行政院國家科學委員會補助專題研究計畫成果報告

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※ RESVERATROL 對大花鼠腦梗塞之療效及其機轉的研究 ※
※ Resveratrol Reduction of Infarct Size in Long-Evans Rats ※
※ Subjected to Focal Cerebral Ischemia ※
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□國際合作研究計畫國外研究報告書一份

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Abstract

Resveratrol is found in a wide variety of plant species. It is present in the seeds and skin of grapes and constitutes one of the major components of red wine. This study was undertaken to evaluate whether resveratrol could effectively suppress infarct size from the damaging effects of focal cerebral ischemia. The middle cerebral artery was occluded for 1 hr and 24 hr reperfusion in anesthetized Long-Evans rats. In pretreatment or treatment groups, resveratrol, at dosages of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg, was intravenous injected 15 minutes before middle cerebral artery (MCA) occlusion or when the common carotid arteries clips were removed respectively. Pretreatment or treatment of resveratrol (10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg) did not produce any changes in pH, blood gases, heart rate or mean arterial blood pressure, but it significantly reduced the total volume of infarction at the doses 10^{-6} and 10^{-7} g/kg. Resveratrol is a potent neuroprotective agent in focal cerebral ischemia. Its beneficial effects may be related to its anti-platelet aggregation activity, vasodilating effect, antioxidant property or by all mechanisms together.

Key words: resveratrol, red wine, stroke, focal cerebral ischemia, infarct maturation

Resveratrol (fig. 1), a natural phytoalexin (3,5,4'-trihydroxystilbene) is found in a wide variety of plant species. It is abundantly present in the seeds and skin of grapes and constitutes one of the major components of red wine (1). Resveratrol has been reported to have several biologic effects such as, an anti-inflammatory activity attributed to cyclooxygenase inhibition (2, 3), an estrogenic activity (4), and an anti-platelet activity (5). Recently, resveratrol was found to prevent lipid peroxidation (6) and lipid peroxidation-induced cell death (7). Resveratrol was also found to stimulate nitric oxide (NO) production in endothelial cells and vasodilatory effect on blood vessels was demonstrated (8).

A number of pharmacological substances have been shown to protect the brain against ischemia reperfusion injury. Among these are drugs with anti-platelet, vasodilation, or antioxidant effect. Vasodilators may improve brain perfusion and anti-platelet agents prevent thrombosis form. Several lines of evidence have reported the roles of nitrogen and oxygen free radicals in the pathogenesis of brain ischemia-reperfusion injury (9). This receives further support from the evidence that a variety of free radical scavengers and antioxidants are capable of ameliorating ischemia reperfusion injury (10).

The anti-platelet activity, vasodilating effect and antioxidant property of resveratrol prompted us to investigate whether it has any protective effects in brain ischemia reperfusion injury. So far studies have concentrated on considering the polyphenol fraction of red wine from the point of view of being responsible for the effects of red wine in the prevention of atherosclerosis and coronary heart disease. However it has never been considered that it may possibly be utilized as a preventive or treatment medicine in brain ischemia reperfusion injury. The present experiment was undertaken to evaluate whether resveratrol could effectively suppress infarct size from the damaging effects of focal cerebral ischemia.

Materials and Methods

Animals

The present investigation conforms to the Guide for the Car and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Male Long-Evans rats (National Lab. Animal Breeding and Research Center) weighing 270-350g were used throughout this study. These animals were housed in a room with controlled temperature (24±1°C) and humidity (55±5%) under a 12:12 h light-dark cycle. They were allowed free access to food and water.

Surgical procedure

Our technique was a modification of the method of Koketsu et al. (11). In brief, each male Long-Evans rat was anesthetized with halothane (1% to 3.5% in a mixture of 70% N_2O and 30% O_2) with the use of a mask. Body temperature was maintained during surgery at 37±0.5°C with a heating pad servo-controlled by a rectal probe. The right femoral artery was cannulated with PE-50 polyethylene catheters for continuous monitoring of heart rate and mean arterial blood pressure (MABP) by Statham P23 XL transducer and displayed on a Gould RS-3400 physiological Recorder (Gould, Cleveland, OH, USA) and blood sampling for analysis of blood gases by Blood Gas Analyzer (GEM-5300 I.L. CO, USA). Measurements were performed before, during and after (reperfusion 10 min and 24 hr) unilateral middle cerebral artery occlusion.

Focal ischemic infarcts were made in the right lateral cerebral cortex in the territory of the middle cerebral artery (MCA). Both common carotid arteries were exposed by midline anterior cervical incision. The animal was placed in a lateral position, and a skin incision was made at the midpoint between the right lateral canthus and the anterior pinna. The temporal muscle was retracted, and a small (3-mm diameter) craniectomy was made at the junction of the zygoma and squamosal bone using a drill (Dremel Multipro+5395, Dremel com. USA) cooled with saline solution. Using a

dissecting microscope (OPMI-1, ZISS, Germany), the dura was opened with fine forceps, and the right MCA was ligated with 10-0 monofilament nylon ties. Both common carotid arteries were then occluded by microaneurysm clips for 1 hr. After removing the clips, return of flow was visualized in the arteries.

Infarct volume analysis

Twenty-four hours after cerebral infarction, animals were anesthetized and killed by rapid decapitation. Brain were removed, inspected visually for the anatomy of the MCA and for signs of hemorrhage or infection, immersed in cold saline solution for 10 minutes, and sectioned into standard coronal slices (each 2-mm thick) using a brain matrix slicer (JACOBOWITZ Systems, Zivic-Miller Laboratories INC, Allison park, USA). Slices were placed in the vital dye 2,3,5-triphenyltetrazolium chloride (TTC, 2%; Sigma, USA) at 37°C in the dark for 30 minutes, followed by 10% formalin at room temperature overnight. The outline of right and left cerebral hemispheres as well as that of infarct tissue, clearly visualizable by a lack of TTC staining (12), was outlined on the posterior surface of each slice using an image analyzer (color image scanner, EPSON GT-9000, connected to an image analysis system (AIS software, Imaging research INC, Canada) run on a personal computer, AMD K6-2 3D 400. Infarct volume was calculated as the sum of infarct area per slice multiplied by slice

thickness. Both the surgeon and image analyzer operator were blinded to the treatment given each animal.

Drug administration

Resveratrol (Sigma, USA) was dissolved in 40% (v/v) propylene glycol to the desired concentrations in normal saline. Final concentration of propylene glycol in the infected resveratrol solution was 4×10^{-3} % (v/v). At this concentration, propylene glycol had no effect on the infarct size of focal cerebral ischemia. In pretreatment or treatment groups, resveratrol solution of 0.3 ml was administered at four different doses (10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg) via intravenous injection 15 minutes before MCA occlusion or when the common carotid arteries clips were removed. Rats injected with 0.3 ml normal saline with 4×10^{-3} % (v/v) propylene glycol were used as controls. Animals were randomly allocated to each drug treatment and control groups.

Statistics

Data are expressed as mean ± standard error of mean (SEM). Statistical analysis of differences in volume of infarcts between control and resveratrol treatment groups was carried by unpaired, two-tailed *t* tests, and by two-way analysis of variance (ANOVA) for combined data. Physiological measurements were compared among

control and resveratrol-treated animals by unpaired, two-tailed *t* tests for each experiment using the Bonferroni correction for multiple pairwise comparisons.

Results

Effects of resveratrol on physiological parameters

Tables 1 and 2 give a summary of physiological parameters measured before, during and after MCA occlusion. Pretreatment or treatment of resveratrol (10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg) did not produce any changes in pH, blood gases, heart rate or mean blood pressure.

Infarct Volume

There were easily identifiable areas of infarction within the territory of the occluded middle cerebral artery. Intravenous injection of resveratrol 15 minutes before MCA occlusion (fig. 2) or intravenous injection when the common carotid arteries clips were removed (fig. 3), significantly reduced the total volume of infarction, in a dose-dependent manner, in rats after 1 hr MCA occlusion and 24 hr reperfusion. Statistically significant decreases in infarct area were detected at the doses 10^{-6} and 10^{-7} g/kg.

Discussion

In this study we provide evidence that pretreatment or treatment with resveratrol possesses robust neuroprotection properties during focal cerebral ischemia in rats. Resveratrol (10^{-6} and 10^{-7} g/kg) provides reduction of injury in this model. In the observation (fig. 2 and fig. 3) that the 10^{-7} g/kg dose seemed more effective than the 10^{-6} g/kg dose, but the difference did not reach statistical significant. We thought that resveratrol has the steady state effect at the dose of 10^{-7} g/kg and 10^{-6} g/kg.

Resveratrol is found in a wide variety of plant species. It is present mostly in the seeds and skin of grapes and constitutes one of the major components of red wine (1). It is the active component of Kojokon prepared from roots of Polygonum species and used in Asian traditional medicine to treat several diseases (13).

The definite mechanism for the beneficial effects of resveratrol may be multifactorial. A number of pharmacological substances, e.g., calmodulin antagonist (14), vasorelaxant, calcium channel blocker (15), immunosuppressant (16), thrombin inhibitor (17) and platelet activating factor antagonist (18) have been shown to protect injury in focal cerebral ischemia. Resveratrol had been found to possess several pharmacological activities such as anti-inflammation (2,3), vasodilating effect (8),

anti-platelet aggregate (5) and protective action against lipid peroxidation (6). It is possible that endothelium dependent vasodilation, anti-platelet aggregation activity and antioxidant effect are contributory to reduce infarct size of focal cerebral ischemia of resveratrol.

In pretreatment of resveratrol groups, we showed that in anesthetized rats the administration of resveratrol 15 min prior to MCA occlusion, resveratrol (10^{-6} and 10^{-7} g/kg) provided reduction of infarct size in this model. We think its anti-platelet aggregation activity prevents vessel thrombosis. This is similar to the situation in humans, in whom focal cerebral ischemia is associated with a massive production of intravascular free radicals. Ischemic insults to central nervous system (CNS) derange oxidative homeostasis most likely via generation of reactive oxygen species and nitric oxide (19). The free radical scavenge effect of resveratrol can prevent this injury.

In treatment of resveratrol groups, we showed that in anesthetized rats the administration of resveratrol when the common carotid arteries clips were removed, doses 10^{-6} and 10^{-7} g/kg provided reduction of infarct size in this model. We think its vasodilating effect may improve brain perfusion, by anti-platelet aggregation activity to prevent vessel thrombosis while antioxidant effect scavenges the free radicals

which are formed during reperfusion period.

The concentration of resveratrol found to be effective in our study was 0.1 μg/kg. It is difficult to establish a relationship between the 0.1 μg/kg concentration found effective in our experiments and average red wine resveratrol concentration. This is because the levels of resveratrol present in red wines vary considerably according to the geographic origin of the grape cultivar, as well as the different fermentation process used. For instance, the value of red wine resveratrol content range from 0.065 mg/100ml in some United States wines to 0.8 mg/100ml in a Pinot noir variety of Spanish red wine (20). Bertelli et al. have established the pharmacokinetic profile of resveratrol determined by oral administration of red wine to rats. This study has demonstrated that chronic oral administration of resveratrol in red wine to rats dose result in a significant plasma bioavailability (21). Further studies to determine whether chronic oral administration of resveratrol is capable of achieving sufficient plasma concentration to exert neuroprotective effects similar to those observed in this study will be undertaken.

In conclusion, the present report represents the first evidence that resveratrol can reduce the size of cerebral infarction. Our results indicate that the beneficial effect of

resveratrol is neuroprotection may be due to its anti-platelet aggregation, vasodilating effect, antioxidant activity or by all these mechanisms together.

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TABLE 1

Effects of pretreatment of resveratrol on hemodynamic and blood gas parameters before, during and after unilateral middle cerebral artery occlusion

	Vehicle	Resveratrol 10^{-6} g/kg	10^{-7} g/kg	10^{-8} g/kg	10^{-9} g/kg
Baseline					
pH, U	7.38±0.04	7.41±0.03	7.41±0.02	7.39±0.02	7.40±0.01
P_{O_2}, mm Hg	97.8±5.4	95.2±6.8	94.3±8.5	93.8±6.1	98.4±2.9
P_{CO_2}, mm Hg	42.1±2.3	41.6±3.6	45.8±4.5	41.9±2.4	43.2±2.1
Heart rate, bpm	433±24	456±25	473±16	459±20	403±50
MABP, mm Hg	113.7±5.2	111.7±v3.9	113.9±3.4	119.1±4.1	113.7±11.3
Ischemia					
pH, U	7.43±0.02	7.46±0.02	7.44±0.04	7.47±0.06	7.42±0.03
P_{O_2}, mm Hg	94.0±5.0	89.0±5.7	92.0±13	87.5±15	94.6±2.4
P_{CO_2}, mm Hg	40.5±3.5	38.7±0.9	44.0±3.0	41.5±5.0	38.9±4.3
Heart rate, bpm	403±48	444±14	451±17	467±16	414±52
MABP, mm Hg	120.0±6.8	116.1±5.0	123.3±3.9	125.1±4.4	130.3±9.2
Reperfusion (10 min)					
pH, U	7.42±0.04	7.39±0.04	7.43±0.01	7.42±0.03	7.39±0.02
P_{O_2}, mm Hg	98.0±10.0	94.5±11.5	98.3±5.8	104.0±19.0	90.0±3.0
P_{CO_2}, mm Hg	48.5±2.5	48.5±1.5	46.7±1.7	41.5±12.5	49.0±3.0
Heart rate, bpm	382±40	415±29	441±17	437±23	386±52
MABP, mm Hg	101.7±5.4	91.7±4.5	95.4±2.7	96.3±5.1	97.7±4.9
Reperfusion (24 hr)					
pH, U	7.43±0.09	7.44±0.02	7.44±0.02	7.47±0.06	7.48±0.06
P_{O_2}, mm Hg	108.0±7.0	112.0±2.6	99.5±9.1	98.7±11.9	107.0±5.0
P_{CO_2}, mm Hg	39.0±2.3	37.3±4.0	37.8±3.7	43.0±4.2	31.5±12.5
Heart rate, bpm	471±26	494±19	466±19	469±17	431±50
MABP, mm Hg	107.3±10.8	101.7±5.8	102.1±4.7	103.3±4.8	90.5±10.5

Seven rats each were pretreated with vehicle or resveratrol.

Values are mean ± SEM. Differences in physiological parameters among groups were not statistically significant ($p>0.05$).

MABP indicates mean arterial blood pressure.

TABLE 2

Effects of treatment of resveratrol on hemodynamic and blood gas parameters before, during and after unilateral middle cerebral artery occlusion

		Resveratrol			
	Vehicle	10^{-6} g/kg	10^{-7} g/kg	10^{-8} g/kg	10^{-9} g/kg
Baseline					
pH, U	7.38±0.03	7.41±0.01	7.37±0.03	7.42±0.02	7.39±0.03
P_{O_2}, mm Hg	96.5±4.5	98.6±7.8	96.1±8.5	97.1±6.4	97.4±2.7
P_{CO_2}, mm Hg	41.3±2.9	44.3±4.8	46.2±5.3	42.8±3.6	42.1±4.6
Heart rate, bpm	433±24	456±25	473±16	459±20	403±50
MABP, mm Hg	113.7±5.2	111.7±3.9	113.9±3.4	119.1±4.1	113.7±11.3
Ischemia					
pH, U	7.39±0.05	7.42±0.01	7.37±0.03	7.43±0.05	7.4±0.03
P_{O_2}, mm Hg	93.5±1.5	91.7±3.6	97.1±6.5	93.5±8.8	94.6±2.4
P_{CO_2}, mm Hg	40.0±5.6	42.3±2.4	45.1±3.5	40.5±6.0	38.9±4.3
Heart rate, bpm	432±18	470±7	466±14	436±25	481±13
MABP, mm Hg	126.3±4.1	130.9±4.2	129.7±4.3	122.0±5.6	132.3±3.4
Reperfusion (10 min)					
pH, U	7.39±0.06	7.40±0.02	7.46±0.04	7.43±0.05	7.42±0.01
P_{O_2}, mm Hg	98.5±8.5	102.5±3.5	102.5±3.5	93.5±8.8	91.5±0.4
P_{CO_2}, mm Hg	45.5±1.5	42.5±0.5	42.0±0.01	43.5±6.0	46.5±2.0
Heart rate, bpm	428±29	445±13	424±21	436±25	420±38
MABP, mm Hg	113.3±3.0	99.6±3.6	92.2±4.3	112.0±5.6	100.0±8.6
Reperfusion (24 hr)					
pH, U	7.45±0.06	7.47±0.04	7.51±0.02	7.48±0.07	7.49±0.07
P_{O_2}, mm Hg	115.0±9.2	112.0±2.6	106.0±0.71	115.3±2.3	126.0±9.0
P_{CO_2}, mm Hg	31±2.3	35.3±6.6	38.5±1.8	32.3±8.2	37.0±7.0
Heart rate, bpm	489±15	484±24	470±17	469±27	452±12
MABP, mm Hg	95.7±9.0	93.8±4.3	107.4±6.2	96.3±6.3	96.0±7.1

Seven rats each were treated with vehicle or resveratrol.

Values are mean ± SEM. Differences in physiological parameters among groups were not statistically significant ($p>0.05$).

MABP indicates mean arterial blood pressure.

Legends for Figures

Fig. 1

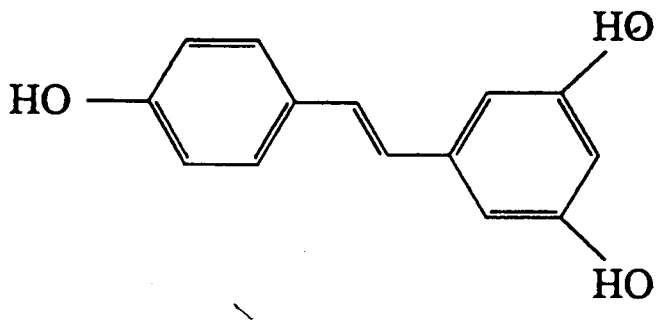
Chemical structure of resveratrol.

Fig. 2

Infarct volumes assessed after 1 hr MCA occlusion and 24 hr reperfusion by vital TTC staining. Rats were pretreated with vehicle or resveratrol at a dose of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg for 15 min before MCA occlusion. Each experimental group is seven rats. Results are expressed as mean ± SEM. *, $p<0.05$ versus control.

Fig. 3

Infarct volumes assessed after 1 hr MCA occlusion and 24 hr reperfusion by vital TTC staining: Rats were treated with vehicle or resveratrol at a dose of 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} g/kg when the common carotid arteries clips were removed. Each experimental group is seven rats. Results are expressed as mean ± SEM. *, $p<0.05$ versus control.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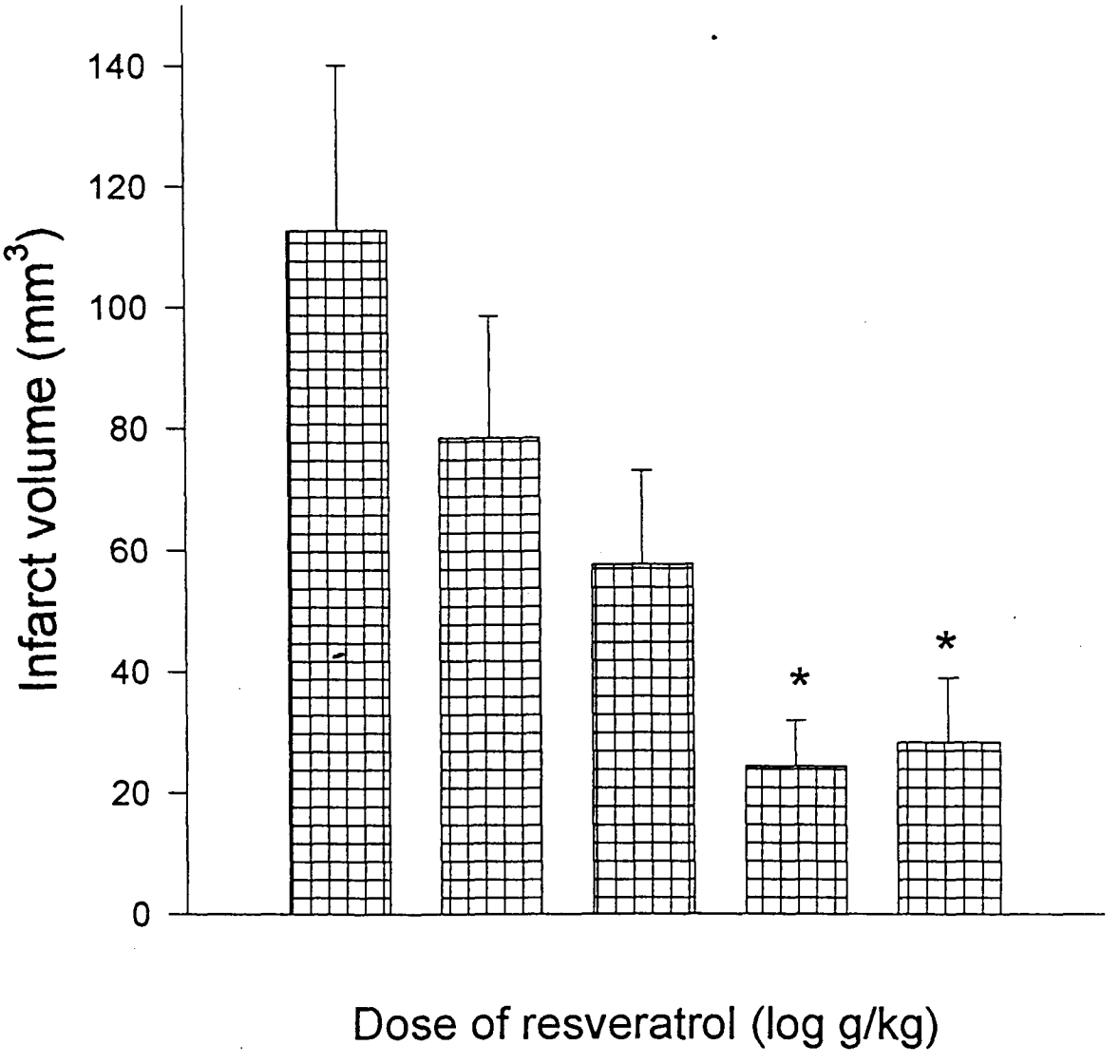
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Infarct volume (mm3)
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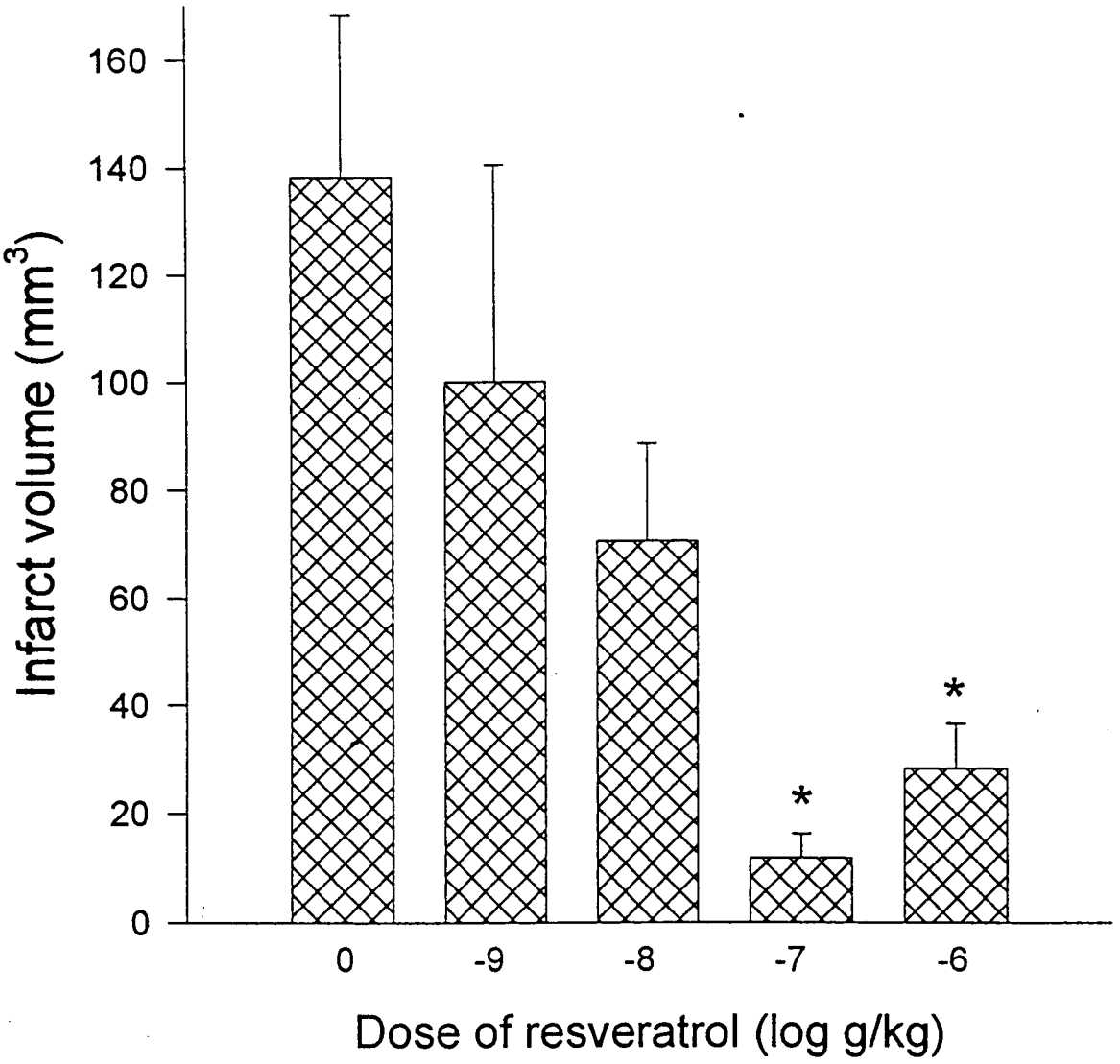
Dose of resveratrol (log g/kg)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140
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Infarct volume (mm3)
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0
-9
-8
-7
-6
Dose of resveratrol (log g/kg)