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# Neuroprotective effects of safranal in a rat model of traumatic injury to the spinal cord by anti-apoptotic, anti-inflammatory and edema-attenuating

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ABSTRACT

Studies on the pathology of spinal cord injury (SCI) have focused on inflammation-associated neuronal apoptosis. The traditional Chinese medicine safranal has been studied extensively and found to have various beneficial health effects. However, study of its potential role in neuroprotection and the underlying mechanism of action in SCI models has been limited. We investigated the effect of safranal on neurologic functions and histopathologic changes after SCI and the mechanism underlying its neuroprotective effects. First, the most effective safranal dose for SCI was evaluated with the Basso, Beattie, and Bresnahan Locomotor Rating Scale and H&E staining: 100 mg/kg was the most effective dose of safranal for SCI. Histopathologic changes were evaluated by performing Nissl staining, which indicated an increased number of neurons after safranal administration. In terms of the mechanism of action, anti-apoptotic effect, downregulation of inflammation, and edema-attenuating effects were detected. TUNEL staining and electron microscopy revealed that safranal treatment inhibited injury-induced apoptosis, and affected the expression of the apoptosis-related genes *Bax* and *Bcl-2*, which indicated an anti-apoptotic role after SCI. Safranal treatment suppressed immunoreactivity and expression of the inflammatory cytokines IL-1β, TNF-α, and p38 MAPK, and increased expression of IL-10 after SCI, suggesting an anti-inflammatory effect. Safranal treatment suppressed expression of AQP-4, which is related to spinal-cord edema, suggesting an edema-attenuating effect. These data suggest that safranal promotes the recovery of neuronal function after SCI in rats, and that this effect is related to its anti-apoptotic, anti-inflammatory, and edema-attenuating effects.



## 1. Introduction

Spinal cord injury (SCI) is a serious condition that often results in neural dysfunction. Despite many promising experimental studies, effective treatment that can overcome secondary damage after SCI is lacking. During SCI, inflammation is the major contributor to secondary degeneration (Can et al., 2009; Beck et al., 2010; Nakajima et al., 2010). Infiltration of neutrophils, activated macrophages, and microglia can break the blood–spinal cord barrier, which is closely associated with edema formation (Perry and Gordon, 1988). In addition, SCI initiates a massive immune response, which may result in the apoptosis of neurons and oligodendrocytes (Byrnes et al., 2007; Cittelly et al., 2008). Recently, inflammation, edema, neural apoptosis, and their interactions have received increasing attention because of their potential role in the pathology of SCI (Samantaray et al., 2008; Gál et al., 2009).

Increasingly, traditional Chinese medicine (TCM) is being recognized as a new therapeutic option for SCI (Liu et al., 2011; Fan et al., 2006; Khanna et al., 2009). Recently, attention has been focused on *Crocus sativus* L. (commonly known as saffron). *C. sativus* L. is used as an antispasmodic, eupeptic, anticatarrhal, nerve sedative, carminative, expectorant, aphrodisiac, and emmenagogic agent in TCM (Rios et al., 1996; Pitsikas et al., 2008). Studies have demonstrated that *C. sativus* L. and its active constituent, safranal, have anti-inflammatory (Hosseinzadeh and Younesi, 2002; Xu et al., 2009) and anti-apoptotic (Soeda et al., 2001; Ochiai et al., 2004; Shen et al., 2004) effects. However, safranal has never been studied in the context of SCI.

Here, we provide further evidence that safranal has a neuroprotective role in SCI through its anti-inflammatory,

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edema-attenuating, and anti-apoptotic properties. These data may provide evidence supporting the use of TCM therapy in SCI.

## 2. Materials and methods

### 2.1. Animals

This study was carried out in accordance with *Guidelines for the Use of Experimental Animals* (National Institutes of Health [Bethesda, MD, USA]). All animal protocols were approved by the Animal Care and Use Committee of Xi'an Jiao Tong University (Xi'an, China). All surgical procedures were performed under anesthesia and all efforts were made to minimize the animals' suffering. Adult male Sprague-Dawley rats (250–300 g; 14–16 weeks) were obtained from the Experimental Animal Center of Xi'an Jiao Tong University. The rats were bred and maintained under a 12-h–12-h light–dark cycle with free access to food and water. Room temperature was set at 25 ± 3 °C and relative humidity at 60 ± 15%.

### 2.2. Surgical procedure

The rats were not allowed to drink water on the morning of surgery. A rat model of SCI was established in accordance with a model of experimental SCI established by Allen et al. (Wrathall et al., 1985). Anesthesia was induced with sodium pentobarbital (50 mg/kg, i.p.). The rats were placed in the prostrate position on the operating table. Fur around the chest and abdomen was shaved. After disinfection, a 3-cm incision was made in the center of the eighth thoracic spine to expose the dura mater. The severity of injury was set at 25 g cm (10 g × 2.5 cm), which can lead to moderate damage to the spinal cord. The diameter of the impounder was 3 mm and the impounder was in opposition to the dura mater. After the hit, the striking instrument was removed quickly, and the wound was sutured. A model was considered to be successfully established if the following were observed: ischemia around the spinal cord; edema around the wound; formation of a tail sway reflex; flicking of body and legs; and sluggish. The animals received prophylactic injections of gentamicin (0.03 mg/kg body weight, i.m.) once per day for three days to prevent infection. Temperatures were maintained strictly. The rats were housed individually with free access to food and water. Padding in each cage was changed every day to keep it dry. After injury, bladder massage was undertaken twice a day until onset of reflexive voiding.

### 2.3. Determination of the most effective dose of safranal

#### 2.3.1. Experimental protocol and tissue processing

Thirty-six rats were assigned randomly to six groups of six each. The sham group underwent laminectomy alone. The model group underwent laminectomy followed by SCI and did not receive medication. Safranal A group underwent laminectomy followed by SCI and received safranal (25 mg/kg, i.p.; Sigma–Aldrich, Saint Louis, MO, USA). Safranal B group underwent laminectomy followed by SCI and received safranal (50 mg/kg, i.p.). Safranal C group ($n = 6$) underwent laminectomy followed by SCI and received safranal (100 mg/kg, i.p.). Safranal D group ($n = 6$) underwent laminectomy followed by SCI and received safranal (200 mg/kg, i.p.). All medications were administered three times per day for three days after SCI.

#### 2.3.2. Evaluation of neurologic function

Seventy-two hours after SCI, recovery of neuronal function was assessed. The rats were examined by two observers blinded to group treatments. Motor function in Hind limbs was evaluated according to the Basso, Beattie, and Bresnahan (BBB) Locomotor Rating Scale (Basso et al., 1996). Twenty-one score values were possible, ranging from 0 (complete paralysis) to 21 (normal locomotion). BBB scores were used to categorize combinations of hind-limb movements; joint movement; weight support; fore/hindlimb coordination; position and stability of the trunk; stepping; paw placement; toe clearance; and tail position. These combinations represented the sequential recovery stages attained by rats after SCI. Rats were allowed to move freely and scored over 4 min.

#### 2.3.3. Histopathology

Seventy-two hours after SCI, the rats were anesthetized and perfused transcardially with 200 mL of 0.1 mol/L phosphate-buffered saline (PBS; pH 7.4), followed by 400 mL of PBS (pH 7.4) with 4% paraformaldehyde. Upon dissection, the dura was marked at the injury epicenter, the entire spinal cord was dissected, and the rostral/caudal orientation labeled. A 3-cm-long piece of tissue, centered on the injury epicenter, was excised from the spinal cord. This spinal tissue was immersed in 4% paraformaldehyde in 0.1 mol/L phosphate buffer and stored at 4 °C for one week. Specimens were embedded in paraffin and sliced into 5-μm-thick sections. To assess histopathologic changes, the sections were further subjected to hematoxylin and eosin (H&E) staining.

### 2.4. Neuroprotective effects and mechanism of action of safranal

#### 2.4.1. Experimental protocol and tissue processing

Sixty rats were assigned randomly into three groups with 20 in each. The sham group underwent laminectomy alone. The model group underwent laminectomy followed by SCI and did not receive medication. The safranal group underwent laminectomy followed by SCI and received safranal with the most effective dose (i.p.). Safranal was administered three times a day for three days after the injury. At 24, 48, and 72 h after injury, blood was harvested from the vena caudalis 1 h after the last dose of safranal had been administered, and serum was isolated. All rats were killed 72 h after SCI, and each group subdivided into three subgroups. Subgroup 1 was subjected to biochemical analyses (seven rats in each group). Subgroup 2 was subjected to histologic assessment (seven rats in each group). Subgroup 3 was subjected to assessment by electron microscopy (six rats in each group). All of the serum and spinal cord tissues were harvested and stored at −80 °C for the experiments detailed below.

#### 2.4.2. Nissl staining

Spinal cords were fixed, embedded, and sliced as described above. Nissl staining was performed as described previously (Putt, 1948). Briefly, slides were dewaxed, rehydrated, and stained in warm (50 °C) 0.5% solution of toluidine blue for 10 min and rinsed quickly in distilled water thrice. Subsequently, the slides were dehydrated and clarified in xylene twice and mounted using neutral gum. Images were captured under a light microscope at 200× from five random fields. Quantification of normal motor neurons and abnormal motor neurons in VIII–IX regions of the cornu anterius medullae spinalis were analyzed with image-processing software (Media Cybernetics, Rockville, MD, USA).

#### 2.4.3. Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining

TUNEL staining (Ben-Izhak et al., 2008) was done on paraffin sections using an *in situ* cell death detection kit (Jiancheng Bioengineering Institute, Nanjing, China) according to manufacturer instructions. Sections were counterstained with hematoxylin. Quantitation was carried out by counting the number of positive cells in five randomly chosen fields of view on each slide at a