Treatment with ginseng total saponins improves the neurorestoration of rat after traumatic brain injury

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Materials and methods: The modified Feeney’s method was used to induce a TBI in rats. Ginseng total saponins (GTS) were treated intraperitoneally twice a day for 1 week after the TBI. The neurological functions, including learning and memory, were assessed. The effects of GTS treatment at 20, 40, 60, and 80 mg/kg were better than the effects of GTS at 5 and 10 mg/kg. GTS treatment alleviated the secondary brain injury and ameliorated the neurological functions, including learning and memory. These effects of GTS might provide a foundation for the use of ginseng as a medicinal herb to enhance intelligence, reduce the aging process, and prolong life in the traditional medicine.

Keywords:
Ginseng total saponins
Neuroprotection
Neural stem/progenitor cell
Neurorestoration
Traumatic brain injury

1. Introduction

Traumatic brain injury (TBI) refers to brain tissue injury caused by trauma, including falls, motor vehicle-traffic injury, being struck (by/against), assault, and unknown/other causes (Maas et al., 2008; Coronado et al., 2011). TBI is a major global problem and affects approximately 10 million people annually; therefore, it has a substantial impact on the healthcare system throughout the world (Hyder et al., 2007; Corrigan et al., 2010; Gean and Fischbein, 2010). TBI not only produces high mortality and morbidity, but also significantly affects the socioeconomic lives...
of survivors, some of which even have long-term disabilities (Hyder et al., 2007; Corrigan et al., 2010; Gean and Fischbein, 2010; Feigin et al., 2013). TBI can result in significant motor, sensory, cognitive and emotional impairments. Even mild TBI can be associated with headache, nausea/vomiting, dizziness, tinnitus, vision changes, impaired balance and coordination, mood and memory changes, difficulty with memory and attention, and fatigue and/or sleep disturbances (Winston, 1979).

TBI includes the primary injury produced by transient mechanical and the secondary brain injury caused by many molecular and cellular responses triggered by a series of subsequent cascades (Rovegno et al., 2012). Due to the irreversibility of the primary injury, many treatment strategies for TBI have been tested to prevent and reduce the secondary injury after TBI, but the results of clinical trials on neuroprotective agents have been disappointing (Xiong et al., 2010a). There is currently no specific treatment available for TBI other than supportive care (Maas et al., 2010; Rovegno et al., 2012). Recent preclinical data suggest that neurorestorative strategies that promote angiogenesis (formation of new blood vessels from pre-existing endothelial cells), axonal remodelling (axonal sprouting and pruning), neurogenesis (generation of new neurons) and synaptogenesis (formation of new synapses) provide promising opportunities for the treatment of TBI (Picard-Riera et al., 2004; Richardson et al., 2010; Xiong et al., 2010a, b). However, there are many factors that impact the neuroregeneration of brain tissue (Picard-Riera et al., 2004; Richardson et al., 2010). The involved mechanisms are very complicated, and there have been no substantial breakthroughs (Picard-Riera et al., 2004; Richardson et al., 2010).

The transplantation of cells or tissues tested to promote neural regeneration, the reconstruction of neurovascular units and brain tissue repair after injury includes peripheral nerve graft, Schwann cells, embryonic brain and spinal cord tissue, olfactory ensheathing cells, embryonic and neural stem cells, bone marrow stromal cells, activated macrophages, and others (Chiu et al., 2009; Richardson et al., 2010; Vaquero and Zurita, 2010; Shear et al., 2011), but the involved methodology is in its infancy, and the efficacy of transplantation is unreliable (Xiong et al., 2010b; Gögel et al., 2011; Jablonska and Lukomska, 2011). An alternative method is to promote the endogenous neural stem cell proliferation (Leker, 2009; Yoneyama et al., 2011) and to help these cells migrate to the injured area, repair the damaged brain tissue and restore the neurological functions. This method is still a long way from being used in clinical applications (Leker, 2009; Xiong et al., 2010b).

Ginseng, the root of Panax ginseng C.A. Meyer (Araliaceae), is a traditional medicinal herb. Through its effects of reinforcing vitality, strengthening the bodily resistance to pathogenic factors, engendering body liquids and allaying thirst, relieving uneasiness of the body and mind and benefiting intelligence, reducing body weight and prolonging life, ginseng have been widely used in Asia for the treatment of many diseases with symptoms such as consciousness uneasiness, insomnia, palpitations, fatigue and lack of strength, lack of appetite, thirst, and prostration (Attele et al., 1999; Ng, 2006; Chen et al., 2008). Ginsenosides are the most important biologically active substances extracted from Panax ginseng. Meyer, and ginseng total saponins (GTS) are a mixture of ginsenosides with various ginsenoside ratios (Attele et al., 1999). Many reports have suggested a prominent neuroprotective effect of ginsenosides on the brain subjected to ischaemia or trauma (Lim et al., 1997; Ji et al., 2005) and on the neurons deprived of oxygen/glucose (Jiang et al., 2000, 2001; Jiang and Jiang, 2003) or damaged under other conditions (Rudakewich et al., 2001; Liao et al., 2002). It has been reported that ginsenosides might exert a neurotrophic effect (Liang et al., 2010) and promote neural stem cell transformation into neurons or glial cells (Shi et al., 2005; Liu et al., 2007). Shen and Zhang (2004) found that ginsenoside Rg1 promoted the proliferation of hippocampal progenitor cells. Ginsenosides could also increase the viability of hippocampal neurons (Gong et al., 2011; Liu et al., 2011) and promote neurite outgrowth and neuronal network formation (Todha et al., 2005; Wang et al., 2006). Zheng et al. (2011) recently reported that GTS enhanced neurogenesis and might contribute to functional recovery after focal cerebral ischaemia.

No studies have been published regarding whether GTS could facilitate neurogenesis and neuronal regeneration after TBI and contribute to the recovery of neurological functions including learning and memory. The present study was performed primarily to investigate the neural stem/progenitor cell (NSC) proliferation-promoting effect in the hippocampal formation while documenting the neuroprotective effect and the effective dosage of ginseng total saponins used in rats after traumatic brain injury.

2. Materials and methods

2.1. Animals and materials

Male Sprague-Dawley rats (250–300 g body weight) were obtained from the Experimental Animal Center of Nantong University, Nantong, China. All the procedures were in strict accordance with the institutional guidelines of Nantong University, which complies with international rules and policies. Ethics in accordance with the ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines were followed in the animal experiments and approved by the Animal Care and Use Committee of Nantong University, Nantong, China (Permit number: 20120210-04). All the surgeries were performed under anaesthesia, and all efforts were made to minimise suffering. One hundred and sixty nine rats were one hundred and sixty nine rats were randomly allocated to each experimental group (Table 1). Due to anaesthetic accidents and anaesthetic side effects such as dynamic ileus, some rats died during the experiments, and a portion of the sample was lost in each group (Table 1). The data obtained from these animals were excluded.

Ginseng total saponins, extracted from the stem and leaf of Jilin Panax ginseng, were purchased from the Department of Organic Chemistry, Medical School of Jilin University, Changchun, China.

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**Table 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Table 2</th>
<th>Fig. 2</th>
<th>Fig. 3</th>
<th>Fig. 4</th>
<th>Fig. 5</th>
<th>Fig. 6</th>
<th>Fig. 7</th>
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The content of each main ginsenoside (HPLC analysis) was as follows: Re 26.85%, Rg2 19.18%, Rd 19.05%, Rg1 9.04%, Rb2 7.19%, Rh1 5.64%, Rc 4.56%, Rf 1.97%, Rb1 1.94%, Rh2 1.48%, and other minor ginsenosides.

Rabbit antibodies of anti-nerve growth factor (NGF)-β, anti-neural cell adhesion molecule (NCAM), anti-neurite outgrowth inhibitor A (Nogo-A), and anti-Nogo-B and anti-tenascin-C (TN-C) were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China), and the mouse antibody of anti-β-actin was purchased from Sigma-Aldrich Corporation (Saint Louis, USA). HRP-labelled goat anti-rabbit and anti-mouse IgG (H+L) were provided by the Beyotime Institute of Biotechnology (Haimen, China).

Goat anti-glia cell line derived-neurotrophic factor (GDNF) was purchased from R&D systems, Inc. (Minneapolis, USA), and mouse anti-NGF fibrillary acidic protein (GFAP) was purchased from Thermo (Milford, USA). Rhodamine (TRITC)-conjugated donkey anti-goat IgG (H+L) and fluorescein (FITC)-conjugated affiniPure donkey anti-mouse IgG (H+L) were provided by the Proteintech Group Inc. (Chicago, USA).

5-Bromo-2-deoxy-uridine (BrdU), mouse anti-BrdU antibody, rabbit anti-nestin antibody, TRITC-conjugated goat anti-mouse IgG (whole molecule), and FITC-conjugated goat anti-rabbit IgG (whole molecule) were purchased from Sigma (St. Louis, USA). The kit for the detection of BrdU was provided by Roche (Basel, Switzerland).

2.2. Traumatic brain injury

TBI was induced following a previously described model of cortical contusion trauma in the rat (Feeney et al., 1981). The rats were anaesthetised with 10% chloral hydrate (400 mg/kg, i.p.). The rectal temperature was maintained within the range of 37 ± 0.5 °C with a heating pad. A right parietal craniotomy (diameter 5 mm) was conducted with a drill under aseptic conditions. The centre of the craniotomy was 3.5 mm posterior and 2.5 mm lateral to the bregma. The parietal contusion was produced by allowing a steel rod weighing 20 g with a flat end and diameter of 3.5 mm to drop onto a piston resting on the dura from a height of 30 cm through a Teflon guide tube. The piston was allowed to compress the brain tissue to a depth of 2.5 mm and was removed immediately after the contusion. The sham-operated rats were surgically treated with right parietal craniotomy alone without brain injury. After trauma, the rats were returned to their cages, and the room temperature was maintained at 25 ± 0.5 °C.

2.3. Evaluation of neurological functions

The beam balance and prehensile traction tests were used to evaluate the neurological functions. The animals underwent pre-training for 3 days prior to randomisation. These two tests were performed on days 1, 2, 3, 7, and 14 after the TBI. All the assessments were performed by an observer blinded to the individual treatments. The neurological deficit scores were calculated by adding the scores from the two tests and were inversely related to the neurological functions. A higher score represents more severe neurological deficit, ranging from a minimum score of 0 for near-normal rats, to a maximum score of 6, indicating severe impairment.

For the beam balance test, a narrow wooden beam (1.5 cm wide) was positioned horizontally 40 cm above a foam pad to cushion the falling rat. The rat was placed at the centre of the beam. The time that the rat spent on the beam was recorded, up to a maximum of 60 s. The same test was repeated again, and the mean score of the two tests was calculated. The beam balance test score was obtained according to a 3-points scoring standard for the rat on the beam: 3, unable to stay on the beam; 2, holding on with four paws for ≥ 60 s; 1, stay stably for ≥ 60 s; and 0, walk easily and turn quickly (Combs and D’Alecy, 1987; DeGraba et al., 1994).

For the prehensile traction test, a 60 cm long straw rope (with a diameter of 1.5 cm) was fixed horizontally 40 cm above a foam pad. After holding the rope with the forepaws, the rat was released, and the time until falling was measured. The prehensile traction test score was obtained according to a 3-points scoring standard for the rat holding the rope: 3, unable to hang on the rope; 2, holding on with forepaws for ≥ 60 s; 1, holding on with hindpaws and maintaining stability for ≥ 60 s; and 0, holding on with hindpaws and climbing up easily (Combs and D’Alecy, 1987; DeGraba et al., 1994).

The learning and memory functions were tested with a Morris water maze (RD1101-MWM-HG, Mobiledatum Co. Ltd., Shanghai, China). Briefly, on day 6 to day 2 before the TBI, the rats were placed for 30 s on a platform (12 cm in diameter) that was hidden 2 cm below the water surface in a pool 1.6 m in diameter filled with 25 °C water. The rats were then placed in one of the three quadrants without the platform, and the time to find the platform (up to 90 s) was recorded. This training was repeated four times per day, and the rats were trained for consecutive 5 days. On day 1 before the TBI, the platform was moved away from the Morris water maze, the rats were placed in one of the four quadrants, and the swimming tracing and the time to enter the location of the platform were recorded. Before the TBI, the learning and memory abilities did not display any significant differences in the different groups (data not shown). After the TBI, the training was repeated on days 9–13, and the recording of the swimming tracing and the time to enter the location of the platform was repeated on day 14.

2.4. Brain tissue slicing

The brains were removed and post-fixed for 24 h in the same fixative. The post-fixed brains were cryoprotected in PBS containing 25% sucrose for 24 h and then in PBS containing 30% sucrose for 48 h. The brain tissue was sectioned coronally with 30 μm or 5 μm thickness as needed from 2.3 mm posterior to the bregma with a cryostat slicer (CM1900, Laica, Bensheim, Germany) and stored at −20 °C. The 30-μm or 5-μm sections were processed for Nissl staining or immunofluorescent imaging, respectively.

2.5. Nissl staining

The sections were mounted with neutral balsalt and blotted onto slides before being processed through different baths in the following order (and time): chloroform (30 min), acetone (15 min), 100% ethanol (30 s), 95% ethanol (30 s), 70% ethanol (30 s), distilled water (30 s, three times), cresyl violet (20 min), distilled water (30 s, three times), 70% ethanol (1 min), 95% ethanol (1 min), 100% ethanol (1 min), chloroform (5 min), differentiator (95% ethanol with the addition of glacial acetic acid to pH 4.1, 1 min), 95% ethanol (30 s), 100% ethanol (30 s, twice), xylene (1 min), xylene (2 min), xylene (3 min). The sections were then covered with a coverslip.

After Nissl staining, the neuronal cells of the hippocampal area CA3 ipsilateral to the trauma were identified under a high-magnification (× 400) light microscope and counted by an investigator blinded to the grouping. For each rat, the mean number of neurons was obtained by examining three serial coronal sections. In each section, the neuronal cells were counted in one visual field.
of the hippocampal CA3 area. Only intact neurons with a clearly defined cell body and nucleus were counted.

2.6. Immunofluorescent imaging

For the immunofluorescent observation of BrdU positive cells, the rats were intraperitoneally injected with a BrdU solution (50 mg/kg, b.i.d., i.p.) for 3 days. Twenty-four hours after the last injection, the rats were sacrificed under deep anesthesia and their brains were processed as above for slicing. A series of sections (200 μm apart, 5 μm thick) of the brain were obtained. For the BrdU staining, all the sections were incubated for 30 min at 37°C in 2 N HCl. The following procedures were the same for BrdU, nestin, GFAP or GDNF staining. The brain sections were washed 3 times in PBS and blocked in 10% goat serum overnight at 4°C. They were then incubated overnight with the primary antibody at 4°C. The sections were washed in PBS 3 times and incubated with the secondary antibody at 4°C for 12 h. The antibodies were used with the following dilution ratios: rabbit anti-nestin antibody (1:100), mouse anti-BrdU antibody (1:100), goat anti-GFAP antibody (20 μg/mL), mouse anti-GFAP (1:150), FITC-conjugated goat anti-rabbit IgG (whole molecule, 1:160), TRITC-conjugated goat anti-mouse IgG (whole molecule, 1:64), TRITC-conjugated donkey anti-goat IgG (H+L, 1:20) and FITC-conjugated affinitypurified donkey anti-mouse IgG (H+L, 1:20). After washing with PBS, the sections were mounted with neutral glycerol buffer and covered with a cover-slip.

The number of GDNF positive cells in the dentate gyrus ipsilateral to the trauma was counted in one visual field under a high-magnification (×200) fluorescent microscope. The BrdU/nestin positive cells were observed with a confocal microscope (TCS-SP2, Leica Microsystems, Heidelberg, Germany) in the hippocampal CA3 area and in the dentate gyrus ipsilateral to the trauma; these cells were counted in one visual field. The mean value of one rat was obtained by examining three serial coronal sections. Cell counting after immunofluorescent imaging was performed by investigators blinded to the treatment groups.

2.7. Western-blot analysis

Seven days after the TBI, the hippocampal formation ipsilateral to the injured side of each rat was removed under anesthesia and homogenised in lysis buffer. The homogenate was centrifuged at 12,000 rpm at 4°C for 10 min to collect the supernatant. The protein concentration of the supernatant was measured with a bicinchoninic acid protein assay kit. The supernatant of each sample equivalent to 40 μg protein was used for sodium dodecyl sulphate-polyacrylamide gel electrophoresis at 110 V with a size marker. The protein on the gel was transferred to a 0.2% polyvinylidene fluoride transfer membrane in a buffer containing methanol, glycine and Tris base. The membrane was placed in 5% fat-free milk for 1.5 h at room temperature to block non-specific binding. After blocking, the membrane was incubated overnight at 4°C with the primary antibodies (anti-NGF-β 1:100, anti-NCAM 1:100, anti-Nogo-A 1:100, anti-Nogo-B 1:100, anti-TN-C 1:100, or anti-β-actin 1:10,000). The next day, after washing with PBS, the membrane was incubated 1.5 h with the secondary antibodies (HRP-labelled goat anti-rabbit IgG (H+L) 1:1000, or HRP-labelled goat anti-mouse IgG (H+L) 1:1000). The signal was detected with chemoluminescence reagents. The stripped blots were scanned and quantified by Scion Image grey scale scanning software. The relative level was calculated as the ratio to β-actin.

2.8. Administration of GTS

For observation of the dose-dependent effect of GTS, different doses of ginseng total saponins (5, 10, 20, 40, 60 and 80 mg/kg) were injected intraperitoneally 6 h after the TBI and the GTS treatment was continued twice per day for 1 week. GTS doses were chosen according to our preliminary experimental results, our previous study (Xia et al., 2012), and those other researchers have reported (Zhao et al., 2009, 2011; Zheng et al., 2011). The GTS treatment (20 mg/kg) was started 6 h after the TBI for the measurement of nerve growth-related factors and the observation of NSCs, and the treatment was maintained twice per day for 1 week. Ginseng total saponins were dissolved in normal saline (0.9% NaCl). The rats in the sham-operated and TBI groups received injections of normal saline alone with the volume equal to the GTS group (5 ml/kg). A diagrammatic protocol is shown in Fig. 1.

2.9. Statistical analysis

All the data were presented as the mean ± standard deviation. The data in Table 2 and in Fig. 3A were analysed with two-way ANOVA, and other data were analysed with one-way ANOVA. Post-hoc comparisons between the experimental groups were performed with Fisher’s LSD. The significance level was set at P < 0.05.

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3. Results

3.1. Dose-dependent neuroprotective effect of GTS

The neurological functions were measured in rats at days 1, 2, 3, 7 and 14 after the TBI. As shown in Table 2, the neurological deficit score increased remarkably at 1 day after the TBI and decreased gradually with time in the TBI group. After treatment with GTS, the neurological deficit score decreased more markedly with time after the TBI in a dose-dependent manner. At 14 days after the TBI, the neurologica
deficit score in rats after different treatments.

Table 2

<table>
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<tr>
<th>Group (n=10)</th>
<th>Before TBI</th>
<th>After TBI</th>
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<tr>
<td></td>
<td>1 day</td>
<td>2 days</td>
</tr>
<tr>
<td>Sham-operated</td>
<td>0.30 ± 0.48</td>
<td>0.10 ± 0.32**</td>
</tr>
<tr>
<td>TBI</td>
<td>0.30 ± 0.48;***</td>
<td>5.20 ± 0.42</td>
</tr>
<tr>
<td>GTS (mg/kg)</td>
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<td>5.00 ± 0.71</td>
</tr>
<tr>
<td>5</td>
<td>0.31 ± 0.48;***</td>
<td>5.00 ± 0.71</td>
</tr>
<tr>
<td>10</td>
<td>0.33 ± 0.49;***</td>
<td>4.92 ± 0.51</td>
</tr>
<tr>
<td>20</td>
<td>0.33 ± 0.49;***</td>
<td>4.83 ± 0.58</td>
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<tr>
<td>40</td>
<td>0.33 ± 0.49;***</td>
<td>4.82 ± 0.40</td>
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<tr>
<td>60</td>
<td>0.27 ± 0.47;***</td>
<td>4.91 ± 0.70</td>
</tr>
<tr>
<td>80</td>
<td>0.27 ± 0.47;***</td>
<td>4.91 ± 0.70</td>
</tr>
</tbody>
</table>

** P < 0.01 vs. TBI group.
* P < 0.05 vs. 5 mg/kg GTS group.
*** P < 0.01 vs. 5 mg/kg GTS group.
# P < 0.05 vs. 1 day after TBI of the same group treated with vehicle or GTS.
## P < 0.01 vs. 1 day after TBI of the same group treated with vehicle or GTS.

3.2. Improvement of the learning and memory functions by GTS

The efficacy of GTS treatment on the learning and memory abilities of rats was determined after a TBI. As shown in Fig. 3, the time to find the platform during the training days (day 9 to day 13) was notably prolonged, and the time to enter the platform location on the test day (day 14) was prolonged after the TBI (P < 0.01), suggesting that the learning and memory abilities of the rats were reduced after the TBI. Treatment with GTS significantly decreased the time to find the platform during the training days and the time to enter the platform location on the test day (P < 0.05 or 0.01, Fig. 3), suggesting that GTS treatment could improve the learning and memory abilities of the rats after the TBI.

3.3. Effect of GTS on the expression of nerve growth-related factors

The expression levels of NGF, NCAM, Nogo-A, Nogo-B and TN-C were measured with western-blots analysis, and the expression level of GDNF was measured using an immunofluorescent method in the hippocampal formation ipsilateral to the trauma 7 days after the TBI. The effects of GTS treatment on these variables were observed.

As shown in Fig. 4, the relative level of NGF expression after the TBI was increased from 0.61 ± 0.05 in the sham-operated group to 0.74 ± 0.05 in the TBI group (P < 0.01). After GTS treatment, the expression of NGF was further elevated to 0.85 ± 0.05 (P < 0.01, Fig. 4B). A similar change was found in the expression of NCAM (Fig. 4C and D). NCAM was divided into three isoforms with molecular weights of 120 kd, 140 kd and 180 kd. The expression levels of all three isoforms of NCAM were elevated after the TBI (P < 0.01) and further enhanced by GTS treatment (P < 0.05 or 0.01, Fig. 4D). The value of the NCAM 120 kd isofrom was elevated from 0.15 ± 0.03 in the sham-operated group to 0.48 ± 0.08 in the TBI group (P < 0.01), and further to 0.85 ± 0.12 after GTS treatment (P < 0.01, Fig. 4D).

Immunofluorescent observation of the co-expression of GDNF/GFAP in the hippocampal formation revealed that GDNF-positive cells were mainly GFAP-positive astrocytes (Fig. 5). Seven days after the TBI, the activation of astrocytes was still observed (Fig. 5D), and GDNF-positive cells were increased (Fig. 5E), leading to an increase in the number of GDNF/GFAP co-expressing cells (Fig. 5F). GTS treatment did not significantly change the number of GFAP-positive astrocytes (Fig. 5G) but increased the expression of GDNF (Fig. 5H) and the number of GDNF/GFAP co-expressing cells (Fig. 5I). As shown in Fig. 5, the number of GDNF/GFAP co-expressing cells was increased from 16.2 ± 2.7 in the sham-operated group to 43.7 ± 4.2 in the TBI group (P < 0.01) at 7 days after TBI, and it was further increased to 61.3 ± 3.9 after GTS treatment (P < 0.01).

Changes in the expression levels of Nogo-A, Nogo-B, and TN-C were somewhat different from the levels of NGF, NCAM, and GDNF (Fig. 6). Seven days after the TBI, the expression of Nogo-A in the TBI group was higher than that of the sham-operated group (1.22 ± 0.03 vs. 0.65 ± 0.06, P < 0.01), but the level was reduced to 0.86 ± 0.10 after GTS treatment (P < 0.01, Fig. 6B). The expression of Nogo-B was elevated from 0.37 ± 0.03 in the sham-
Fig. 2. Morphological observations of the hippocampal formation in rats treated with different doses of GTS (n = 6 rats in each group). A–H Examples of Nissl staining of the hippocampal formation in the rats of sham-operated, TBI, GTS 5, 10, 20, 40, 60 and 80 mg/kg groups, respectively. I–P Magnification of the hippocampal CA3 areas ipsilateral to the contusion side and corresponding to A–H, respectively. Q Cell counting of the hippocampal CA3 area corresponding to each group. *P < 0.05, **P < 0.01, vs. TBI group; *P < 0.05, **P < 0.01, vs. GTS 5 mg/kg group. Scale bar, 2 mm (A–H), 50 μm (I–P).
operated group to 0.95 ± 0.03 in the TBI group ($P < 0.01$), but it was reduced by GTS treatment to 0.80 ± 0.11 ($P < 0.01$, Fig. 6D). There are two isoforms of TN-C with molecular weights of 200 kD and 220 kD. The expression levels of the two isoforms of TN-C were elevated 7 days after the TBI ($P < 0.01$) but were reduced after GTS treatment ($P < 0.01$, Fig. 6F).

### 3.4. Improvement of GTS on NSC proliferation in the hippocampal formation

To determine the NSC proliferation-promoting effect of GTS treatment in rats after a TBI, the NSC proliferation in the CA3 area and dentate gyrus of the hippocampal formation were observed 7 days after the TBI.

In the dentate gyrus of the hippocampal formation, BrdU-labelled cells and nestin-positive neurons were increased after the TBI (Fig. 7D and E), leading to an increase in the number of BrdU/nestin co-expressing cells (Fig. 7F), which suggests that the proliferation of NSCs is activated after a TBI. Following GTS treatment, the BrdU-labelled, nestin-positive and BrdU/nestin co-expressing cells were increased in the dentate gyrus, including the subgranular zone (Fig. 7G, H and I). The number of BrdU/nestin co-expressing cells was increased from 2.5 ± 0.5 in the sham-operated group to 9.4 ± 3.4 ($P < 0.01$); the number was further increased to 26.3 ± 4.5 after GTS treatment ($P < 0.01$, Fig. 7J).

In the hippocampal CA3 area, few BrdU/nestin co-expressing cells were found in the sham-operated group (Fig. 8C). BrdU/nestin co-expressing cells were increased 7 days after the TBI (Fig. 8F).
GTS treatment further enhanced the number of BrdU/nestin co-expressing cells (Fig. 8I). The mean value of the number of BrdU/nestin co-expressing cells was increased from 4.2 ± 1.5 in the sham-operated group to 10.4 ± 4.2 in the TBI group (P < 0.05); the number was further increased to 21.3 ± 3.5 after GTS treatment (P < 0.01, Fig. 8J).

4. Discussion

The present results show that GTS treatment reduced the neuronal loss caused by TBI in the hippocampus and promoted the recovery of neurological functions, including the learning and memory abilities. These results suggest that GTS has a neuroprotective effect, which is consistent with the reports of Ji et al. (2005) and our previous report (Xia et al., 2012). In our previous study (Xia et al., 2012), it was confirmed that treatment with GTS after a TBI could reduce the secondary brain injury by inhibiting oxidative and nitrative stresses, attenuating inflammatory responses, and reducing apoptotic neuronal death. In the present study, we further found that the neuroprotective effect of GTS was dose-dependent. GTS treatments at doses of 5–80 mg/kg were notably effective, but the effect of GTS at 20–60 mg/kg was more prominent.

Recent developments in neurobiology research have revealed that neurogenesis occurs in all mammals throughout adult life at a...
low rate. The paradigm of replacing the lost neurons after a TBI has been suggested to promote tissue repair of the injured brain and to restore the neurological functions (Richardson et al., 2010). Because exogenous stem/progenitor cell transplantations have many ethical and host-vs.-donor rejection issues (Xiong et al., 2010b; Yoneyama et al., 2011), promoting endogenous NSC proliferation is likely a better alternative method for neuronal regeneration in the damaged portion of the brain (Leker, 2009; Yoneyama et al., 2011). Increasing data suggest that neurogenesis in various regions of the adult mammalian brain increases in response to mechanical or ischaemic brain injury (Richardson et al., 2007; Yoneyama et al., 2011). In the present study, the number of BrdU/nestin co-expressing NSCs in the hippocampal formation was increased after a TBI, and this result was consistent with other reports (Rice et al., 2003; Sun et al., 2005; Richardson et al., 2007). The number of new neurons that develop after a TBI in adult and aged animals is not sufficient to meet the need of significant neurorestoration (Richardson et al., 2007; Yoneyama et al., 2011). The number of new neurons that develop after a TBI in adult and aged animals is not sufficient to meet the need of significant neurorestoration (Richardson et al., 2007). The number of new neurons that develop after a TBI in adult and aged animals is not sufficient to meet the need of significant neurorestoration (Richardson et al., 2007). The number of new neurons that develop after a TBI in adult and aged animals is not sufficient to meet the need of significant neurorestoration (Richardson et al., 2007). The number of new neurons that develop after a TBI in adult and aged animals is not sufficient to meet the need of significant neurorestoration (Richardson et al., 2007).

For this purpose, we treated TBI rats with ginseng total saponins based on previous studies that suggest that ginsenosides have a neurotrophic effect, promote nerve growth and enhance neurogenesis (Shen and Zhang, 2004; Zheng et al., 2011). GTS treatment notably increased the number of BrdU/nestin co-expressing NSCs in the hippocampal formation observed 7 days after a TBI, suggesting a facilitating effect of GTS on NSC proliferation. This effect of ginsenosides has been reported by Shen and Zhang (2004) in the hippocampus of normal adult mice, by Shen and Zhang (2003, 2007) in the dentate gyrus of adult gerbils after global ischaemia and by Zheng et al. (2011) in the subventricular zone and in the infarct area of rats after permanent focal cerebral ischaemia, but it has not been reported in a mouse or rat model of TBI. Present results are consistent with these reports, and the dose of GTS we used in this study is also similar to that used by Zheng et al. (2011).

To investigate the mechanisms underlying this promoting effect of GTS on the proliferation of NSCs, we observed the expression of some endogenous peptides in the ipsilateral hippocampal formation of rats after a TBI; these endogenous peptides are likely implicated in the endogenous neurogenic process (Leker, 2009). We found that the expression of NGF, NCAM, and GDNF in the ipsilateral hippocampal formation was elevated 7 days after TBI in the present study, and the expression increased further after treatment with GTS. These elevations by GTS were positively related to the increase in the number of BrdU/nestin co-expressing NSCs in the hippocampal formation. It has been reported that NCAM expression induces neurogenesis in vivo (Boutin et al., 2009) and that NGF and GDNF are important factors that promote neurogenesis (Leker, 2009). The increases in the expression levels of NGF, GDNF and NCAM in the hippocampal formation of TBI rats after GTS treatment might underlie the NSC proliferation-promoting effect of GTS that we found in the present study.

The expression levels of Nogo-A, Nogo-B and TN-C in the ipsilateral hippocampal formation were observed in the present study. The expression of some endogenous peptides in the ipsilateral hippocampal formation of rats after a TBI; these endogenous peptides are likely implicated in the endogenous neurogenic process (Leker, 2009; Richardson et al., 2010).

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study. Seven days after the TBI, the expression levels of these three factors were enhanced, but the levels were reduced after GTS treatment. Nogo-A is one of the most studied and most potent myelin-associated growth inhibitory molecules, which inhibit axonal growth (Schwab, 2004; Cafferty and Strittmatter, 2006; Yiu and He, 2006). The 66-amino acid region in the C-terminal domain of Nogo-A (Nogo66) binds to the Nogo66 receptor and induces myelin inhibition of neurite outgrowth. Other Nogo splice variants, including Nogo-B, also contain Nogo66, and blockade of the Nogo66 receptor leads to enhanced regeneration and nerve fibre growth associated with increased functional recovery in the adult CNS after injury (Li et al., 2004; Schwab, 2004; Joset et al., 2010). Garcion et al. (2004) reported that TN-C inhibited neurogenesis in cells derived from neural stem cells. It has been suggested that NGF, GDNF and NCAM are important factors that favour neurite outgrowth (Hannila and Kawaja, 1999; Walsh et al., 1999; Schmid and Maness, 2008). The present results suggest that GTS treatment, through inhibiting the upregulation of Nogo-A, Nogo-B and TN-C, after TBI and increasing the expression of NGF, GDNF and NCAM, is likely to ameliorate the microenvironment for NSC proliferation, the differentiation towards neurons, neuronal regeneration, and tissue repair of the injured brain, leading to functional recovery better than control rats after a TBI.

It has been reported that ginsenoside could reduce memory loss in aged mice (Zhao et al., 2009, 2011) and improve the spatial learning in mice (Mook-Jung et al., 2001) and in rats (Liu et al., 2011). NSCs in the dentate gyrus differentiate into newborn neurons, integrate into the existing circuitry and receive functional input (Ming and Song, 2005). Functionally, neurogenesis in the adult hippocampus is closely related to learning and memory.
abilities. Many studies have demonstrated that spatial learning (Kempermann and Gage, 2002) and memory (Jessberger et al., 2009) are facilitated in animals with more newborn neurons. Any agents that promote neurogenesis in the hippocampal formation are supposed to improve learning and memory abilities. Improvement of NSCs proliferation in the hippocampal formation after GTS treatment in the present study might contribute to improved recovery of the learning and memory abilities in TBI rats. This suggestion is consistent with other studies with GTS at similar doses (Zhao et al., 2009, 2011).

The present study confirmed that GTS treatment in rats after a TBI alleviated the secondary brain injury and ameliorated the neurological functions with an effective dose limit of 5–80 mg/kg. GTS regulated the expression of nerve growth-related factors and improved the proliferation of neural stem/progenitor cells, which might facilitate neural regeneration and tissue repair, and might contribute to the recovery of neurological functions, including learning and memory (Fig. 9). These effects of ginseng total saponins found in the present study might provide a foundation for the use of ginseng as a medicinal herb to enhance intelligence, reduce the aging process and prolong life in the traditional medicine.

**Author disclosure statement**

No conflicting financial interests exist.

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Fig. 8. Immunofluorescent staining of BrdU- and nestin-expressing cells in the hippocampal CA3 area ipsilateral to the contusion side (n = 5 rats in each group). A, D and G Examples of BrdU positive cells in the hippocampal CA3 area of the sham-operated, TBI and GTS-treated rats, respectively (red). B, E and H Examples of nestin positive cells in the same slice corresponding to A, D and G, respectively (green). C, F and I Merge of A and B, D and E, and G and H, respectively, to reveal the BrdU and nestin co-expressing cells (yellow). J Cell counting of BrdU/nestin co-expressing cells in the three different groups. *P < 0.05, **P < 0.01, vs. sham-operated group; ***P < 0.01, vs. TBI group. Scale bar 100 μm (A–I).
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**Reference**


Zhao, H.F., Li, Q., Li, Y., 2011. Long-term ginsenoside administration prevents memory loss in aged female C57BL/6J mice by modulating the redox status and up-regulating the plasticity-related proteins in hippocampus. Neuroscience 183, 189–202.