both the gray and the white matter and in the neurovascular unit as well.[9,10,11,23] The findings indicated that **1** offered beneficial neuroprotection by improving rCBF and by protecting against early increase in the BBB permeability due to the ischemic insult, which further justifies its actions for improving late increase in the BBB permeability and final functional recovery after stroke.

However, it should be stressed that numerous other possible mechanisms of action on neurovascular unit after stroke, including the effect of **1** on the postischemic activation of circulatory leukocytes and cerebral microglia, the activation of proteases such as matrix metalloproteinases, and the TJ of endothelia cells, need further evaluation.[8,24]

In summary, the treatment of rats with **1** (3, 10, and 30 mg/kg) at the initiation time of occlusion significantly improved rCBF, reduced brain swelling, improved the preservation of the BBB integrity, and decreased the neurological deficits following permanent focal cerebral ischemia, indicating that **1** is beneficial for the ischemic neurovascular unit. Further study on the potential of **1** in the treatment of ischemic stroke is of necessity.

3. Experimental

3.1 Resource and preparation of drug

(−)-Pinocembrin (**1**) was first isolated by the Department of Natural Product Chemistry, Institute of Materia Medica, Chinese Academy of Medical Sciences. Racemic of **1** ((+)-pinocembrin:(−)-pinocembrin = 1:1, 99% purity) used in the present study was synthesized and processed using a sterile injection powder by the Department of New Drug Development in the Institute of Materia Medica, Chinese Academy of Medical Sciences. It was dissolved in 0.9% NaCl before use.

3.2 Animal preparation and drug administration

Male Sprague–Dawley rats weighing 240–280 g were obtained from Vital River Experimental Animal and Technology Co., Ltd (Beijing, China). They were housed under natural light/dark (12/12 h) cycle with food and water available *ad libitum*. All procedures were performed in accordance with institutional guidelines for laboratory animals, and all efforts were made to minimize the suffering and the number of rats used. After permanent MCAO, rats were randomly assigned into five groups: model (vehicle-treated) group; 3, 10, and 30 mg/kg of **1** groups and 3 mg/kg edaravone group (as positive control[19]). All drugs were injected via tail vein at 0, 8, and 16 h after occlusion. Control rats received vehicle (0.9% NaCl i.v.).

3.3 Experimental model

Rats were anesthetized with intraperitoneal injection of chloral hydrate (Tianjin, China) at a dose of 380 mg/kg. Permanent focal brain ischemia model was reproduced by the occlusion of MCA as described previously.[20] Briefly, 4-0 monofilament nylon suture with a round tip was inserted from the right external carotid artery into the lumen of the internal carotid artery to occlude the origin of the MCA. Sham operation was performed for control rats by omitting the occlusion.

3.4 rCBF and StO_2 monitoring

Laser Doppler flowmetry (Periflux PF 5000, Perimed Company, Sweden) was used for measuring the rCBF. The scalp was incised along the midline, and a 1.5-mm diameter area in right parietal bone was thinned 2 mm lateral and 1 mm caudal to the bregma. rCBF was measured at four time points: 10 min prior to, 10, 90 min, and 24 h after the occlusion, i.e. just before decapitation. The rCBF data were expressed as a percentage of the baseline values, and the percent oxygen saturation (StO_2) was detected non-invasively by using the ODISseyTM Tissue Oximeter

(ViOptix, CA, USA) at the same time points of rCBF determination.

3.5 Measurement of neurological deficit score and infarct size

After 24 h of occlusion, the neurological deficit score of each rat was recorded according to Longa's method[21] by a single experimenter, who was blinded to the experimental treatment groups. Neurological findings were scored on a five-point scale: no neurological deficit = 0, failure to extend right paw fully = 1, circling to right = 2, falling to right = 3, and did not walk spontaneously and had depressed levels of consciousness = 4.

After scoring, rats were decapitated and the coronal sections (2 mm) of the cerebrum were prepared and stained with 0.1% 2,3,5-triphenyltetrazolium chloride solution at 37°C for 30 min, and were then fixed using 4% formaldehyde in PBS. The size of the unstained infarcted area per section was analyzed (Olympus Micro Image Lite 4.0 system, Tokyo, Japan). The infarct size was calculated from the sum of the thickness (2 mm) of the infarct areas (six sections in all). The degree of infarction was expressed as a percent volume of the whole coronal section.

3.6 Measurement of EB/NF leakage

To determine the BBB disruption, EB and NF extravasations were quantified. Just after the MCAO, 0.25 ml of 0.5% EB and NF mixture (mixed the night before to allow maximal binding of EB to albumin), in 0.9% saline, was injected intravenously via tail vein. Twenty-four hours after the occlusion, rats were perfused with 0.9% saline to discard redundant dyes, and then decapitated, and the brains were immediately removed, divided into ischemic and non-ischemic hemispheres. Each hemisphere was weighed and mechanically homogenized in 7.5% (w/v) trichloroacetic acid at 3 ml/g wet brain weight, and the resulting suspension was divided into two aliquots. One-milliliter aliquot was neutralized with 52 μl 5 N NaOH and was measured by fluorimetry on a microplate reader (excitation 485 nm and emission 535 nm; SpectraMax M5, Molecular Devices, CA, USA for NF determination). The other aliquot was centrifuged at 12,000*g* for 20 min at 4°C, and the supernatant was measured by absorbance spectroscopy at 620 nm for EB determination.[22,23] Results are presented as micrograms of EB/NF per gram of wet brain weight by comparison with a standard solution.

3.7 Morphology of BBB disruption

For microscopic studies, rats ($n = 3$ for each group) were prepared as described above. Brains were removed, snap-frozen in dry ice/isopentane, and immediately stored at −80°C until cutting. Ten micrometers of coronal sections were mounted onto gelatin-coated slides with VectashieldTM (Vector Laboratories, Burlingame, CA) for visualization on a fluorescence microscopy (NIKON ECLIPSE 80i, × 400) and photographed by ACT-2U NIKON Imaging System.

3.8 Statistical analysis

Statistical analysis was performed using Prism software (GraphPad Software Inc., San Diego, CA). All data are presented as means ± SEM. Ischemic lesion volumes were compared by one-way ANOVA. $P < 0.05$ was considered significant.

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