Effects of resveratrol on secondary damages after acute spinal cord injury in rats

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KEY WORDS resveratrol; spinal cord injuries; edema; lactate dehydrogenase; Na^+-K^+- exchanging ATPase; malondialdehyde; ultrastructure

ABSTRACT

AIM: To study the effects of resveratrol (Res) on secondary spinal cord edema , the activity of lactate dehydrogenase (LDH) , Na^+, K^+-ATPase, and malondialdehyde (MDA) content in experimental spinal cord injured (SCI) rats. **METHODS:** The weight-dropping method was used to produce the experimental SCI in adult rats. Res (50, 100 mg/kg) and methylprednisolone (MPSS) 100 mg/kg were injected ip immediately after the induction of SCI. The effects of Res on edema, LDH, Na^+, K^+-ATPase, and MDA were determined at 1 h, 24 h, and 48 h after SCI compared with MPSS. The electron microscope was employed to investigate the ultrastructural effects of Res on axons, neurons, and subcellular organelles after SCI. **RESULTS:** Res obviously inhibited the secondary spinal cord edema with the most remarkable suppressing rate by 11.5 % at 48 h. Res significantly suppressed the activities of the lactate dehydrogenase with the highest suppressing rate > 40 % at 24 h. Res markedly improved the Na^+, K^+-ATPase activities that were promoted to the biggest extent of 60 % at 48 h. At the same time, Res (50 and 100 mg/kg) obviously reduced MDA production in the injured spinal cord tissue in comparison with the SCI model, the most remarkable effect of Res was detected at 48 h with the inhibitory rate >40 %. The ultrastructural findings suggested that SCI caused profound spinal cord damage, which could be protected or improved by injection of Res and MPSS. **CONCLUSION:** Both Res and MPSS effectively protected the spinal cord from secondary spinal cord injures. But the effects of Res 50 and 100 mg/kg were stronger in improving the energy metabolism system and inhibiting the lipid peroxidation in the local injured spinal cord after SCI than MPSS at the dose of 100 mg/kg. Res might have greatly potent therapeutic effects on SCI.

INTRODUCTION

It has been known that spinal cord injury (SCI) is a disastrous trauma for which there is no effectively therapeutic methods and thus its prognosis is difficult to be measured for SCI to date[1]. The greatest harmfulness of SCI was severe nerve injuries that result from two mechanisms, primary trauma, and secondary damage. The primary trauma induced mechanical compression, bleeding, electrolyte disturbance, etc, which could lead to irreversible nerve injury. The secondary damage contained edema, hemorrhage, inflammatory reaction, lipid peroxdative reaction, energy metabolism system disorder, and ischemia, etc, which could give rise to reversible nerve injury. In the meantime, the secondary lesion also further produced cascade

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amplifying biological reaction so as to aggravate the nerve injury. However, the secondary damage could be regulated and also the key to treact SCI[2-4], though there was still no effectively reversing protocol in the clinic up to date.

Resveratrol (Res, 3,4',5-trihydroxystilbene) is a natural phenolic compound which is extensively present at high levels in various herbs, especially in the grape and *Polygonum cuspidatum* sieb et zucc[5,6]. Res reduces oxidation of low density lipid by chelating Cu^{2+} and phagocytizing oxygen free radical, inhibits platelet aggregation and prevents thrombus formation and atherosclerosis so as to a potential cardiovascular disease preventive and therapeutic agent[7,8]. Res produces anti-inflammatory and anti-anaphylatic effects by influencing arachidonic acid metabolism, possesses antibacterial, anti-cancer, and anti-mutation, and inhibits protein kinase activities, indicating that Res could affect the pathophysiological processes of inflammatory or traumatic reactions[5,9]. The present study was to investigate the effects of Res on the secondary spinal cord edema, the LDH activity, Na^+,K^+-ATPase activity, and lipid peroxidation after acute SCI in rats in order to look for potential therapeutic drugs and methods for SCI.

MATERIALS AND METHODS

Reagents Coenzyme I (Co I) was purchased from Sigma Chemical Co (St Louis, USA). Methylprednisolone (MPSS) was supplied by Nanfang Hospital, Guangzhou, China (Pharmacia & Uprohn, Belgium). Res was obtained with the purity over 99.5 % from Xi'an Aosaisi Bio-tech Engineering Co, China. Res was dissolved in a vehicle containing 2 % Me_2SO and 98 % saline. Supplies for LDH activity and MDA content assay and Na^+, K^+-ATPase kit for measurement of Na^+, K^+-ATPase were purchased from Nanjing Tiancheng Bioengineering Institute, Nanjing, China.

Animals Health Sprague-Dawley rats of either sex (weighing 180-220 g, 3-4 months old), were provided by the Experimental Animal Center of First Military Medical University (Grade II, Certificate No 2001A059, conferred by Guangdong Medical Laboratory Animal Administration Committee).

Animal model of SCI[10,11] Rats were anesthetized with ip injection of sodium phenobaribital (30 mg/kg), and allowed to breathe room air. Body temperature was maintained between 37-38 ºC with a heating pad during all procedures. Surgery was performed using sterile technique. With the aid of a dissecting microscope, a complete T6-T10 level laminectomy was performed in each rats, the spinal cord contusion damage was performed by dropping a rod weighing 10 g from a 25-mm height directly to the intact dura at T8 vertebral level. The wound was closed in the layers and the animal was allowed to recover from anesthesia and injected sc 10 mL of saline to compensate for poor oral water intake in the perioperative period. Surgeries were carried out each day between 8:00 and 13:00. Manual expression of bladders was performed twice daily. The animals were housed 4-5 in a cage with free access to tap water and foods in a 12-h light-dark cycle (lights on from 7:00 to 19:00). No prophylactic antibiotics were given to avoid possible drug interactions.

Experimental protocol The study included 15 groups of animals (n=6 rats per group), Sham operation group (Sham), SCI model-control group (SCI), injected Res 50 and 100 mg/kg or MPSS 100 mg/kg groups each at 1, 24, and 48 h immediately after spinal cord injury, respectively. Res and MPSS dissolved in 2 % Me_2SO were injected ip immediately after injury. Sham and SCI groups received vehicle of 2 % Me_2SO. In the Sham-operated groups, only a laminectomy was performed and the dura was exposed, but no lesion was produced. The spinal cord tissues (20 mm) were collected at 1, 24, and 48 h following lesion production from the reanesthetized rats and were washed by cooled saline. The sample was dried by filter paper and then dissected into 2 blocks at the center of lesion site. Each block of sample was about 8-10 mm in length. One block of the sample was stored at -70 ºC for water content measurement, and the other was homogenized in a cold saline solution for measurement of LDH activity, Na^+, K^+-ATPase activity, and MDA content. The protein content was measured by the method of Bradford.

Measurement of spinal cord water content The sample was weighed in a humidity chamber and the wet weight (W) was obtained immediately. Then the sample was put into the electric constant temperature oven at (110±1) ºC for 48 h to constant weight for determination of dry weight (D). Ellicot formula was used to calculate the sample's percent water content to reflect the spinal tissue edema: H_2O %=[(W-D)/W]× 100 %.

Measurement of LDH activity The LDH activity was measured by colorimetric method, because LDH

could catalysis lactic acid to form acetonic acid which was able to combine 2,4-dinitrophenylhydrazine to produce acetonic acid-clinitrophenylhydrazone that was shown brownish red in the basic solution. Briefly, 20 μL of 2 % sample homogenate was added into 250 μL of PBS buffer (pH 7.4), and then, mixed with 50 μL CoI, and incubated at 37 °C for 15 min. After 250 μL of 2,4-dinitrophenylhydrazine was added into above reaction system, mixed and incubated at 37 °C for 15 min again, 2.5 mL of sodium hydroxide (0.4 mol/L) was added misce bene at room temperature for 3 min. Absorbance (*A*) value was measured at 440 nm with the spectrophotometer (Shanghai Analysis Equippment Factory). Measurement control tube was performed by replacing CoI with distilled water. Standard tube was acetonic acid (2 mmol/L) instead of sample, standard control tube was distilled water. The total volume of reaction system was 3.07 mL. The LDH activity was calculated by the following equation:

LDH activity (U/g protein)=[(Test tube−Test control tube)/(Standard tube−Standard control tube)]× concentration of standard tube (2 mmol/L)÷protein content (10^3×g protein/L)

Measurement of Na^+, K^+-ATPase activity Na^+, K^+-ATPase activity was measured by detecting inorganic phosphorus content because Na^+, K^+-ATPase could decomposite ATP to ADP and inorganic phosphorus. The measurement was performed strictly according to the directions in kit. The unit of Na^+, K^+-ATPase was represented by inorganic phosphorus millimole content in decomposited per gram tissue protein in 1 h, which was mmol Pi· g^{-1} (protein)· h^{-1}.

Measurement of MDA content MDA assay was performed by the thiobarbituric acid method[12].

Ultrastructural experiment of the injured spinal cord Another 3 rats were arranged for each group at each time point for the electron microscopic experiment. The experimental protocol was the same as above. The samples of injured spinal cord were obtained at 1 h, 24 h, and 48 h after trauma at the 3-mm length for ultrastructural examination with an electron microscope (JEM 1200EX, Jeol, Tokyo, Japan).

Statistics Data were expressed as mean±SD. Statistical analysis was carried out by SPSS 10.0 software through Post Hoc multiple comparisons of one-way analysis of variance (ANOVA). Suppressing rate (SR) or increasing rate (IR) was calculated by the following formula: SR or IR=[(administration group−SCI group)/SCI group]×100 %

RESULTS

Effect of Res on tissue edema After being injured, spinal tissue water content of SCI group at each time point most significantly differed from that of Sham group ($P<0.05$, or $P<0.01$), indicating that spinal tissue suffered from a serious tissue edema. At 1 h after SCI, Res (50 and 100 mg/kg) and MPSS had no significant influence on tissue water content ($P>0.05$). However, Res 100 mg/kg significantly reduced spinal cord edema at 24 h and at 48 h. In the meantime, MPSS still had no remarkable effect. These results demonstrated that Res 100 mg/kg could markedly inhibit tissue edema after SCI and the greatest suppressing rate detected at 48 h was above 11.47 % (Tab 1).

Effect of Res on LDH activity Post-traumatic LDH activity in the injured cord was significantly increased at each time point compared with Sham ($P<0.05$ or 0.01), suggesting that SCI could seriously destroy the normal LDH state. LDH activity in Res 50 and 100 mg/kg or MPSS group was reduced at 1 h and 24 h after trauma than SCI group ($P<0.05$ and 0.01, respectively). At 48 h after SCI, however, only Res100 mg/kg could significantly affect LDH activity compared with SCI ($P<0.01$). The greatest suppressing rate was 43.5 % at 24 h. The results showed that Res effectively improved the destroyed LDH state after SCI (Tab 2).

Effect of Res on Na^+, K^+-ATPase activity Post-traumatic Na^+, K^+-ATPase activity alteration was a time-lag process. The statistical differences were shown at 24 h ($P<0.05$) and at 48 h ($P<0.01$) between SCI and Sham group. At 1 h after SCI, Res and MPSS had no significantly influence on Na^+, K^+-ATPase ($P>0.05$). At 24 h, Res (100 mg/kg) and MPSS could markedly affect Na^+, K^+-ATPase activity ($P<0.05$), and at 48 h, Res 50 mg/kg and 100 mg/kg also significantly improved Na^+, K^+-ATPase activity ($P<0.05$ and 0.01, respectively). The highest increasing rate was 63.16 % in Res 100 mg/kg at 48 h, much greater than that of MPSS group (Tab 3).

Effect of Res on cord MDA content After trauma, the lipid peroxidation in the injured spinal cord was very strong. MDA content of SCI group was significantly greater than that of Sham group at each time point ($P<0.01$). Compared with SCI group, Res had no significant effect on cord MDA content ($P>0.05$) at 1 h but significantly reduced cord MDA content at 24 h and 48 h ($P<0.05$ or 0.01). Otherwise, MPSS could

Tab 1. Effect of resveratrol on the H_2O content in the injured spinal cord in rats. *n*=6 rats in each group. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* Sham group. $^eP<0.05$ *vs* SCI group.

Group	Dose /mg· kg^{-1}	1 h H_2O content/%	1 h SR/%	24 h H_2O content/%	24 h SR/%	48 h H_2O content/%	48 h SR/%
Sham	-	66±4	-	66±5	-	65±3	-
SCI	-	75.2±2.6^c	-	72.4±0.3^c	-	73.0±2.0^b	-
MPSS	100	71.7±2.0	4.68	68.7±0.6	5.15	67±7	8.70
Res	50	73.1±1.5	2.84	70.8±1.2	2.25	69±3^e	5.78
	100	72.55±0.24	3.55	67±4^e	7.07	65±6^e	11.47

SR: suppressing rate.

Tab 2. Effect of Res on LDH activities in the injured spinal cord in rats. *n*=6 rats in each group. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* Sham group. $^eP<0.05$, $^fP<0.01$ *vs* SCI group.

Group	Dose /mg· kg^{-1}	1 h LDH activities /U· g^{-1} (protein)	1 h SR/%	24 h LDH activities /U· g^{-1} (protein)	24 h SR/%	48 h LDH activities /U· g^{-1} (protein)	48 h SR/%
Sham	-	1775±358	-	1883±524	-	2001±244	-
SCI	-	2602±405^c	-	3316±1319^b	-	2580±324^b	-
MPSS	100	1910±325^e	26.6	1860±914^f	43.9	2399±339	7.0
Res	50	1882±54^e	27.7	1841±679^f	44.5	2198±368	14.8
	100	1952±695^e	25.0	1873±895^f	43.5	1873±231^f	27.4

Tab 3. Effect of Res on Na^+, K^+-ATPase activities in the injured spinal cord in rats. *n*=6 rats in each group. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* Sham group. $^eP<0.05$, $^fP<0.01$ *vs* SCI group.

Group	Dose /mg· kg^{-1}	1 h Na^+,K^+-ATPase activities/mmolPi· g^{-1} (protein)· h^{-1}	1 h IR/%	24 h Na^+,K^+-ATPase activities/mmolPi· g^{-1} (protein)· h^{-1}	24 h IR/%	48 h Na^+,K^+-ATPase activities/mmolPi· g^{-1} (protein)· h^{-1}	48 h IR/%
Sham	-	2.27±0.16	-	2.3±0.6	-	3.0±0.3	-
SCI	-	1.6±0.5	-	1.6±0.6^b	-	1.71±0.21^c	-
MPSS	100	1.9±0.7	19.38	2.18±0.21^e	33.74	1.9±0.4	11.70
Res	50	1.86±0.22	16.25	1.79±0.22	9.82	2.1±0.6^e	24.56
	100	2.1±0.8	37.75	2.2±0.3^e	27.61	2.8±0.6^f	63.16

IR: increasing rate.

inhibit MDA formation only at 24 h (P<0.01). Effect of Res gradually increased to the greatest level at 48 h by the suppressing rate of 52.12 % (Tab 4).

Effect of Res on ultrastructure of the injured spinal cord All rats in sham group showed normal ultrastructure with intact axons, neurons, and subcellular organelles such as mitochondrion and nucleus (Fig 1 A and B). In SCI group, the similar ultrastructural morphological abnormities were seen in all rats at 1 h, 24 h, and 48 h after SCI (Fig 1C and D). The abnormities included heavier cytoplasmic edema, empty appearance of the axons, lysosis and thin of myelin sheath,

Tab 4. Effect of Res on MDA content in the injured spinal cord in rats. *n*=6 rats in each group. Mean±SD. [c]*P*<0.01 *vs* Sham group. [f]*P*<0.01 *vs* SCI group.

Group	Dose /mg· kg^{-1}	1 h MDA/mmol· g^{-1} (protein)	1 h SR/%	24 h MDA/mmol· g^{-1}(protein)	24 h SR/%	48 h MDA/mmol· g^{-1}(protein)	48 h SR/%
Sham	-	4.0±1.0	-	3.7±0.8	-	3.1±0.6	-
SCI	-	6.8±0.4[c]	-	6.6±1.1[c]	-	7.1±1.8[c]	-
MPSS	100	5.4±1.9	18.56	4.4±1.0[f]	33.28	6.1±1.4	14.16
Res	50	5.3±0.9	22.68	5.0±0.4[f]	25.11	4.5±0.8[f]	35.98
	100	4.8±0.6	29.46	5.0±0.4[f]	25.11	3.4±0.8[f]	52.12

spare neurofilaments and vesicles and large vacuoles, nucleus membrane defect, loss of intracytoplasmic organelles such as edematous and empty mitochodrias, which were not seen in Sham group (Fig 1C and D). In MPSS-treated group, the much more slight abnormal changes at post-trauma 1 h, 24 h, and 48 h were observed compared with SCI group. All samples of MPSS-treated group showed completely ultrastructure of axons, neurofilaments, myelin sheath, mitochodria and nucleus, but still had relatively mild edema and very fewer edematous mitochodria and damaged myelin sheath (Fig 1 E and F). Res-treated, like MPSS-treated group, soundly maintained the ultrastructure of the injured spinal cord in the relatively good appearance (Fig 1G and H).

DISCUSSION

In this study, we reported for the first time that Res, like MPSS, strongly affects the secondary pathophysiological reaction through investigating the effects of Res on the cord edema, energy metabolism system such as LDH activity and Na^+, K^+-ATPase activity, and lipid peroxidation. The experimental results demonstrated that after SCI, there were many traumatic changes occurring in the injured cord tissue, such as edema, increase of LDH activity, inhibition of Na^+, K^+-ATPase activity, and activation of lipid peroxidation, which were obviously related to the extent of SCI. However, Res could protect the spinal cord from these secondary damages.

It had been known that after SCI, local microcirculation was seriously disturbed and volume of blood flow was gradually reduced, which led to a disturbance of energetic metabolism in spinal cord tissue and serious edema[11,13]. Spinal cord edema happened at the center of the cord firstly and then spread to the peripheral cord tissue to affect white matter, in which the most disastrous timing window was within 24 h after SCI, and SCI could be greatly deteriorated by the tissue edema suppressing and abnormality of local electrolytic equilibrium[14-16], which was consistent with the present results. In the present study, the effect of Res on edema formation was also assessed. The result of reduction of edema in the injured cord suggests that Res is an effective therapeutic agent for vasogenic edema in experimental SCI.

At the same time, we assessed the effect of Res and MPSS on energy metabolism system disturbance through examining changes in LDH and Na^+, K^+-ATPase activities. Local energy metabolism reduced linearly and then sustained for a long time[17]. Energy metabolism change was shown by gradual depletion of high-energy phosphate and changes of lactic acid metabolism[18,19]. Because LDH and Na^+, K^+-ATPase were the important enzymes of energy metabolism, which occurred in various other body cells, their activities were the most important indices for energetic metabolism. Thus, investigating their changes and relationship with the cord edema and lipid peroxidation indicated by MDA content after SCI was very beneficial for drug research, which could be employed as markers of drug actions and effects for the therapeutic drug research of SCI. Levels of LDH activity and Na^+, K^+-ATPase activity could act as the marks of energy metabolism changes in the injured tissues and their quantity and quality could directly affect body's energy metabolism. When tissues and organs were injured, LDH activity abnormally increased and Na^+, K^+-ATPase activity was greatly inhibited[20,21]. The present experimental results suggested

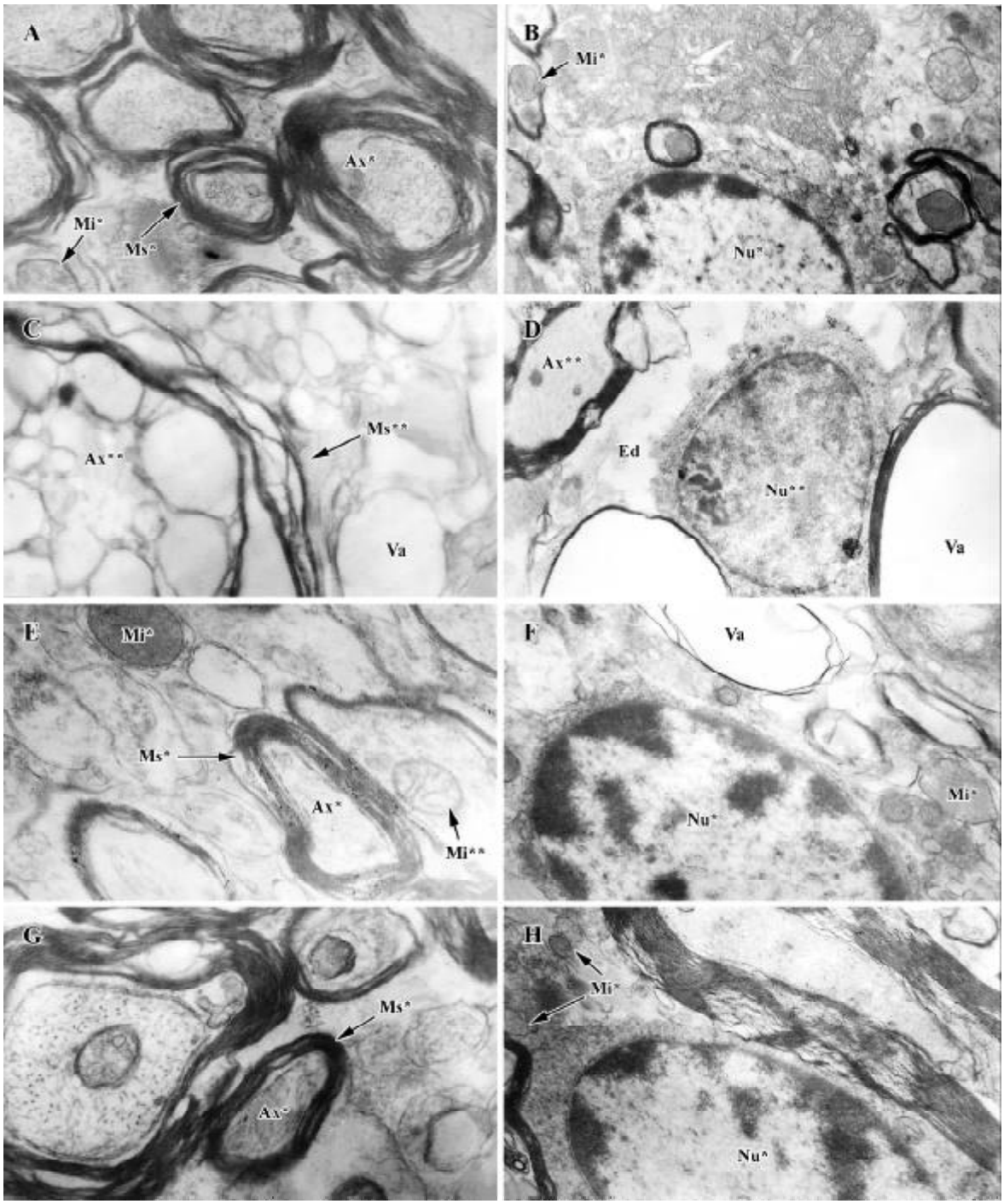

Fig 1. Electron microscopy shows ultrastructural changes in the local injured spinal cord after 48 h of SCI. A and B: Sham group. C and D: SCI group. E and F: MPSS-treated group. G and H: Res-treated group. *: normal; **: abnormal; Ax: axon; Ms: myelin sheath; Mi: mitochondria; Nu: nucleus; Ed: edema; Va: vacuoles. (×8000) .

that Res at the dose of 100 mg/kg could effectively increase Na^+,K^+-ATPase activity and reduce LDH activity in the local injured tissue, whereas MPSS had no obvious effect at the same dose.

Previous data provided by Sun and his colleagues also showed that tissue injury and cell death were a free radical-mediated process with loss of cell viability and increased LDH activity, and Res had a protective effect against LDH increasing and lipid peroxidation[22]. Hung *et al* also reported that a Res analogue, astringinin, strongly prevented myocardial ischemia and infarction through increasing nitric oxide (NO) and decreasing

LDH levels in the carotid blood[23]. Therefore, Res may play an important role in the observed improvement of LDH activity after tissue trauma, and benefit for injured nerve after SCI.

On the other hand, free radical generation has been considered as a major cause of the secondary spinal cord injury in a variety of abnormalities after SCI. The present study demonstrated that MDA content in the injured spinal cord was increased markedly, which was similar to other related reports. Recently, it was indicated that one of the plausible ways to prevent the free radical-mediated tissue injury was pharmaceutical augmentation of endogenous antioxidant defense capacity. Res has been recently proposed as a potential antioxidant that could obviously inhibit free radical generation in red cell membrane, heart, liver, brain, kidney and so on, *in vitro*, which reflected beneficial effects to prevent functional injury and improve nerve function and promote restoration after trauma. Sun *et al* also reported that Res protected the brain from neuronal damage due to chronic ethanol administration and suggested that Res might be used as a therapeutic agent to ameliorate neurodegenerative processes[24]. In addition, the experiments *in vitro* also demonstrated that Res could effectively protect DNA from oxidative damages, so as to assure cell proliferation, differentiation, and function to be normal[25]. Ray *et al* found that Res possessed cardioprotective effects through its peroxyl radical scavenging activity and inhibiting lipid peroxidation to reduce MDA content[26,27]. The result indicated that Res possessed beneficially neuroprotective effects after SCI.

From the ultrastuctural investigation, it has been suggested that both Res and MPSS have neuroprotective effects, including protecting axon, neuron, myelin, and subcellular organelles such as nucleus and mitochondrion, and reducing local spinal tissue edema, which were in accordance with above results. Compared with MPSS, however, Res exerts greatly more remarkable neuroprotection in the aspects of reducing edema, and improving Na^+, K^+-ATPase activities and anti-lipid-peroxidative action. The statistical differences occurring in the MPSS group were low and almost all at the time point of 24 h, fewer at 1 h and 48 h, whereas Res had statistical different values, almost at all time points on all results that the greatest values were near at 48 h. The main reason is that the neuroprotective effect of MPSS was mediated through the direct membrane action, completely unlike Res, and had nothing to do with glucocorticoid receptor-mediated activity. The reason why MPSS has been the best drug to cure SCI in clinic at present is related to its extensively pharmacological actions, especially anti-inflammation, stabilization of lysosomal membranes, enhancement of spinal cord blood flow, and suppression of vasogenic edema etc[13,28,29].

In summary, it seems reasonable to conclude that after acute SCI, Res effectively protected spinal cord from the secondary damages through inhibiting edema, improving energy metabolism system, and suppressing lipid peroxidation to prevent nerve functions to be exacerbated progressively, which is most important for restoring nerve function, preventing mitigating nerve damage, and maybe promoting nerve regeneration. The therapeutic values and mechanisms of Res on SCI should be studied further.

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KEY WORDS resveratrol; spinal cord injuries; edema; lactate dehydrogenase; Na^+-K^+- exchanging ATPase; malondialdehyde; ultrastructure

ABSTRACT

AIM: To study the effects of resveratrol (Res) on secondary spinal cord edema , the activity of lactate dehydrogenase (LDH) , Na^+, K^+-ATPase, and malondialdehyde (MDA) content in experimental spinal cord injured (SCI) rats. **METHODS:** The weight-dropping method was used to produce the experimental SCI in adult rats. Res (50, 100 mg/kg) and methylprednisolone (MPSS) 100 mg/kg were injected ip immediately after the induction of SCI. The effects of Res on edema, LDH, Na^+, K^+-ATPase, and MDA were determined at 1 h, 24 h, and 48 h after SCI compared with MPSS. The electron microscope was employed to investigate the ultrastructural effects of Res on axons, neurons, and subcellular organelles after SCI. **RESULTS:** Res obviously inhibited the secondary spinal cord edema with the most remarkable suppressing rate by 11.5 % at 48 h. Res significantly suppressed the activities of the lactate dehydrogenase with the highest suppressing rate > 40 % at 24 h. Res markedly improved the Na^+, K^+-ATPase activities that were promoted to the biggest extent of 60 % at 48 h. At the same time, Res (50 and 100 mg/kg) obviously reduced MDA production in the injured spinal cord tissue in comparison with the SCI model, the most remarkable effect of Res was detected at 48 h with the inhibitory rate >40 %. The ultrastructural findings suggested that SCI caused profound spinal cord damage, which could be protected or improved by injection of Res and MPSS. **CONCLUSION:** Both Res and MPSS effectively protected the spinal cord from secondary spinal cord injures. But the effects of Res 50 and 100 mg/kg were stronger in improving the energy metabolism system and inhibiting the lipid peroxidation in the local injured spinal cord after SCI than MPSS at the dose of 100 mg/kg. Res might have greatly potent therapeutic effects on SCI.

INTRODUCTION

It has been known that spinal cord injury (SCI) is a disastrous trauma for which there is no effectively therapeutic methods and thus its prognosis is difficult to be measured for SCI to date[1]. The greatest harmfulness of SCI was severe nerve injuries that result from two mechanisms, primary trauma, and secondary damage. The primary trauma induced mechanical compression, bleeding, electrolyte disturbance, etc, which could lead to irreversible nerve injury. The secondary damage contained edema, hemorrhage, inflammatory reaction, lipid peroxdative reaction, energy metabolism system disorder, and ischemia, etc, which could give rise to reversible nerve injury. In the meantime, the secondary lesion also further produced cascade

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amplifying biological reaction so as to aggravate the nerve injury. However, the secondary damage could be regulated and also the key to treact SCI[2-4], though there was still no effectively reversing protocol in the clinic up to date.

Resveratrol (Res, 3,4',5-trihydroxystilbene) is a natural phenolic compound which is extensively present at high levels in various herbs, especially in the grape and *Polygonum cuspidatum* sieb et zucc[5,6]. Res reduces oxidation of low density lipid by chelating Cu^{2+} and phagocytizing oxygen free radical, inhibits platelet aggregation and prevents thrombus formation and atherosclerosis so as to a potential cardiovascular disease preventive and therapeutic agent[7,8]. Res produces anti-inflammatory and anti-anaphylatic effects by influencing arachidonic acid metabolism, possesses antibacterial, anti-cancer, and anti-mutation, and inhibits protein kinase activities, indicating that Res could affect the pathophysiological processes of inflammatory or traumatic reactions[5,9]. The present study was to investigate the effects of Res on the secondary spinal cord edema, the LDH activity, Na^+,K^+-ATPase activity, and lipid peroxidation after acute SCI in rats in order to look for potential therapeutic drugs and methods for SCI.

MATERIALS AND METHODS

Reagents Coenzyme I (Co I) was purchased from Sigma Chemical Co (St Louis, USA). Methylprednisolone (MPSS) was supplied by Nanfang Hospital, Guangzhou, China (Pharmacia & Uprohn, Belgium). Res was obtained with the purity over 99.5 % from Xi'an Aosaisi Bio-tech Engineering Co, China. Res was dissolved in a vehicle containing 2 % Me_2SO and 98 % saline. Supplies for LDH activity and MDA content assay and Na^+, K^+-ATPase kit for measurement of Na^+, K^+-ATPase were purchased from Nanjing Tiancheng Bioengineering Institute, Nanjing, China.

Animals Health Sprague-Dawley rats of either sex (weighing 180-220 g, 3-4 months old), were provided by the Experimental Animal Center of First Military Medical University (Grade II, Certificate No 2001A059, conferred by Guangdong Medical Laboratory Animal Administration Committee).

Animal model of SCI[10,11] Rats were anesthetized with ip injection of sodium phenobaribital (30 mg/kg), and allowed to breathe room air. Body temperature was maintained between 37-38 ºC with a heating pad during all procedures. Surgery was performed using sterile technique. With the aid of a dissecting microscope, a complete T6-T10 level laminectomy was performed in each rats, the spinal cord contusion damage was performed by dropping a rod weighing 10 g from a 25-mm height directly to the intact dura at T8 vertebral level. The wound was closed in the layers and the animal was allowed to recover from anesthesia and injected sc 10 mL of saline to compensate for poor oral water intake in the perioperative period. Surgeries were carried out each day between 8:00 and 13:00. Manual expression of bladders was performed twice daily. The animals were housed 4-5 in a cage with free access to tap water and foods in a 12-h light-dark cycle (lights on from 7:00 to 19:00). No prophylactic antibiotics were given to avoid possible drug interactions.

Experimental protocol The study included 15 groups of animals (n=6 rats per group), Sham operation group (Sham), SCI model-control group (SCI), injected Res 50 and 100 mg/kg or MPSS 100 mg/kg groups each at 1, 24, and 48 h immediately after spinal cord injury, respectively. Res and MPSS dissolved in 2 % Me_2SO were injected ip immediately after injury. Sham and SCI groups received vehicle of 2 % Me_2SO. In the Sham-operated groups, only a laminectomy was performed and the dura was exposed, but no lesion was produced. The spinal cord tissues (20 mm) were collected at 1, 24, and 48 h following lesion production from the reanesthetized rats and were washed by cooled saline. The sample was dried by filter paper and then dissected into 2 blocks at the center of lesion site. Each block of sample was about 8-10 mm in length. One block of the sample was stored at -70 ºC for water content measurement, and the other was homogenized in a cold saline solution for measurement of LDH activity, Na^+, K^+-ATPase activity, and MDA content. The protein content was measured by the method of Bradford.

Measurement of spinal cord water content The sample was weighed in a humidity chamber and the wet weight (W) was obtained immediately. Then the sample was put into the electric constant temperature oven at (110±1) ºC for 48 h to constant weight for determination of dry weight (D). Ellicot formula was used to calculate the sample's percent water content to reflect the spinal tissue edema: H_2O %=[(W-D)/W]× 100 %.

Measurement of LDH activity The LDH activity was measured by colorimetric method, because LDH

could catalysis lactic acid to form acetonic acid which was able to combine 2,4-dinitrophenylhydrazine to produce acetonic acid-clinitrophenylhydrazone that was shown brownish red in the basic solution. Briefly, 20 μL of 2 % sample homogenate was added into 250 μL of PBS buffer (pH 7.4), and then, mixed with 50 μL CoI, and incubated at 37 °C for 15 min. After 250 μL of 2,4-dinitrophenylhydrazine was added into above reaction system, mixed and incubated at 37 °C for 15 min again, 2.5 mL of sodium hydroxide (0.4 mol/L) was added misce bene at room temperature for 3 min. Absorbance (*A*) value was measured at 440 nm with the spectrophotometer (Shanghai Analysis Equippment Factory). Measurement control tube was performed by replacing CoI with distilled water. Standard tube was acetonic acid (2 mmol/L) instead of sample, standard control tube was distilled water. The total volume of reaction system was 3.07 mL. The LDH activity was calculated by the following equation:

LDH activity (U/g protein)=[(Test tube−Test control tube)/(Standard tube−Standard control tube)]× concentration of standard tube (2 mmol/L)÷protein content (10^3×g protein/L)

Measurement of Na^+, K^+-ATPase activity Na^+, K^+-ATPase activity was measured by detecting inorganic phosphorus content because Na^+, K^+-ATPase could decomposite ATP to ADP and inorganic phosphorus. The measurement was performed strictly according to the directions in kit. The unit of Na^+, K^+-ATPase was represented by inorganic phosphorus millimole content in decomposited per gram tissue protein in 1 h, which was mmol Pi· g^{-1} (protein)· h^{-1}.

Measurement of MDA content MDA assay was performed by the thiobarbituric acid method[12].

Ultrastructural experiment of the injured spinal cord Another 3 rats were arranged for each group at each time point for the electron microscopic experiment. The experimental protocol was the same as above. The samples of injured spinal cord were obtained at 1 h, 24 h, and 48 h after trauma at the 3-mm length for ultrastructural examination with an electron microscope (JEM 1200EX, Jeol, Tokyo, Japan).

Statistics Data were expressed as mean±SD. Statistical analysis was carried out by SPSS 10.0 software through Post Hoc multiple comparisons of one-way analysis of variance (ANOVA). Suppressing rate (SR) or increasing rate (IR) was calculated by the following formula: SR or IR=[(administration group−SCI group)/SCI group]×100 %

RESULTS

Effect of Res on tissue edema After being injured, spinal tissue water content of SCI group at each time point most significantly differed from that of Sham group ($P<0.05$, or $P<0.01$), indicating that spinal tissue suffered from a serious tissue edema. At 1 h after SCI, Res (50 and 100 mg/kg) and MPSS had no significant influence on tissue water content ($P>0.05$). However, Res 100 mg/kg significantly reduced spinal cord edema at 24 h and at 48 h. In the meantime, MPSS still had no remarkable effect. These results demonstrated that Res 100 mg/kg could markedly inhibit tissue edema after SCI and the greatest suppressing rate detected at 48 h was above 11.47 % (Tab 1).

Effect of Res on LDH activity Post-traumatic LDH activity in the injured cord was significantly increased at each time point compared with Sham ($P<0.05$ or 0.01), suggesting that SCI could seriously destroy the normal LDH state. LDH activity in Res 50 and 100 mg/kg or MPSS group was reduced at 1 h and 24 h after trauma than SCI group ($P<0.05$ and 0.01, respectively). At 48 h after SCI, however, only Res100 mg/kg could significantly affect LDH activity compared with SCI ($P<0.01$). The greatest suppressing rate was 43.5 % at 24 h. The results showed that Res effectively improved the destroyed LDH state after SCI (Tab 2).

Effect of Res on Na^+, K^+-ATPase activity Post-traumatic Na^+, K^+-ATPase activity alteration was a time-lag process. The statistical differences were shown at 24 h ($P<0.05$) and at 48 h ($P<0.01$) between SCI and Sham group. At 1 h after SCI, Res and MPSS had no significantly influence on Na^+, K^+-ATPase ($P>0.05$). At 24 h, Res (100 mg/kg) and MPSS could markedly affect Na^+, K^+-ATPase activity ($P<0.05$), and at 48 h, Res 50 mg/kg and 100 mg/kg also significantly improved Na^+, K^+-ATPase activity ($P<0.05$ and 0.01, respectively). The highest increasing rate was 63.16 % in Res 100 mg/kg at 48 h, much greater than that of MPSS group (Tab 3).

Effect of Res on cord MDA content After trauma, the lipid peroxidation in the injured spinal cord was very strong. MDA content of SCI group was significantly greater than that of Sham group at each time point ($P<0.01$). Compared with SCI group, Res had no significant effect on cord MDA content ($P>0.05$) at 1 h but significantly reduced cord MDA content at 24 h and 48 h ($P<0.05$ or 0.01). Otherwise, MPSS could

Tab 1. Effect of resveratrol on the H_2O content in the injured spinal cord in rats. *n*=6 rats in each group. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* Sham group. $^eP<0.05$ *vs* SCI group.

Group	Dose /mg· kg^{-1}	1 h H_2O content/%	1 h SR/%	24 h H_2O content/%	24 h SR/%	48 h H_2O content/%	48 h SR/%
Sham	-	66±4	-	66±5	-	65±3	-
SCI	-	75.2±2.6^c	-	72.4±0.3^c	-	73.0±2.0^b	-
MPSS	100	71.7±2.0	4.68	68.7±0.6	5.15	67±7	8.70
Res	50	73.1±1.5	2.84	70.8±1.2	2.25	69±3^e	5.78
	100	72.55±0.24	3.55	67±4^e	7.07	65±6^e	11.47

SR: suppressing rate.

Tab 2. Effect of Res on LDH activities in the injured spinal cord in rats. *n*=6 rats in each group. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* Sham group. $^eP<0.05$, $^fP<0.01$ *vs* SCI group.

Group	Dose /mg· kg^{-1}	1 h LDH activities /U· g^{-1} (protein)	1 h SR/%	24 h LDH activities /U· g^{-1} (protein)	24 h SR/%	48 h LDH activities /U· g^{-1} (protein)	48 h SR/%
Sham	-	1775±358	-	1883±524	-	2001±244	-
SCI	-	2602±405^c	-	3316±1319^b	-	2580±324^b	-
MPSS	100	1910±325^e	26.6	1860±914^f	43.9	2399±339	7.0
Res	50	1882±54^e	27.7	1841±679^f	44.5	2198±368	14.8
	100	1952±695^e	25.0	1873±895^f	43.5	1873±231^f	27.4

Tab 3. Effect of Res on Na^+, K^+-ATPase activities in the injured spinal cord in rats. *n*=6 rats in each group. Mean±SD. $^bP<0.05$, $^cP<0.01$ *vs* Sham group. $^eP<0.05$, $^fP<0.01$ *vs* SCI group.

Group	Dose /mg· kg^{-1}	1 h Na^+,K^+-ATPase activities/mmolPi· g^{-1} (protein)· h^{-1}	1 h IR/%	24 h Na^+,K^+-ATPase activities/mmolPi· g^{-1} (protein)· h^{-1}	24 h IR/%	48 h Na^+,K^+-ATPase activities/mmolPi· g^{-1} (protein)· h^{-1}	48 h IR/%
Sham	-	2.27±0.16	-	2.3±0.6	-	3.0±0.3	-
SCI	-	1.6±0.5	-	1.6±0.6^b	-	1.71±0.21^c	-
MPSS	100	1.9±0.7	19.38	2.18±0.21^e	33.74	1.9±0.4	11.70
Res	50	1.86±0.22	16.25	1.79±0.22	9.82	2.1±0.6^e	24.56
	100	2.1±0.8	37.75	2.2±0.3^e	27.61	2.8±0.6^f	63.16

IR: increasing rate.

inhibit MDA formation only at 24 h (P<0.01). Effect of Res gradually increased to the greatest level at 48 h by the suppressing rate of 52.12 % (Tab 4).

Effect of Res on ultrastructure of the injured spinal cord All rats in sham group showed normal ultrastructure with intact axons, neurons, and subcellular organelles such as mitochondrion and nucleus (Fig 1 A and B). In SCI group, the similar ultrastructural morphological abnormities were seen in all rats at 1 h, 24 h, and 48 h after SCI (Fig 1C and D). The abnormities included heavier cytoplasmic edema, empty appearance of the axons, lysosis and thin of myelin sheath,

Tab 4. Effect of Res on MDA content in the injured spinal cord in rats. *n*=6 rats in each group. Mean±SD. [c]*P*<0.01 *vs* Sham group. [f]*P*<0.01 *vs* SCI group.

Group	Dose /mg· kg^{-1}	1 h MDA/mmol· g^{-1} (protein)	1 h SR/%	24 h MDA/mmol· g^{-1}(protein)	24 h SR/%	48 h MDA/mmol· g^{-1}(protein)	48 h SR/%
Sham	-	4.0±1.0	-	3.7±0.8	-	3.1±0.6	-
SCI	-	6.8±0.4[c]	-	6.6±1.1[c]	-	7.1±1.8[c]	-
MPSS	100	5.4±1.9	18.56	4.4±1.0[f]	33.28	6.1±1.4	14.16
Res	50	5.3±0.9	22.68	5.0±0.4[f]	25.11	4.5±0.8[f]	35.98
	100	4.8±0.6	29.46	5.0±0.4[f]	25.11	3.4±0.8[f]	52.12

spare neurofilaments and vesicles and large vacuoles, nucleus membrane defect, loss of intracytoplasmic organelles such as edematous and empty mitochodrias, which were not seen in Sham group (Fig 1C and D). In MPSS-treated group, the much more slight abnormal changes at post-trauma 1 h, 24 h, and 48 h were observed compared with SCI group. All samples of MPSS-treated group showed completely ultrastructure of axons, neurofilaments, myelin sheath, mitochodria and nucleus, but still had relatively mild edema and very fewer edematous mitochodria and damaged myelin sheath (Fig 1 E and F). Res-treated, like MPSS-treated group, soundly maintained the ultrastructure of the injured spinal cord in the relatively good appearance (Fig 1G and H).

DISCUSSION

In this study, we reported for the first time that Res, like MPSS, strongly affects the secondary pathophysiological reaction through investigating the effects of Res on the cord edema, energy metabolism system such as LDH activity and Na^+, K^+-ATPase activity, and lipid peroxidation. The experimental results demonstrated that after SCI, there were many traumatic changes occurring in the injured cord tissue, such as edema, increase of LDH activity, inhibition of Na^+, K^+-ATPase activity, and activation of lipid peroxidation, which were obviously related to the extent of SCI. However, Res could protect the spinal cord from these secondary damages.

It had been known that after SCI, local microcirculation was seriously disturbed and volume of blood flow was gradually reduced, which led to a disturbance of energetic metabolism in spinal cord tissue and serious edema[11,13]. Spinal cord edema happened at the center of the cord firstly and then spread to the peripheral cord tissue to affect white matter, in which the most disastrous timing window was within 24 h after SCI, and SCI could be greatly deteriorated by the tissue edema suppressing and abnormality of local electrolytic equilibrium[14-16], which was consistent with the present results. In the present study, the effect of Res on edema formation was also assessed. The result of reduction of edema in the injured cord suggests that Res is an effective therapeutic agent for vasogenic edema in experimental SCI.

At the same time, we assessed the effect of Res and MPSS on energy metabolism system disturbance through examining changes in LDH and Na^+, K^+-ATPase activities. Local energy metabolism reduced linearly and then sustained for a long time[17]. Energy metabolism change was shown by gradual depletion of high-energy phosphate and changes of lactic acid metabolism[18,19]. Because LDH and Na^+, K^+-ATPase were the important enzymes of energy metabolism, which occurred in various other body cells, their activities were the most important indices for energetic metabolism. Thus, investigating their changes and relationship with the cord edema and lipid peroxidation indicated by MDA content after SCI was very beneficial for drug research, which could be employed as markers of drug actions and effects for the therapeutic drug research of SCI. Levels of LDH activity and Na^+, K^+-ATPase activity could act as the marks of energy metabolism changes in the injured tissues and their quantity and quality could directly affect body's energy metabolism. When tissues and organs were injured, LDH activity abnormally increased and Na^+, K^+-ATPase activity was greatly inhibited[20,21]. The present experimental results suggested

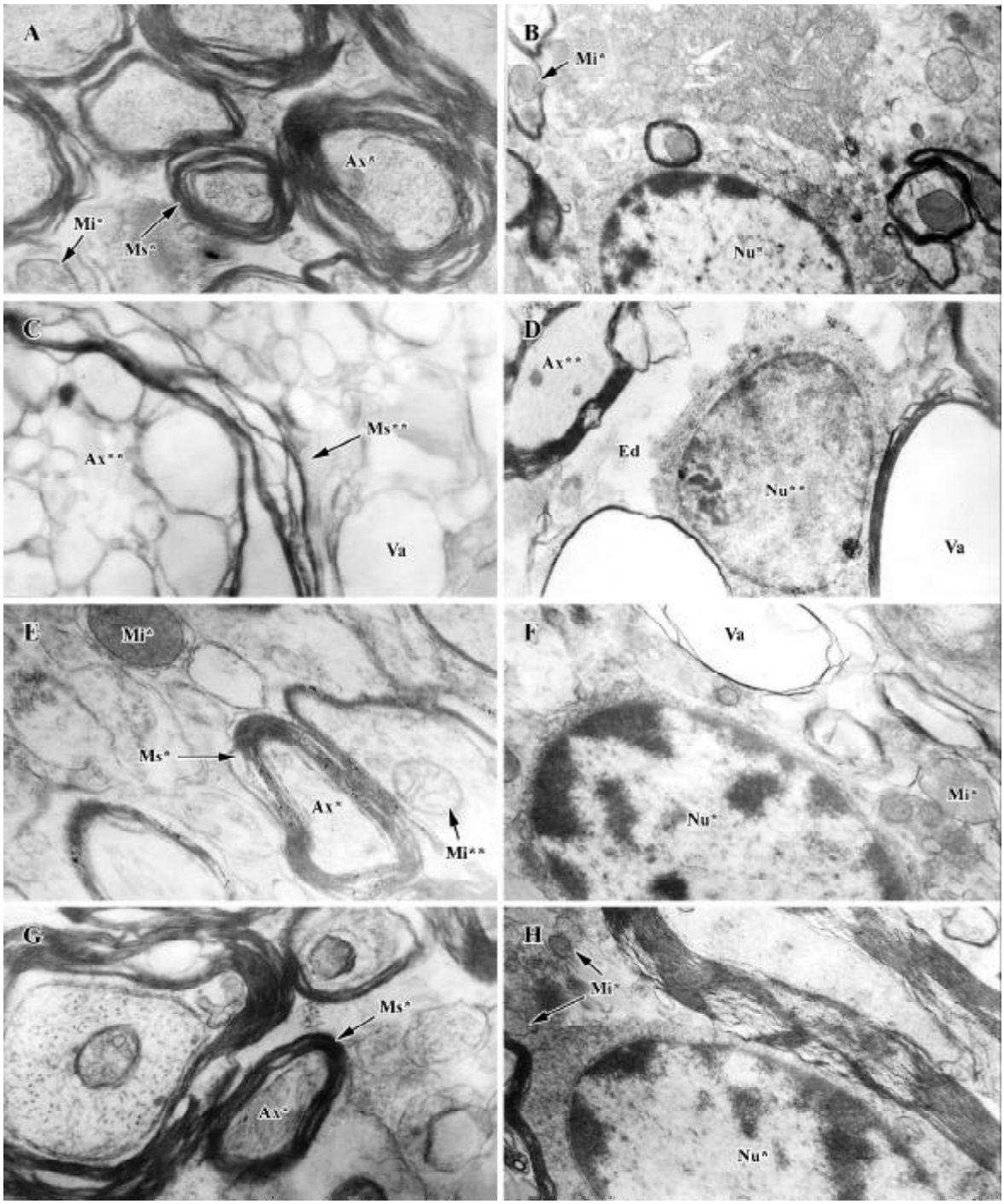

Fig 1. Electron microscopy shows ultrastructural changes in the local injured spinal cord after 48 h of SCI. A and B: Sham group. C and D: SCI group. E and F: MPSS-treated group. G and H: Res-treated group. *: normal; **: abnormal; Ax: axon; Ms: myelin sheath; Mi: mitochondria; Nu: nucleus; Ed: edema; Va: vacuoles. (×8000) .

that Res at the dose of 100 mg/kg could effectively increase Na^+,K^+-ATPase activity and reduce LDH activity in the local injured tissue, whereas MPSS had no obvious effect at the same dose.

Previous data provided by Sun and his colleagues also showed that tissue injury and cell death were a free radical-mediated process with loss of cell viability and increased LDH activity, and Res had a protective effect against LDH increasing and lipid peroxidation[22]. Hung *et al* also reported that a Res analogue, astringinin, strongly prevented myocardial ischemia and infarction through increasing nitric oxide (NO) and decreasing

LDH levels in the carotid blood[23]. Therefore, Res may play an important role in the observed improvement of LDH activity after tissue trauma, and benefit for injured nerve after SCI.

On the other hand, free radical generation has been considered as a major cause of the secondary spinal cord injury in a variety of abnormalities after SCI. The present study demonstrated that MDA content in the injured spinal cord was increased markedly, which was similar to other related reports. Recently, it was indicated that one of the plausible ways to prevent the free radical-mediated tissue injury was pharmaceutical augmentation of endogenous antioxidant defense capacity. Res has been recently proposed as a potential antioxidant that could obviously inhibit free radical generation in red cell membrane, heart, liver, brain, kidney and so on, *in vitro*, which reflected beneficial effects to prevent functional injury and improve nerve function and promote restoration after trauma. Sun *et al* also reported that Res protected the brain from neuronal damage due to chronic ethanol administration and suggested that Res might be used as a therapeutic agent to ameliorate neurodegenerative processes[24]. In addition, the experiments *in vitro* also demonstrated that Res could effectively protect DNA from oxidative damages, so as to assure cell proliferation, differentiation, and function to be normal[25]. Ray *et al* found that Res possessed cardioprotective effects through its peroxyl radical scavenging activity and inhibiting lipid peroxidation to reduce MDA content[26,27]. The result indicated that Res possessed beneficially neuroprotective effects after SCI.

From the ultrastuctural investigation, it has been suggested that both Res and MPSS have neuroprotective effects, including protecting axon, neuron, myelin, and subcellular organelles such as nucleus and mitochondrion, and reducing local spinal tissue edema, which were in accordance with above results. Compared with MPSS, however, Res exerts greatly more remarkable neuroprotection in the aspects of reducing edema, and improving Na^+, K^+-ATPase activities and anti-lipid-peroxidative action. The statistical differences occurring in the MPSS group were low and almost all at the time point of 24 h, fewer at 1 h and 48 h, whereas Res had statistical different values, almost at all time points on all results that the greatest values were near at 48 h. The main reason is that the neuroprotective effect of MPSS was mediated through the direct membrane action, completely unlike Res, and had nothing to do with glucocorticoid receptor-mediated activity. The reason why MPSS has been the best drug to cure SCI in clinic at present is related to its extensively pharmacological actions, especially anti-inflammation, stabilization of lysosomal membranes, enhancement of spinal cord blood flow, and suppression of vasogenic edema etc[13,28,29].

In summary, it seems reasonable to conclude that after acute SCI, Res effectively protected spinal cord from the secondary damages through inhibiting edema, improving energy metabolism system, and suppressing lipid peroxidation to prevent nerve functions to be exacerbated progressively, which is most important for restoring nerve function, preventing mitigating nerve damage, and maybe promoting nerve regeneration. The therapeutic values and mechanisms of Res on SCI should be studied further.

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