Protection of ginsenoside Rg1 on central nerve cell damage and the influence on neuron apoptosis

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Abstract: This paper aimed to verify the function of ginsenoside in the repair of peripheral nerve injury through the model of sciatic nerve injury in rat. The method was to prepare the model of SD rat injury of sciatic nerve, and to conduct treatment with different dose of ginsenoside Rg1. At the same time, the control group was established. The regenerative repair, functional recovery and the situation of target organ, etc. were evaluated by neuromorphic metrology index, fluorescence gold retrograde tag, animal behavior index (sciatic nerve index). The result was the situation of nerve regenerative repair and functional recovery in high dose ginsenoside Rg1 group was obviously superior to other groups, the recovery of sciatic nerve index, target muscle, etc. were fine and mostly close to normal. It was concluded that ginsenoside Rg1 could effectively promote the regenerative repair of peripheral nerve injury, and accelerate the recovery of its nerve function. It could also promote the regeneration of peripheral nerve and the recovery of its nerve function.

Keywords: Ginsenoside Rg1, peripheral nerve injury, nerve regeneration, functional recovery.

INTRODUCTION

Ginsenosides is a kind of sterol compounds, and triterpenoid saponin is its main effective constituent. The current study found that there were dozens of ginsenosides, which could generally be divided into three categories: ginsenoside diol monomer, ginsenoside triol monomer and oleanolic acid monomer. A large number of research and data showed that ginsenoside had the utility of nerve protection and neurotrophy in central nervous system (Na and Naishong, 2009).

Peripheral nerve injury, especially the spinal nerve injury is still one of the difficulties faced by clinic. At present, effective treatment was badly needed so as to actively promote the regenerative repair of peripheral nerve injury. In vitro studies have shown that GS Rg1 (GRg1) has the function of nerve protection. In the model of PC12 neuron apoptosis reduced by dopamine, GRg1 could reduce neuron apoptosis by inhibiting oxidative stress reaction (Sigrid C and Johannes, 2010). GRg1 could also reduce the toxic effect from rotenone or toxic rotenone on the neurons in the substantia nigra by inhibiting the pathway of mitochondrial apoptosis. Moreover, GRg1 could reduce the deficiency of neurons in the substantia nigra caused by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) by inhibiting the expression of inducible nitrogen oxide (Jianping et al., 2009).

In addition, GRg1 could promote the appreciation and migration of RSC96 SCs through the signal path of insulin-like growth factor-I (IGF-I) and fibroblast growth factor-2 (FGF-2) (Francesca et al., 2010). The above research results showed that GRg1 played an important role in the protection of central neuron in vitro. At the same time, its function of promoting SCs hinted that GRg1 also played an important role in the protection of peripherall nerve system (Mo’onica et al., 2010). However, there is no relevant research on the repair of peripherall nerve injury in vivo currently. This research was aimed at verifying the function of GRg1 in the repair of peripherall nerve injury in the model of the injury of sciatic nerve in rat in vivo.

MATERIALS AND METHODS

Materials

Sprague-Dawley (SD) needed in this experiment was provided by laboratory culture. The main experimental reagent and equipment are shown in table 1.

Table 1: Main reagent and equipment

<table>
<thead>
<tr>
<th>Fluoro-gold</th>
<th>Biotium, USA</th>
</tr>
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<tbody>
<tr>
<td>Mecobalamin</td>
<td>Eisai, Japan</td>
</tr>
<tr>
<td>Dimethylbenzene, Ethyl alcohol absolute</td>
<td>Tianjin Fuyu Refining Company</td>
</tr>
<tr>
<td>Transmission Electron Microscopy</td>
<td>JEM-2000EX, Japan</td>
</tr>
<tr>
<td>Electronic scales</td>
<td>Shimadzu, Japan</td>
</tr>
<tr>
<td>Fluorescence microscope (Olympus BX-60)</td>
<td>Olympus, Japan</td>
</tr>
</tbody>
</table>

Methods

Nerve injury model and its grouping

There were 32 male rats with the weight of 200–220g. 1.0% (w/v) pentobarbital sodium (40mg/kg, intraperitoneal injection) was used to anesthetize animals.
The left posterior incision was took and cut layer by layer until the sciatic nerve was shown up. The nerve in sciatic nerve bifurcate near-end 5mm was clamped three times by clamp (10s every time, with 10s interval). The model of injury of sciatic nerve was prepared. Incision was rinsed and sewed. The shown-up operation was shown in fig. 1. The animals were randomly divided into 4 groups after operation, and each group had 8 ones. These animals were raised in cages, and the treatment of intraperitoneal injection of drug or normal saline were given according to the grouping situation, one times per day with 4 weeks continuously. The method of grouping and drug administration: normal saline group; Mecobalamin group, 100µg/kg; low dose GRg1 group, 1mg/kg; high dose GRg1 group, 5mg/kg. The general animal situation, foot skin ulcer and side limb function were closely observed after operation.

Therein, PL refers to the distance between heel and the third tip toe; TS refers to the distance between the first toe and the fifth toe; IT refers to the distance between the second toe and the fourth toe; E is experimental limb; N is non-operated limb (fig. 2).

![Fig. 1: Sciatic nerve appeared during operation (indicated by the arrows)](image1)

**Collection of footprint and calculation of index of the sciatic nerve**

In order to evaluate the recovery condition of animal movement function after nerve injury in different groups, the animal behavior index (sciatic functional index) was detected at 1w, 2w, 3w and 4w before and after operation. Animals were trained in advance before operation, so they could get through the homemade gait-recording device by themselves (the passageway was 50cm long and 7cm wide). The animal back feet were smeared non-toxic red dye at 1w, 2w, 3w and 4w after operation. White paper was tiled on the bottom of passageway for printing and dyeing footprint. Then animals were asked to pass the gait-recording device and the bilateral footprints were recorded. More than 5 complete and clear footprints for each animal were needed to record, and then its SFI was calculated, the calculation formula was as follows:

\[
SFI = \frac{-38.3 \times (EPL - NPL)}{NPL} + \frac{109.5 \times (ETS - NTS)}{NTS} + \frac{13.3 \times (EIT - NIT)}{NIT} - 8.8
\]

**Fluorescence gold retrograde tracing tag**

Fluorescence gold retrograde tracing tag was conducted in 25 days after operation, thus determined the regenerative repaired neuron number through the injured part. 1.0% (w/v) pentobarbital sodium (40mg/kg, intraperitoneal injection) was used to anesthetize animals and the sciatic nerve was shown up. The nerve injury part was determined under microscope. Sciatic nerve 2µl and 4% fluorescence gold solution were injected into sciatic nerve in the injury part far-end. Then that part was conventionally closed. Animals was raised in cages for 3 days, and during this period, fluorescence gold could be retrograde transported into nerve cells soma by fluorescence gold. Para formaldehyde was irrigated through abdominal aorta for fixation. L4-6 and dorsal root ganglion (DRG) of corresponding segment was cut and put in 4% (w/v) para formaldehyde and fixed for 4h. Then it was soaked in sucrose solution (30%) overnight under 4°C, so as to settle the organization into the bottom of the container. The frozen embedding medium was used to embed the organization. Spinal cord tissue cross-sectional slices of 25µm thickness and DRG longitudinal slices of 20µm were prepared. Then Phosphate Buffered Saline (PBS) was used to rinse them for 3 to 5 times, and glycerol was used to seal the slices. They were observed under the microscope (ultraviolet excitation wavelength), thus counted the number of positive neurons, which were tagged by fluorescence gold.

**Quantitative analysis of neuromorphology**

4 weeks after operation, sciatic nerve organizations of various animals were obtained and they were fixed by 3% glutaraldehyde. After that they were fixed by 1% osmium tetroxide solution, then dehydrated and embedded. The tissue slices needed by transmission electron microscopy were prepared. The concrete operations were as follows:
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Table 2: The sciatic nerve index of various groups at different time points after operation

<table>
<thead>
<tr>
<th>Time after operation</th>
<th>Normal saline</th>
<th>Low dose GRg1 group</th>
<th>High dose GRg1 group</th>
<th>Mecobalamine group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-74.10±6.16</td>
<td>72.80±7.10</td>
<td>71.78±6.85</td>
<td>72.39±7.93</td>
</tr>
<tr>
<td>2</td>
<td>-67.41±5.74</td>
<td>62.45±7.04*</td>
<td>57.95±5.95</td>
<td>61.39±5.29</td>
</tr>
<tr>
<td>3</td>
<td>51.88±5.16</td>
<td>47.63±5.38*</td>
<td>42.56±5.29</td>
<td>46.92±4.75</td>
</tr>
<tr>
<td>4</td>
<td>43.05±4.69</td>
<td>39.01±4.73*</td>
<td>35.21±4.16</td>
<td>38.37±3.35</td>
</tr>
</tbody>
</table>

(1) 0.1M phosphoric acid bleaching lotion was used to rinse these organizations for 3 times, and each time for 15 min;
(2) 1% osmium tetroxide solution was used for fixation for 1h under indoor temperature;
(3) then 0.1M phosphoric acid bleaching lotion was used to rinse these organizations for 3 times, and each time for 15 min;
(4) Ethanol and acetone of graded concentration were used to dehydrated these organizations: in 4°C refrigerator, 50% ethanol, 70% ethanol, 90% ethanol, 90% acetone and 90% acetone were used orderly to dehydrated these organizations, finally, 100% acetone was used to dehydrated them under indoor temperature, for 15 min each time;
(5) The samples were embedded: they were incubated for 4h in embedding solution 1 under indoor temperature (acetone: embedding solution=2:1), then incubated overnight in embedding solution 2 (acetone: embedding solution=1:2);
(6) Solidification: after finishing the above procedures, they were stayed at oven overnight under 37°C. Then the temperature in oven was risen to 45°C and solidified for 12h. After the finishing of final step for 12h, then transmission electron microscopy embedding block could be prepared. 1.0µm semi-thin section and 50nm ultra-thin section was prepared. 1% toluidine blue was used to dye the semi-thin section, and the organization structure was observed under optical microscope. Uranyl acetate and lead citrate were used to dye the ultra-thin section, and the neuromechanisms were observed under transmission electron microscope. The index of observation and record includes the area of the regenerative nerve, the number of myelinated nerve fibers in nerve organization of per unit area, the diameter of myelinated nerve fibers and its myelinization degree.

STATISTICAL ANALYSIS

X±S was used to express the obtained various data. SPSS 17.0 software was used to conduct statistical analysis of data. The one-way ANOVA was adopted to conduct index comparison among groups. When P<0.05, LSD-t was used to conduct two-two sample test. When P<0.05, then it was of significant differences.

RESULTS

Behavioristics index (Sciatic Nerve Index)
The animal footprints were collected at 1w, 2w, 3w, 4w after operation respectively and SFI was calculated, thus to evaluate the movement function and recovery condition of various animals. The results showed that the SFI in low dose GRg1 group, high dose GRg1 group and mecobalamine group were obviously superior to that in normal saline group (table 2, in high dose GRg1, P<0.01; in low dose GRg1 group and mecobalamine group, P<0.05). It showed that low dose GRg1, high dose GRg1 and mecobalamine all had certain treatment effect on the recovery of neural function. At the same time, the SFI in high dose GRg1 group was obviously superior to that in low dose GRg1 group and mecobalamine group (P<0.05) after operation in 2w, 3w and 4w, which indicated that high dose GRg1 had better treatment effect on neural injury and was more beneficial to the recovery of neural function.

Fluorescence gold retrograde tracing tag
The results of fluorescence gold retrograde tracing tag showed that fluorescence gold tag positive neuron condition of motor neuron were visible in spinal cord of various groups at 4 week after operation, as shown in fig. 3. After the counting and statistical analysis of the number of fluorescence gold positive neuron, the number of fluorescence gold tag positive neuron in various group could be seen as shown in fig. 4. The number of fluorescence gold tag positive neuron in high dose GRg1 group, low dose GRg1 group and mecobalamine group were obviously higher than that in normal saline group at 4week after operation, which indicated that low dose GRg, high dose GRg1 and mecobalamine all could accelerate the speed of nerve regenerative repair. At the same time, they could increase the survival rate of injured neuron. In addition, the number of fluorescence gold tag positive neuron in high dose GRg1 group, low dose GRg1 group and mecobalamine group were obviously higher than that in normal saline group at 4week after operation, which indicated that high dose GRg1 in promoting neural repair was superior to that of low dose GRg1 and mecobalamine, and its protective effect and treatment effect on neuron were better.
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Fig. 3: Fluorescence gold tag positive neuron (motor neuron) with 200 times magnification
A: normal saline Group; B: mecobalamine Group; C: low dose GRg1 Group; D: high dose GRg1 group;

E: the number of fluorescence gold tag positive neuron in various groups. Magnification times: ×200 times. * refers to compare with normal saline group, P<0.05; ** refers to compare with normal saline group, P<0.01; # refers to compare with low dose GRg1 group and mecobalamine group, P<0.05.

Fig. 4: the number of fluorescence gold tag positive neuron in various groups
Among which, A: normal saline group; B: mecobalamine group; C: low dose GRg1 group; D: high dose GRg1 group

Neural morphology metrology
The results of neural morphology analysis was showed in fig. 5, the column diagram in fig. from left to right are respectively: high dose GRg1 group, low dose GRg1 group, mecobalamine group and normal saline group. The regenerative nerve area, the number of myelinated nerve fibers, and the diameter of myelinated nerve fibers in high dose GRg1 group, low dose GRg1 group and mecobalamine group were all higher than that in normal saline group. It indicated that both GRg1 and mecobalamine could promote the regeneration of axon. The myelinization degree of regenerative nerve in high dose GRg1 group was superior to that in normal saline group, which suggested that high dose GRg1 could promote the myelinization of regenerative nerve. At the same time, the regenerative nerve area, the number of myelinated nerve fibers, and the diameter of myelinated nerve fibers in high dose GRg1 group were all higher than that in low dose GRg1 group and mecobalamine group. It showed that the function of high dose GRg1 group on the promotion of axon regeneration was superior to that in low dose GRg1 group and mecobalamine group. In addition, the myelinization degree of regenerative nerve in low dose GRg1 group was of no significant difference with normal saline group, which indicated that low dose GRg1 and mecobalamine could not promote the myelinization of regenerative nerve.

Fig. 5: The analysis of morphology metrology
Among which: A: the area of regenerative nerve, B: the number of myelinated nerve fibers, C: the diameter of myelinated nerve fibers, D: the myelinization degree of regenerative nerve. * Refers to compare with normal saline group, P<0.05; ** refers to compare with normal saline group, P<0.01; # refers to compare with low dose GRg1 group and mecobalamine group, P<0.05.

DISCUSSION
As a kind of potential neuroprotective agents, GRg1 may have the function of promoting the repair of peripheral nerve injury. In order to verify the possibility of this potential, this research conducted the above relevant experiments. The results indicated that in rat sciatic nerve injury model, GRg1 could strengthen nerve regeneration and accelerate the recovery of neural function. The treatment effect of low dose GRg1 (1mg/kg) was fairly
equal to that of mecobalamine. However, the regenerative ability to nerve regeneration of high dose GRg1 was apparently higher than that of low dose GRg1 and mecobalamine. According to the above research results, we considered that GRg1 has enormous potential, application value and prospect advantages in the drug treatment of the regenerative repair of peripheral neural injury.

The electrophysiological examination and analysis of nerve was one of the important methods in the evaluation of the functional recovery situation after nerve injury (Jun et al., 2011). After nerve injury, the incubation period of CMAPs prolonged, the peak value of CAMPs decreased, the nerve conduction velocity increased (Weiyan et al., 2014). The in vivo experiment results of Delgado-Garcia, et al. showed that after axon was cut off, the time of incubation period was prolonged and the speed of nerve conduction was decreased, which signified the nerve function was not good. In this study, after the application of GRg1 or mecobalamine, the time of incubation period was shortened and the speed of nerve conduction was increased, which indicated that GRg1 and mecobalamine could promote the recovery of neural function.

In this research, the number of fluorescence gold positive neuron in GRg1 group was obviously higher than that in the control group. The existed researches had shown that GRg1 could increase the expression of BDNF in rat hippocampal area (Ming et al., 2012). Thus, GRg1 could promote nerve regeneration by up-regulating the expression of BDNF of injured nerve. In addition, GRg1 could play its function in nerve protection by inhibiting the necrotic apoptosis. After nerve injury, many apoptosis signals were been activated, which caused the apoptosis of injured neuron. Many existed in vitro researches had shown that GRg1 could inhibit the apoptosis pathway of many injured neurons, such as the pathway of caspase-3 activation or mitochondria apoptosis (Qingyong et al., 2013). Thus, the function of inhibiting apoptosis by GRg1 in this research might be one of the mechanisms of its function in nerve protection. Currently, further researches are needed to define the potential mechanism of GRg1 on the function of nerve protection in vivo.

SCs is the glial cell in the peripheral nervous system. After nerve injury, SCs provides environmental conditions for neural regeneration. Thus, it plays an important role in the repair of nerve injury. After nerve injury, GRg1 would proliferate, migrate and composite into NTFs and extracellular matrix, and provide conditions for axon regeneration. Researches showed that (Yingbo et al., 2013) GRg1 could regulate the proliferation and migration of SCs in RSC 96 cell line by activating correlated signal pathways. It indicated that GRg1 might regulate cell behavior of SCs by activating various signal pathways. The regulation of GRg1 to SCs might include proliferation, migration and NTFs composition, which might be one of the mechanisms of promoting the recovery effect on nerve injury. However, further experiment is needed to verify this speculation.

The researches showed that if rat was given ginsenoside in the senescence accelerated mouse mode, then the GSH in its body would increase. While GSSG would decrease, the specific value of GSH/GSSG would increase, and the activity of its body antioxidant system would increase. The correlated researches showed that ginsenoside can decrease the neuron injury in Parkinson’s disease model. It was speculated that its possible mechanism was to inhibit the expression of Bax mRNA and increase the expression of Bal-2mRNA, thus to decrease cell apoptosis. And it also pointed out that ginsenoside could prevent calcium influx by inhibiting neurons voltage-gated calcium ions, then protected neuron and avoided neuron death caused by calcium influx. In the PC12 cell apoptosis models induced by dopamine, GRg1 also could increase cell apoptosis by inhibiting oxidative stress reaction (David and Peter, 2013). GRg1 could inhibit mitochondrial apoptosis so as to decrease the toxic effect of rotenone/ toxic deguelin on the substantia nigra of primary culture. The above research results proved that GRg1 in vitro played an important role in the protection of central neuron. At the same time, its function on the promotion of SCs hinted that GRg1 might play an important role in peripheral nervous systems. Therefore, we speculated that GRg1 might have the effect on the promotion of the repair of peripheral nervous system and the protection of neuron.

CONCLUSION
The experimental results showed that in the model of rat sciatic nerve injury, GRg1 could strengthen nerve regeneration and accelerate the recovery of nerve function. The therapeutic effect of low dose GRg1 was fairly equal to that of mecobalamine. However, the regenerative ability of promoting nerve regeneration by high dose GRg1 was significantly higher than that by low dose GRg1 and mecobalamine. According to these research results, it was believed that GRg1 could promote the nerve regeneration and accelerate the recovery of neural function. It showed that GRg1 might be applied to the treatment of the repair of nerve injury, and to the drug treatment of the regenerative repair of peripheral nerve injury in future. GRg1 has great potential and its application value is of good prospect.

REFERENCES
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