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Original Research Article

Liquiritin alleviates spinal cord injury through suppression of inflammation, oxidative stress, and cell apoptosis in a rat model

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Abstract

Purpose: *Liquiritin is an extract from Glycyrrhiza Radix, one of the oldest traditional Chinese herbal medicines, which is commonly used to treat various injuries and swellings. This study is aimed to determine whether liquiritin can protect spinal cord injuries (SCIs) from secondary injuries.*
Methods: *A rat SCI model was established. After liquiritin treatment, the neural-function of Rats was determined by Basso, Beattie and Bresnahan (BBB) scores, paw withdrawal threshold (PWT), and thermal withdrawal latency (PWL). The effects of anti-inflammation, anti-oxidation, and anti-apoptosis of liquiritin were also examined in the rats with SCI. Moreover, the activities of several signaling elements, such as, inflammation-associated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), toll-like receptor 4 (TLR4), proliferative-related p38 mitogen-activated protein kinases (MAPK) and myeloid differentiation primary response 88 (MyD88) which was involved in the TLR4 signaling, were used for further investigation of the underlying molecular mechanisms.*
Results: *Liquiritin improved locomotor function recovery, alleviated allodynia and hyperalgesia, and decreased water content of spinal cord in SCI rats. Also, liquiritin reduced SCI–induced inflammatory responses by decreasing the levels of tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and IL-6. Liquiritin inhibited SCI–induced oxidative stress by decreasing malondialdehyde (MDA) level and increasing the levels of uperoxide dismutase (SOD) ($p < 0.05$), glutathione (GSH) ($p < 0.01$), and GSH-PX ($p < 0.001$). In addition, liquiritin alleviated spinal cord injury (SCI) –induced apoptosis of neural cells by decreasing the expression of cleaved caspase-9, -3 and cleaved poly ADP-ribose polymerase (PARP). Finally, liquiritin decreased spinal cord injury (SCI) -induced up-regulation of TLR4/MyD88/NF-κB and p38 MAPK signaling cascades.*
Conclusion: *Liquiritin exerts protective role in SCI by reducing excessive inflammation, suppressing oxidative stress, and inhibiting neural cell apoptosis in a rat model of SCI. Thus, the agent can potentially be used for the management of SCI*

Keywords: *Liquiritin, Spinal cord injury (SCI), Inflammation, Oxidative stress, Apoptosis*

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INTRODUCTION

Spinal cord injury (SCI) is a neurological trauma that results in a loss of neurobehavioral function and causes acute disability and mortality [1]. Therapeutic measures such as hormonotherapy and cell transplantation, have been applied clinically for SCI treatment [2]. However, it is difficult to cure SCI patients with the aggravated neurological defects caused by secondary injuries, such as excessive inflammation, oxidative stress, and neural cell apoptosis [3]. Accumulating evidences have shown that prevention of secondary injuries could improve functional recovery after SCI and contribute to the successful treatment of SCI patients [4,5]. Therefore, developing more effective treatments to protect spinal cords against secondary injuries is a key for SCI therapy.

Liquiritin is an extract from *Glycyrrhiza Radix*, one of the oldest traditional Chinese herbal medicines, which is commonly used to treat various injuries and swelling [6]. Liquiritin has various biological activities, including anti-inflammation, anti-oxidation, anti-tumor, and anti-virus [7]. Liquiritin has protective and neurotrophic effects on hippocampal cells [8] and can alleviate sciatica ischialgia in mice induced by chronic constriction injury [9]. Liquiritin protects against injuries resulting from focal cerebral ischemia/reperfusion by alleviating neural cell apoptosis in mice [10]. However, it is unclear whether liquiritin has a protective effect on SCI.

However, the effect of liquiritin on SCI-induced secondary injuries, including excessive inflammation, oxidative stress, and neural cell apoptosis, has not been reported. Thus, the present study investigated the potential role of liquiritin in a rat model of SCI and the detailed mechanisms underlying was explored by assessing neurobehavioral function and determining the levels of inflammatory, oxidative, and apoptotic biomarkers.

EXPERIMENTAL

Animals and liquiritin administration

Sprague-Dawley (SD) rats (> 8 weeks and 250 - 300 g weight) were purchased from Dashuo (Chengdu, China), housed in plastic chambers in a temperature-controlled room (25 ℃), and provided feed and water *ad libitum*. Several groups of Sprague-Dawley (SD) rats with 10 rats in per group were used in this study: normal control (Control), SD rats with SCI (Sham) and SCI rats administrated with different concentrations of liquiritin (S-527029, GuanDao, Shanghai, China). First, 0.5 % carboxymethyl cellulose sodium (CMC-Na) aqueous solution was used to dissolve the liquiritin. Then, rats were orally administered by liquiritin or CMC-Na aqueous solution. Of note, to abide strictly by the rules of the animal care made by the National Institutes of Health (NIH) [12], all rats were breed under conditions of sterility. This study was approved by the Institutional Animal Care and Use Committee of Gansu Provincial Hospital of Traditional Chinese Medicine.

Establishment of the rat SCI model, and Basso, Beattie and Bresnahan (BBB) assay

SCI models were established using SD rats as described previously and BBB assays were performed [13,14]. The locomotor function of the rats in different groups was evaluated once per week by three separate observers using the BBB locomotor rating scale. Rats were scored from 0 (no observable movement) to 21 (normal movement) [15] and the average from three independent results was determined as the final score.

Paw withdrawal threshold and thermal withdrawal latency assessment

In this study, electronic Von Frey filaments (North Coast Medical, Gilroy, CA, USA) was used to evaluate the mechanical allodynia of paw withdrawal threshold (PWT). In addition, a BME-410C thermal pain stimulator (Chinese Academy of Medical Sciences, Beijing, China) was used to determine the thermal withdrawal latency (PWL) of thermal hyperalgesia. This study repeated three times at 15 min intervals and the average was taken as the final result. This measurement was performed once per week after SCI surgery.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Injured spinal cords were excised 28 days after SCI surgery and TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA. For qRT-PCR, complementary DNA was reverse transcribed from 2 μg of RNA using RNA extraction kit (M-MLV Reverse Transcriptase, Invitrogen). All gene expression was measured by bio-Rad PCR system (BioRad, USA) Notably, GAPDH expression level was used to normalize the target genes expression. Primer pairs used for GAPDH, – and several inflammatory cytokines in this study were described previously [16].

Western blotting

RIPA lysis (Beyotime Biotechnology, Shanghai, China) buffer was used to extract total proteins from injured spinal cords. Then, after unspecific antigens block, primary antibodies (Table 1) were incubated at 4℃ overnight. Finally, The HRP-conjugated secondary antibodies were used to examine proteins expression through Bio-Rad and quantified by ImageJ.

Enzyme-linked immunosorbent assay (ELISA)

Peripheral blood of rats was collected through retro-orbital bleeding 28 days after SCI surgery and stored at -80°C. ELISA kits (Thermo, MA, USA) was used to test the concentrations of several inflammatory cytokines in the supernatant following the manufacturer's instructions.

Measurement of MDA, SOD, GSH, and GSH-PX levels

Serum samples were used to evaluate oxidative stress. Indicated kits were used to test the corresponding proteins expression (Table 1).

Measurement of spinal cord water content

After liquiritin treatment for consecutive 28 days, we sacrifice the rat and collected the spinal cord samples and weighed as wet weight. Briefly, we immediately put the spinal cord samples in the refrigerator at -80°C and samples were dried for 48 h and weighed as dry weight. Spinal cord water content was calculated using the following formula: Water content of spinal cord (%) = [(wet weight − dry weight)/wet weight] × 100%.

Statistical analysis

Data are shown as mean ± standard deviation (SD). One-way ANOVA with Bonferroni's correction was used to analyze the data for comparison of multiple groups. In addition, an independent Student's t-test was applied to analyze the data for comparison between two groups. GraphPad Prism 6.0 software was used for data analysis, and $p < 0.05$ considered as statistically significant.

RESULTS

Liquiritin exerts protective effects on rats with spinal cord injury (SCI)

To investigate the role of liquiritin in the recovery of spinal cord injury (SCI), a SCI rat model was established by moderate contusion of the spinal cord. Rats with SCI were orally administrated with different concentrations of liquiritin (10, 20, and 30 mg/kg). Basso, Beattie and Bresnahan scores at 0, 1, 7, 14, 21, and 28 days after injury were determined to evaluate locomotor function recovery. The results showed that the scores were significant increased in a dose-dependent manner (Figure 1 A).

Mechanical allodynia and thermal hyperalgesia were determined using PWT and PWL. The PWT and PWL values were significantly increased following liquiritin treatment, suggesting that liquiritin attenuated allodynia and hyperalgesia caused by SCI (Figure 1 B and C).

Table 1: Primary antibodies used in this study

Antibody	Catalogue no.	Supplier
Rabbit anti XO	Sc-20991	Santa cruz
Rabbit anti iNOS	13120	Cell signaling technology
Rabbit anti NOX-2	Ab80508	Abcam
Rabbit anti NOX-4	Ab133303	Abcam
Rabbit anti cleaved caspase-9	Ab2324	Abcam
Rabbit anti cleaved caspase-3	Ab2302	Abcam
Rabbit anti NF-Kb	8242	Cell signaling technology
Rabbit anti p-NF-κB	3033	Cell signaling technology
uperoxide dismutase (SOD)	A006-1	Jiancheng
Rabbit anti p-p38	4092	Cell signaling technology
Rabbit anti p38	8690	Cell signaling technology
mouse anti p-JNK	9255	Cell signaling technology
Rabbit anti JNK	9252	Cell signaling technology
Rabbit anti MyD88	4283	Cell signaling technology
uperoxide dismutase (SOD)	A001-3	Jiancheng
Rabbit anti GAPDH	5174	Cell signaling technology
Rat anti PARP	Ab74290	Abcam
malondialdehyde (MDA)	A003-1	Jiancheng

In addition, water content of spinal cord was dramatically decreased following liquiritin administration in SCI rats (Figure 1 D). Thus, liquiritin improved locomotor function recovery, alleviated allodynia and hyperalgesia, and decreased water content of spinal cord in SCI rats.

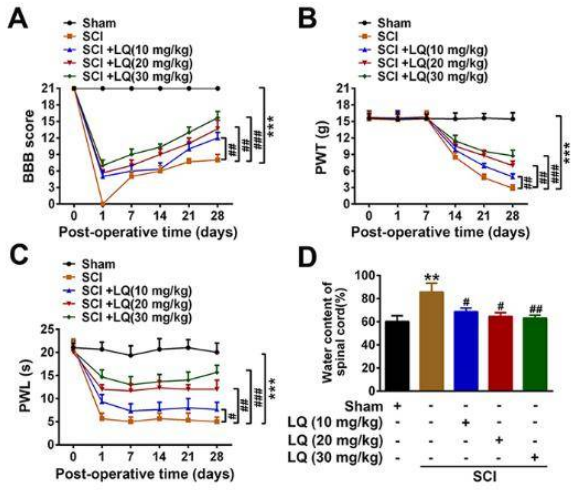

Figure 1: Effects of liquiritin on the neurobehavioral function in rats with spinal cord injury (SCI). Basso, Beattie and Bresnahan (BBB) scores (A), paw withdrawal threshold (PWT) assessment(B) and thermal withdrawal latency (PWL) assessment (C)at 0, 1, 7, 14, 21, and 28 days after SCI in rats following treatment with various concentrations (10, 20, and 30 mg/kg) of liquiritin, (D) The water content of spinal cords from rats following treatment with various concentrations (10, 20, and 30 mg/kg) of liquiritin at 28 days after SCI (n = 10), $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* Sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$ *vs.* SCI group. LQ, liquiritin

Liquiritin reduced spinal cord injury (SCI)–induced inflammatory responses

Compared to the Sham rats, SCI rats exhibited higher levels of pro-inflammatory cytokines, tumor necrosis factor-α, interleukin-1β, and interleukin-6. After liquiritin treatment, the levels of tumor necrosis factor-α, interleukin-1β, and interleukin-6 in both peripheral blood and spinal cords from SCI rats were evaluated. Enzyme linked immunosorbent assay showed that liquiritin significantly reduced the releases of tumor necrosis factor-α, interleukin-1β, and interleukin-6 (Figure 2 A - C). Consistently, qRT-PCR results indicated that the mRNA levels of those cytokines in spinal cord tissues were remarkably decreased in SCI rats following liquiritin treatment (Figures 2 D - F). Therefore, liquiritin significantly reduced inflammation in SCI rats.

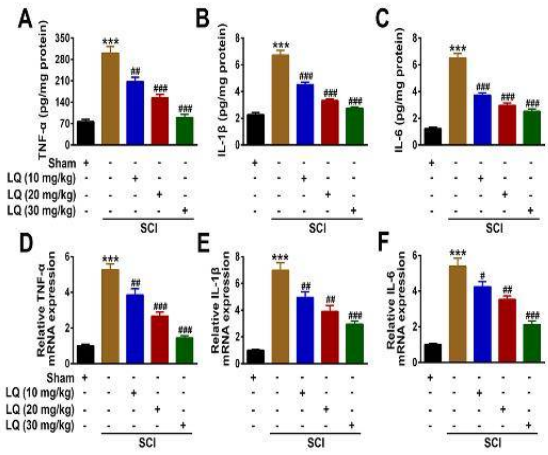

Figure 2: Anti-inflammatory effects of liquiritin on rats with spinal cord injury (SCI). The concentrations of indicated inflammatory cytokines, tumor necrosis factor-α (A), interleukin- 1β (B), and interleukin -6 (C), were evaluated using ELISA assays. The mRNA expression levels of inflammatory cytokines, tumor necrosis factor-α (D), interleukin- 1β (E), and interleukin -6 (F), were determined using qRT-PCR. $^{***}p < 0.001$ *vs.* Sham group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ $^{\#\#\#}p < 0.001$ *vs.*SCI group. LQ = liquiritin; tumor necrosis factor-α (A), interleukin- 1β (B), and interleukin -6 (C)

Liquiritin inhibited spinal cord injury (SCI)–induced oxidative stress

To clarify the effect of liquiritin on oxidative stress, the concentrations and expression levels of oxidative stress-related proteins were measured SCI rats. The data indicated that the level of MDA was increased after SCI and significantly decreased by liquiritin treatment (Figure 3 A). The levels of the anti-oxidants (SOD, GSH, and GSH-PX) were significantly increased following liquiritin treatment (Figures 3 B - D). XO, iNOS, NOX-2, and NOX-4 are oxidative stress-related proteins and could promote reactive oxygen species (ROS) generation [17]. The results showed that their expression levels were increased after SCT treatment, and inhibited by liquiritin administration (Figure 3 E).

Liquiritin alleviated spinal cord injury (SCI) – induced apoptosis of neural cells

The effects of liquiritin on neural cell apoptosis were evaluated. The protein levels of cleaved caspase-9, cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP), a family of proteins involved in programmed cell death, were significantly increased after SCI treatment, and then significantly decreased after liquiritin administration in a dose-dependent manner (Figure 4). These data suggested that liquiritin alleviated neural cell apoptosis in SCI rats.

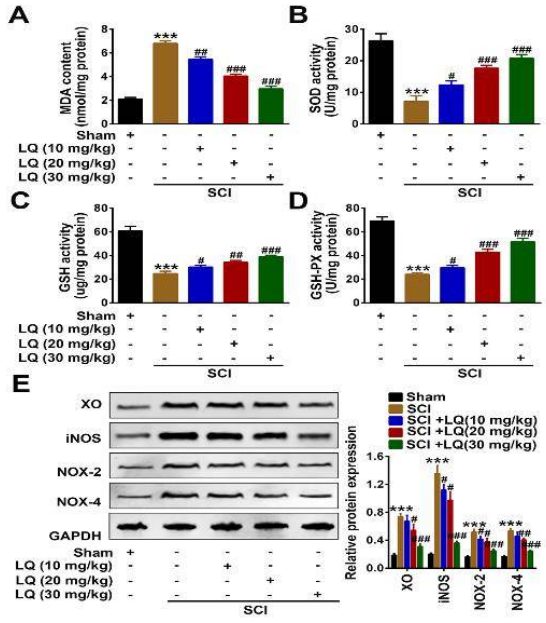

Figure 3: Anti-oxidative effects of liquiritin on rats with spinal cord injury (SCI). Levels of malondialdehyde (MDA) (A), superoxide dismutase (SOD) (B), glutathione (GSH) (C), and GSH-PX (D) in peripheral blood of SCI rats using ELISA assays. (E) Protein expression levels of xanthine oxidase (XO), inducible nitric oxide synthase (iNOS), nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX-2), and NOX-4 in spinal cords were evaluated by western blot (left) and quantified using Image J software (right); ***p < 0.001 *vs.* Sham group. $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01 and $^{\#\#\#}p$ < 0.001 *vs.* SCI group. LQ, liquiritin

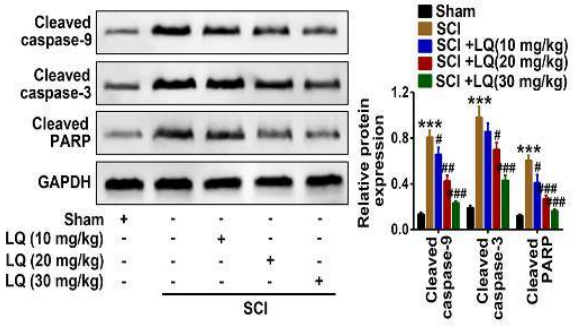

Figure 4: Anti-apoptotic effects of liquiritin on rats with spinal cord injury (SCI). The protein levels of cleaved caspase-9, cleaved caspase-3, and cleaved poly (ADP-ribose) polymerase (PARP) in spinal cords were determined by western blot (left) and quantified using Image J software (right); ***p < 0.001 *vs.* Sham group; $^{\#}p$ < 0.05, $^{\#\#}p$ < 0.01 and $^{\#\#\#}p$ < 0.001 *vs.* SCI group

Liquiritin decreased spinal cord injury (SCI)--induced up-regulation of TLR4/MyD88/NF-κB and p38 MAPK cascades

Liquiritin was reported to alleviate ultraviolet B (UVB)-induced skin injury by modulating toll-like receptor 4 (TLR4)/myeloid differentiation primary response 88 (MyD88)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p38 mitogen-activated protein kinases (p38-MAPK) signaling [11]. Activation of TLR4/MyD88 signaling pathway can lead to direct activation of NF-κB, which promotes the pro-inflammatory cytokines secretion [18]. It raises the possibility that the role of liquiritin in SCI rats may be related to TLR4/MyD88/NF-κB and p38 MAPK signaling pathway. As shown in Figure 5, the protein levels of TLR4, MyD88, and p-NF-κB in spinal cords from SCI rats were elevated and reduced following liquiritin administration.

In addition, p38 MAPK signaling pathway, known to regulate apoptosis [19], was also evaluated in spinal cords from SCI rats. Western blot analysis indicated that p38 and JNK were phosphorylated in SCI rats and that p-p38 and p-JNK levels were reduced following liquiritin administration (respectively; Figure 5). These results indicate that liquiritin inhibited the activation of the p38 MAPK signaling pathway, thus alleviating neural cell apoptosis in SCI rats. Taken together, liquiritin alleviated SCI, at least partially, through inactivation of TLR4/MyD88/NF-κB signaling pathways and downregulation of p-p38 and p-JNK.

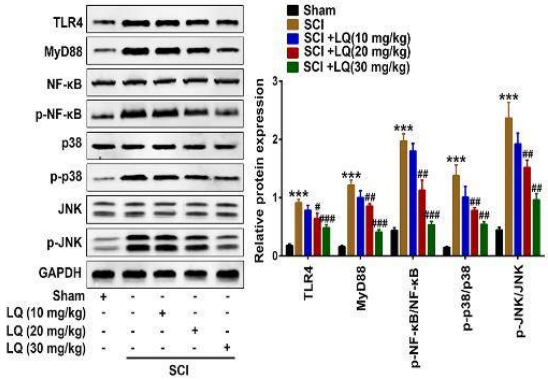

Figure 5: Liquiritin decreased spinal cord injury (SCI) -induced up-regulation of TLR4/MyD88/NF-κB and p38 MAPK signaling cascades. The protein levels of toll-like receptor 4 (TLR4), myeloid differentiation primary response 88 (MyD88), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), p-NF-κB, p38, p-p38, Jun N-terminal kinase (JNK), and p-JNK in spinal cords were determined by western blot (left) and quantified using Image J software (right); ***p < 0.001 *vs.* Sham group. $^{\#\#}p$ < 0.01 and $^{\#\#\#}p$ < 0.001 *vs.* SCI group.

DISCUSSION

Spinal cord injury (SCI) is a neurological trauma that can result in severe disability and mortality due to the complete or incomplete loss of neurobehavioral function [1,2]. The present study indicated that liquiritin alleviated secondary injury

following SCI and contributed to functional recovery in SCI rats. Liquiritin significantly inhibited the excessive inflammation, oxidative stress, and neural cell apoptosis caused by SCI. Further exploration of the molecular mechanisms disclosed that the TLR4/MyD88/NF-κB and p38 MAPKsignaling pathways were involved in this process.

Liquiritin is a traditional Chinese herbal medicine [6], and possesses a variety of biological activities, such as anti-inflammation, anti-oxidation, anti-tumor, and anti-virus [7]. Emerging reports have shown that liquiritin has neuroprotective effects [20,21]. Liquiritin exerts its protective and neurotrophic effects on hippocampal cells [8] and can relieve sciatica ischialgia in mice caused by chronic constriction injury [9]. Liquiritin ameliorated the PC12 cell damage by alleviating nuclear and mitochondrial apoptosis [22].

Liquiritin protects against injuries resulting from focal cerebral ischemia/reperfusion by suppressing neural cell apoptosis in mice [10]. Moreover, liquiritin is reported to have a protective effect on chronic neurologic diseases, such as Parkinson's disease [22], Alzheimer's disease [21], and depression [23]. In this study, liquiritin exerts protective effects on suppressing excessive inflammation, oxidative stress, and neural cell apoptosis in SCI rats.

Following primary injuries, the secondary inflammatory environment of the local damaged tissue further worsens this condition due to pathological changes like cell necrosis and apoptosis [4,24]. Moreover, activation of inflammatory cells triggers the generation of pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin-1β, and interleukin-6 [24]. Previous studies have indicated that activation of the TLR4/MyD88 signaling pathway can lead to the direct activation of NF-κB, resulting in increased expression of tumor necrosis factor-α, interleukin-1β, and interleukin-6 causing aggravation of the SCI [18]. In the present study, liquiritin had an inhibitory effect on the TLR4/MyD88/NF-κB signaling pathway and suppressed inflammation by inhibiting the release of tumor necrosis factor-α, interleukin-1β, and interleukin-6 in SCI rats.

Oxidative stress was also suppressed following liquiritin treatment due to increased antioxidants and decreased oxidants. The protein expression levels of XO, iNOS, NOX-4, and NOX-2, which are crucial for ROS generation [17], were also downregulated in SCI rats. The p38 MAPK pathway participates in the regulation of inflammatory reactions, stress reactions, and apoptosis [25]. The results of this study demonstrated that the p38 MAPK pathway was inhibited by liquiritin treatment. This finding needs further exploration.

CONCLUSION

Due to the nature and impact of secondary injuries following SCI, it is important to develop a clinical therapy for alleviating these secondary injuries. The findings of this study confirm that liquiritin alleviates SCI in rats. Therefore, liquiritin is a promising candidate for SCI therapy due to its ability to prevent excessive inflammation, oxidative stress, and neural cell apoptosis following SCI.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the researchers listed in this article. All liabilities related with the content of this article will be borne by the authors. Yongsheng Luo and Sheihai Chen designed all the experiments and revised the paper. Ting Li and Yonglin Guan formed the experiments. Yongming Liu and Binxiang Ma wrote the paper.

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