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Neuro-protective effect of Linalool against spinal cord injury in rats and the mechanism involved

[Efecto neuroprotector del Linalool contra la lesión de la médula espinal en ratas y el mecanismo involucrado]

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Abstract: The current study was conducted to determine the neuroprotective role and mechanism of action of Linalool (LIN) in SCI. The SCI in Sprague-Dawley (SD) rats was induced by weight-drop contusion model. Results of the suggested that LIN showed improvement in the locomotor function of SCI rats in a BBB scoring analysis. It was found in agreement with histopathological analysis of spinal cord tissue where LIN improves the neuronal architecture of spinal cord tissues, and protect neurons from degeneration. It also reduces oxidative stress via modulating endogenous antioxidants (MDA, SOD, and GSH) and inhibits the generation of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6). In western blot analysis, LIN showed dose-dependent reduction of expression of toll-like receptor (TLR-4) and nuclear factor-kappa B (NF-κB). Our study demonstrated that administration of Linalool alleviated spinal cord injury via anti-inflammatory and antioxidant activities in spinal cord tissues possibly due to inhibition of TLR4/NF-κB activation.

Keywords: Linalool; Spinal cord injury; Inflammation; Oxidatives stress; NF-κB.

Resumen: El estudio actual se realizó para determinar el papel neuroprotector y el mecanismo de acción de Linalool (LIN) en SCI. La LIN en ratas Sprague-Dawley (SD) se indujo mediante el modelo de contusión de caída de peso. Los resultados sugirieron que LIN mostró una mejora en la función locomotora de ratas SCI en un análisis de puntuación BBB. De acuerdo con el análisis histopatológico del tejido de la médula espinal se encontró que LIN mejora la arquitectura neuronal de los tejidos de la médula espinal y protege a las neuronas de la degeneración. También reduce el estrés oxidativo mediante la modulación de antioxidantes endógenos (MDA, SOD y GSH) e inhibe la generación de citocinas proinflamatorias (TNF-α, IL-1β e IL-6). En el análisis de Western blot, LIN mostró una reducción dependiente de la dosis de la expresión del receptor tipo toll (TLR-4) y el factor nuclear kappa B (NF-κB). Nuestro estudio demostró que la administración de Linalool alivió la lesión de la médula espinal a través de actividades antiinflamatorias y antioxidantes en los tejidos de la médula espinal, posiblemente debido a la inhibición de la activación de TLR4/NF-κB.

Palabras clave: Linalool; Lesión de la médula espinal; Inflamación; estrés oxidativo; NF-κB.

INTRODUCTION

The spinal cord is a very significant system of the body that is responsible for mediating the signals that originated from the brain to other body parts. Any injury to it leads to the generation of Spinal cord injury (SCI) which is devastating. The SCI causes loss of motor and sensory functions and affects overall body functioning (Hagen, 2015). Biochemically, the SCI has been categorized into two distinct phases (primary and secondary) which are interrelated. The initial trauma to the spinal cord caused due to physical insult is responsible for the development of primary injury (Ahuja *et al*., 2017). It results in the loss of membrane integrity and damage to the myelin sheath. The primary injury will transcend to secondary injury in few days or will take many weeks. It causes cellular edema, ischemia, inflammation, altered intracellular ion homeostasis, apoptosis, and other changes which contribute to the loss of neuronal activity (Sanchini & Boniolo, 2019). The current therapeutic option to treat SCI is based on controlling the after-effects of secondary injury, but the majority of medicine are failed to achieve high clinical benefit (Kwon *et al*., 2004). The complex multifactorial etiology of SCI has compelled us to discover new drugs that act via multiple pathways against SCI.

Natural products have a long history of medicinal importance and are currently being utilized against many human ailments. Linalool (LIN) is a noncyclic monoterpenoid that is commonly extracted from lavender (*Lavandula* spp.), rose (Rosa spp.), basil (*Ocimum basilicum*), and neroli oil (*Citrus aurantium*) (Kamatou & Viljoen, 2008). Studies have shown that it exerts many pharmacological properties such as antimicrobial, anti-inflammatory, anticancer, anti-oxidant properties and several *in vivo* studies have confirmed various effects of linalool on the central nervous system (Cheng *et al*., 2018; Harada *et al*., 2018; Magnard *et al*., 2018; Gao *et al*., 2019; Kim *et al*., 2019; Sabogal-Guáqueta *et al*., 2019; Liu *et al*., 2020; Zhao *et al*., 2020). However, none of the studies has enumerated the benefit of Linalool against SCI. Thus, prompted by the above, in the present study, we wish to scrutinize the pharmacological effect of LIN against spinal cord injury in rats and the mechanism involved.

MATERIAL AND METHODS

Chemicals

The Linalool (>95% pure) and other chemical used in the present study was procured from Sigma-Aldrich (USA).

Animals

Adult male Sprague-Dawley (SD) rats (240-270 gm) rats (Age: 9 weeks) housed in polypropylene cages under strict hygienic laboratory conditions with alternate light and dark cycles of 12h. The Ethical Committee for Biomedical Experimentation of Baoding First Central Hospital, China has approved the current study (BFCH/ECBE/2020/021).

Establishment of spinal cord injury (SCI)

The SCI injury was introduced in the rats as per modified Allen's method (Allen, 1911). Briefly, following anesthesia, a 2 cm midline incision was performed and laminectomy at the vertebral lamina of T8 was performed. 10 g rod was dropped from a distance of 5 cm onto the spinal cord and letting the rod rest on the lesion site for 3 min for the induction of SCI. The surgical site was then sutured and 5 ml of physiological saline was injected i.p. immediately after the surgery. Until normal urination was resolved, the bladder was evacuated manually. The rats in the sham group received the laminectomy only. The LIN-treated group received different doses, such as 5 mg/kg; 10 mg/kg; and 15 mg/kg via *i.p.* immediately after surgery once a day for 14 days. Rats in the sham and SCI groups received vehicles only. Total 50 animals and divided in 5 groups, where each group contain 10 animals.

Group 1: Sham
Group 2: SCI
Group 3: SCI + LIN (5 mg/kg)
Group 4: SCI + LIN (10 mg/kg)
Group 5: SCI + LIN (15 mg/kg)

Evaluation of motor function

The Basso–Beattie–Bresnahan (BBB) scale was used to evaluate locomotion at baseline (1 day before surgery) as well as day 1, 4, 7, 10, and 14 days post-SCI. It is most widely accepted locomotor rating scale used to test behavioral consequences of spinal cord injury (SCI) to the rat. The rating scale ranges between 0 and 21. The rats are trained twice the week before the surgery to move in an open field which is a molded-plastic circular enclosure with a smooth, nonslip floor. Rats are allowed to move freely and are scored during 4 minutes by two observers for their ability to use their hindlimbs. Joint movements, paw placement, weight support, and fore/hindlimb coordination are judged according to the 21-point

BBB locomotion scale.

The scoring is perfomed as follows as per the previously established protocol (Basso *et al*., 1995; Basso *et al*., 2006).

1. Early Stage (score of 0 - 7): Composed of isolated joint movements with little or no hindlimb movement
2. Intermediate Stage (score of 8 - 13): Intervals of uncoordinated stepping
3. Late Stage (score of 14 - 21): Forelimb and hindlimb coordination

The behaviours of the trunk, tail and hindlimbs of the rats were assessed in an open field. Twenty-one points refer to normal motor capacity and lower scores indicate impairment in motor capacity. The observers were blind to the treatment

Harvesting of spinal cord

The spinal cord tissues (a 10 mm segment containing the injury epicenter) was extracted from the animals after sacrificing in the early morning. Brain and SC tissues were dissected, frozen on dry ice, and stored at −70°C until further use.

Histological examinations

The spinal cord segments near the lesion epicenter were accumulated and fixed in 4% (w/v) PFA for 24 h and embedded in paraffin for transverse sectioning. Then, 5 μm-thick sections were cut for histopathological examination. Sections were stained with hematoxylin and eosin for HE staining as per the manufacturer's instructions. The slides were dehydrated with ethanol series, cleared with xylene, mounted in DPX (Sigma), and observed under microscopy (Nikon).

Biochemical determination

Homogenates of the spinal cord were centrifuged at 15,000 g for 10 min at 4°C. The supernatant was used for the measurement of MDA and GSH levels and the determination of SOD activity according to the protocols in the commercially available kits (Beyotime Biotech., Jiangsu, China).

Enzyme-linked immunosorbent assay (ELISA)

The determination of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 were performed in the supernatants of the spinal cord protein using commercially available ELISA kits as per the manufacturer's instructions.

Western blot assay

Total protein was obtained using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and quantified with a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.). The proteins were separated using SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. The membrane was blocked with 0.1% BSA in PBS for 1 h at room temperature and then incubated with the primary antibodies (1:1,000 dilution) in PBST overnight at 4°C. The membrane was incubated with a goat anti-rabbit secondary antibody (1:2,000 dilution) for 1 h at room temperature. Immunoreactive bands were detected using an enhanced chemiluminescence system (ECL kit), and the images were analyzed with ImageJ 1.42q software.

Statistical analysis

All data are presented as mean ± SEM of three independent experiments. A one-way analysis of variance (ANOVA) with Duncan's multiple range post hoc test was carried out for comparisons between individual groups and to determine if the differences between these groups were statistically significant ($p<0.05$). At the beginning, ANOVA was performed to determine whether there were statistically significant differences among the experimental groups, and when the differences occurred, Duncan's multiple range post hoc test was performed to determine which two means differed ($p<0.05$). In the case when the post hoc analysis revealed any influence of the dose and time on the investigated parameter, the possible interactive and independent effects of LIN was evaluated with the use of a two-way analysis of variance (ANOVA/MANOVA, test F). F values having $p<0.05$ were taken to indicate a statistically significant effect. Statistical software GraphPad Prism 5.0 (California, USA) was used for the statistical analaysis reported in the present paper. The p-value<0.05 was considered statistically significant.

RESULTS

LIN improves motor function in rats

After the SCI induction, the effect of LIN on the motor function in rats was initially studied by BBB scoring. The BBB scale ranges from 0-21 which stands for chronological recovery phases and categorizes alone or together rat functions, such as, rat joint movement, hindlimb movements, stepping, forelimb and hindlimb coordination, trunk position

and stability, paw placement and tail position. The 0 corresponds to complete paralysis and 21 represent normal motor function in hind limbs. As shown in Figure No. 1, the BBB scoring was measured at baseline (1 day before surgery) as well as day 1, 4, 7, 10, and 14 days post-SCI. Results suggested that SCI rats showed complete paralysis of the hind limbs and suggested to have significant disturbance in the motor function as compared to Sham treated rats in the initial experiement period. After administration of LIN, the motor ability of rats has been significantly restored near to normal in a dose-dependent manner as compared with SCI.

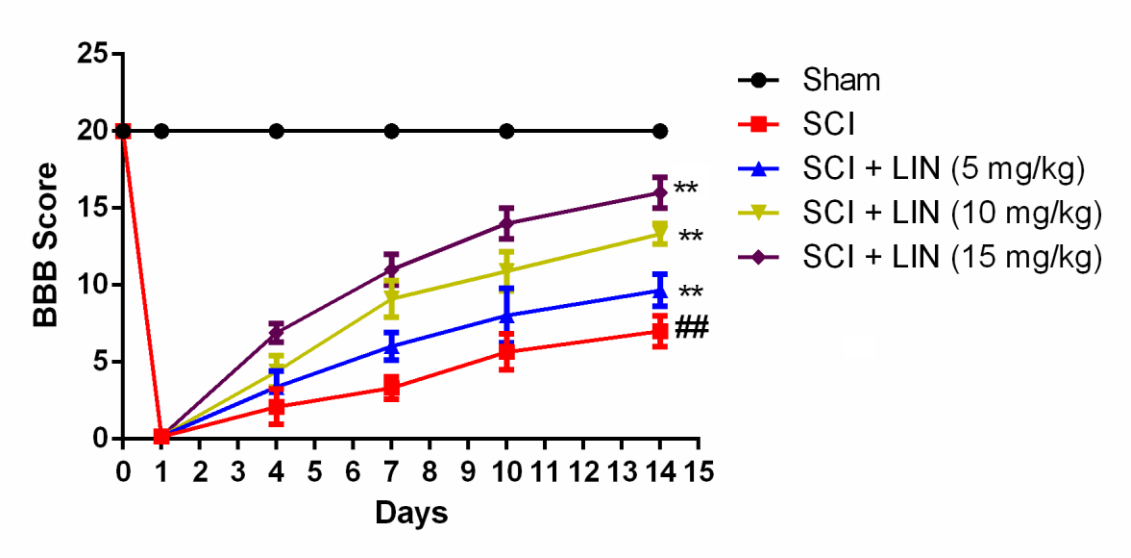

Figure No. 1
LIN improves motor function in SCI rats determined by BBB Score at baseline (1 day before surgery) as well day 1, 4, 7, 10, and 14 days post-SCI. Values represent the mean ± SEM and are representative of three independent experiments. $^{\#\#}p<0.05$ *vs* sham; $^{*}p<0.05$, $^{**}p<0.01$ *vs.* SCI, (n=4)

LIN prevents neuronal damage in the spinal cord

The effect of LIN on neuronal damage was studied using the H and E staining of injured spinal cord tissue. As shown in Figure No. 2, the SCI rats showed the presence of necrosis, cellular edema, and enhanced infiltration of inflammatory cells. However, none of these characters were observed in Sham treated group. The impact of SCI in the LIN treated group was found significantly lowered as compared to the SCI group. The LIN showed a reduction in the injury and alleviated spinal cord edema in mice. It also showed improvement in the histopathology of the spinal cord and the number of neurons observed was protected than that in the SCI group. It has been suggested that LIN showed a significant neuroprotective effect against SCI.

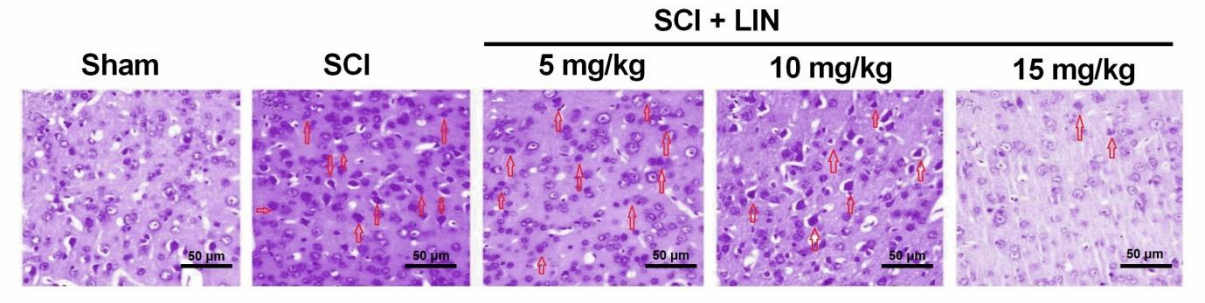

Figure No. 2
LIN showed improvement in neuronal architecture in the spinal cord of SCI rats, (n=4)

LIN reduces oxidative stress in the spinal cord

The level of oxidative stress in rats was quantified in rats after SCI. The SCI-treated rats showed a reduced level of GSH and SOD with a high level of MDA as compared to Sham-treated rats. Moreover, after administering LIN, the level of these studied biomarkers was found restored near to normal which confirms the antioxidant effect of LIN.

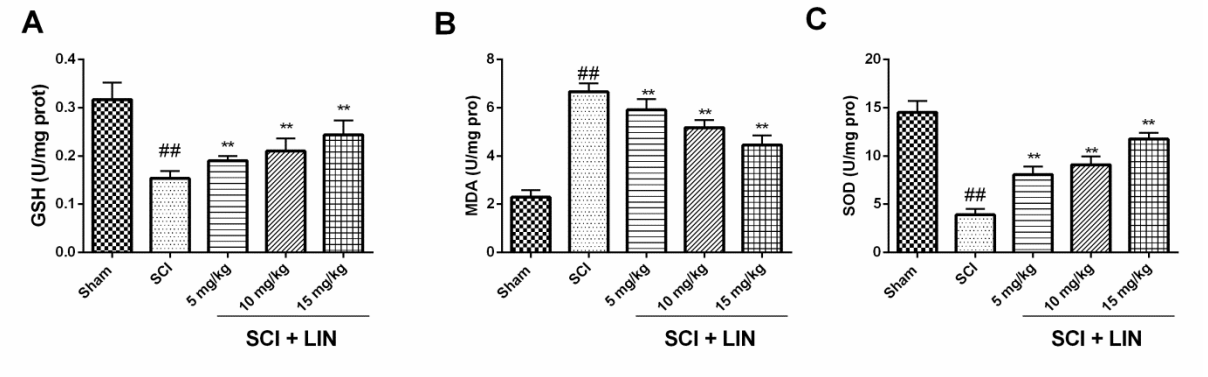

Figure No. 3

LIN showed a reduction in oxidative stress in SCI rats. (A) GSH, (B) MDA, and (C) SOD. Values represent the mean ± SEM and are representative of three independent experiments. $^{\#\#}p<0.05$ *vs* sham; *$p<0.05$, **$p<0.01$ *vs.* SCI, (n=6)

LIN reduces the generation of pro-inflammatory cytokines in the spinal cord

To assess the effect of LIN against inflammation, the level of pro-inflammatory cytokines was studied in the lesion site of the spinal cord in rats. As shown in Figure No. 4, the level of cytokines was found increased in the SCI group as compared to the control. However, a significant decrease was observed in the level of these cytokines (TNF-α, IL-1β, and IL-6) in the LIN-treated group. LIN causes dose-dependent reduction of inflammation as suggested by reduced pro-inflammatory cytokines.

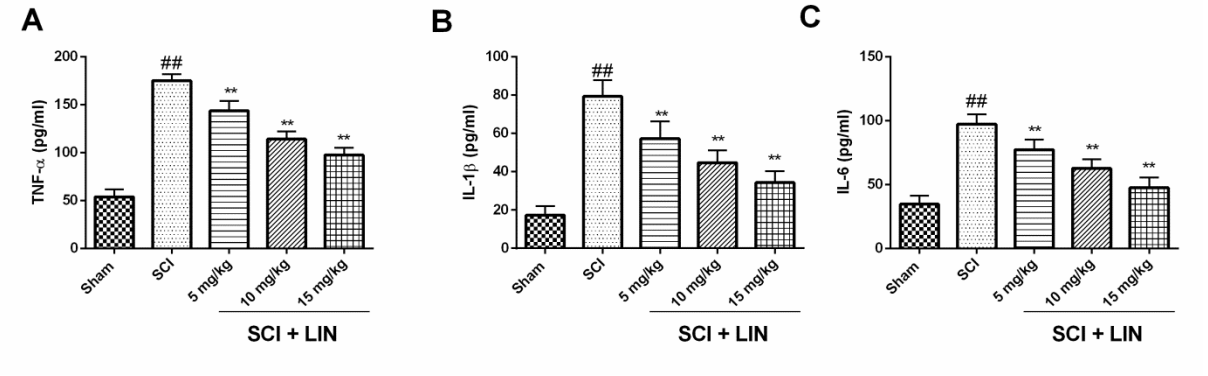

Figure No. 4

LIN reduces the generation of pro-inflammatory cytokines in SCI rats. (A) TNF-α, (B) IL-1β, and (C) IL-6. Values represent the mean ± SEM and are representative of three independent experiments. $^{\#\#}p<0.05$ *vs* sham; *$p<0.05$, **$p<0.01$ *vs.* SCI, (n=6)

LIN inhibited TLR4/NF-κB signaling pathway

The effect of LIN was investigated on the TLR4/NF-κB pathway using the western blot analysis. As shown in Figure No. 5, the SCI group showed increased expression of TLR4 and NF-κB as compared to Sham. However, LIN-treated rats showed a dose-dependent reduction of the expression of TLR4 and NF-κB in rats as compared to the SCI group.

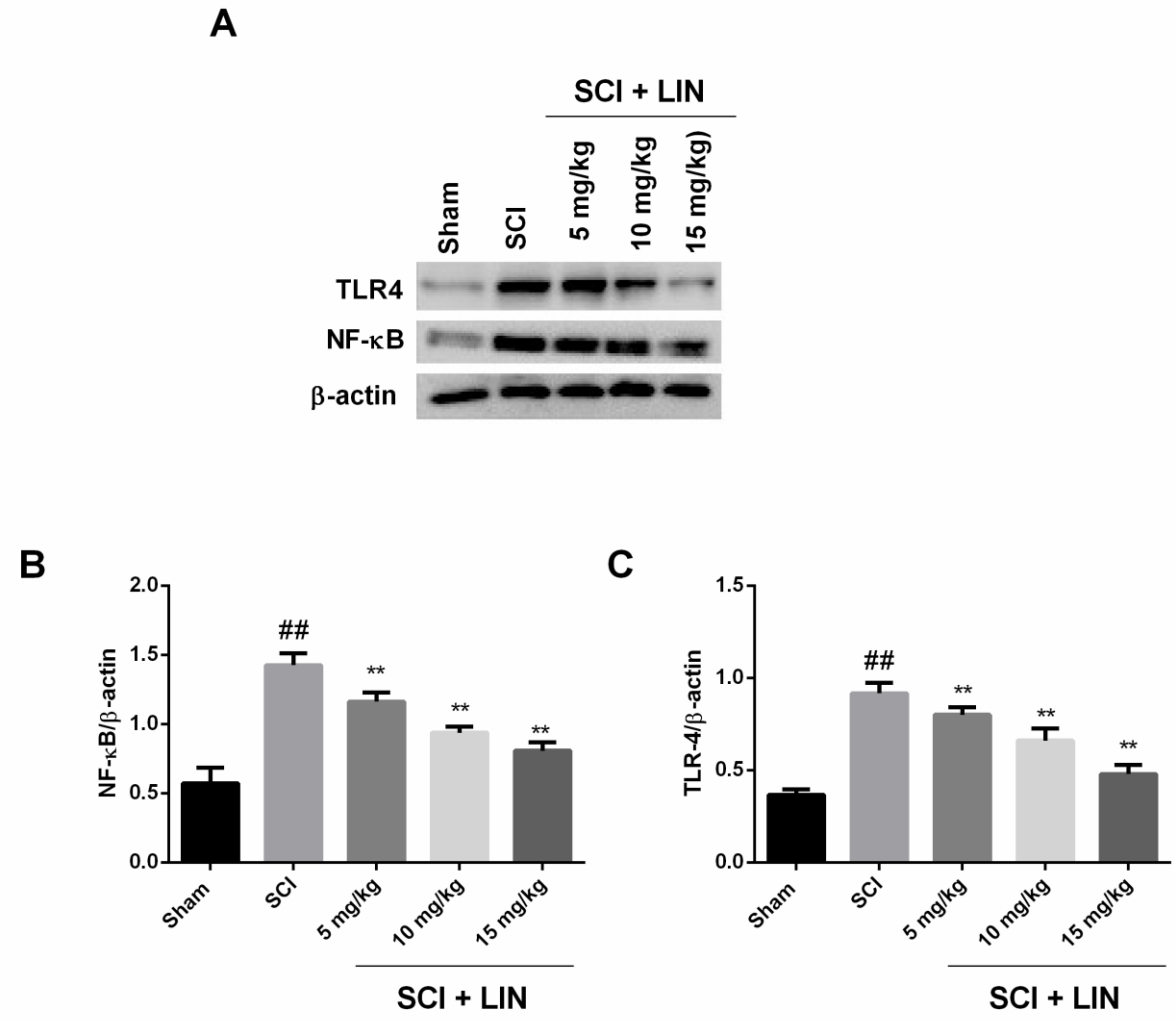

Figure No. 5

LIN inhibited TLR4/NF-κB pathway as shown in western blot analysis. Values represent the mean ± SEM and are representative of three independent experiments. $^{\#\#}p<0.05$ *vs* sham; $^{*}p<0.05$, $^{**}p<0.01$ *vs.* SCI, (n=6)

DISCUSSION

SCI is a critical life-threatening debilitating condition where the spinal cord got damaged due to sudden physical insult. The damage to the spinal cord leads to temporary or permanent changes in its neurological function and sometimes leads to paralysis (McDonald & Sadowsky, 2002; Rabinstein, 2018; Kwiecien *et al*., 2020). Majority of drugs that are used to treat SCI mainly target secondary events such as edema, hemorrhage, ischemia, inflammatory cell infiltration, the release of cytotoxic products, and cell death. Currently, many of drugs/molecules are used in clinical practice against SCI, but each has its merit and demerit (Courtine *et al*., 2011; Stein & Sheth, 2015). Till now, none of the single-agent provides significant benefits against SCI. The multifactorial nature of secondary injury provides impetus to discover newer agents that act by multiple pathways (Delamarter & Coyle, 1999). In our present study, we have demonstrated the significant protective effect of LIN against SCI via inhibition of oxidative stress, inflammation in rats. Motor function is the main critical task mediated by the spinal cord, and any injury to it leads to alteration in motor ability below the level of injury. In general, the higher in the spinal cord an injury occurs, the more function, sensation, and internal body functions will be affected (Dietz & Fouad, 2014; Thietje & Hirschfeld,

2017). In the present study, the BBB score in the sham group stayed at ~20 points which was the highest among these groups. However, upon treatment with LIN, the rats showed improvement in motor ability as suggested by increased BBB score in a dose-dependent manner. To further confirm the neuroprotective effect of LIN, histopathological analysis was conducted on the lesion site of the spinal cord in treated and non-treated rats. The SCI rats showed a significant reduction in neuron, necrosis, edema, and increased infiltration as compared to Sham. However, in LIN-treated rats, the injury was found reduced significantly preserved neurons via reduction of necrosis and edema. This observation indicates the LIN has a significant neuroprotective effect against SCI. Studies have shown excessive release of free radicals generates oxidative stress following SCI (Ali & Whitmore, 2016; Thietje & Hirschfeld, 2017; Kang *et al*., 2018). Oxidative stress is a phenomena where body is unable to clear-off the generated reactive oxygen species (ROS) due to impaired endogenous anti-oxidant defense system. The endogenous antioxidant defense system cataegorised in two type of system: enzymatic (e.g. superoxide dismutase (SOD), Glutathione (GSH), and catalase (CAT), etc) and non-enzymatic (Vit. A, Vit. E, and Vit. C, etc). The SOD is an enzyme that helps break down potentially harmful oxygen molecules in cells. It catalyzes the dismutation of two molecules of superoxide anion ($^{*}O_2$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2), consequently rendering the potentially harmful superoxide anion less hazardous. GSH is one of the most abundant low molecular weight non-protein thiols, modulates physiological levels of ROS and is involved in the cell's oxidative stress response. On the other hand, Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA. Malondialdehyde level is commonly known as a marker of oxidative stress and the antioxidant status SCI patients. ROS causes tissue damage by altering the antioxifdant defense system and promotes DNA damage, lipid peroxidation (through activation of cyclooxygenases and lipooxygenases), protein damage including gingival hyaluronic acid and proteoglycans, and oxidation of important enzymes (e.g., antiproteases, α-antitrypsin, stimulation of proinflammatory cytokine release by monocytes and macrophages by depleting intracellular thiol compounds and activating nuclear factor). These radicals target postsynaptic neurons and activate adjacent astrocytes and microglial cells, resulting in ionic unbalance of nerve cells. The oxidative stress causes lipid peroxidation in the injured neuronal tissues and leads to excitatory toxicity (Winkler *et al*., 2013; Dietz & Fouad, 2014; Ali & Whitmore, 2016; Thietje & Hirschfeld, 2017; Kang *et al*., 2018). This could extend the degeneration period of the *substantia alba medullae spinalis* and accelerate the apoptosis of oligodendroglia cells (Dong *et al*., 2003). In the previous study, LIN showed a strong antioxidant effect in patients with carpal tunnel syndrome (Seol *et al*., 2016), thus we were prompted to analyze the anti-oxidant behavior of LIN in SCI rats. As shown in the present study, LIN causes a reduction of MDA level and increases the activity of GSH level and SOD activity in a dose-dependent manner as compared to the SCI group. These results suggest that LIN significantly mitigates the consequences of oxidative stress and indicated that, its anti-oxidative effect may be involved in the neuroprotective effect against SCI. The oxidative stress activates glial cells and astrocytes and results in the release of inflammatory cytokines and TNF-α which progress the neuro-degeneration in SCI (Esposito & Cuzzocrea, 2011; Anwar *et al*., 2016; Ijaz *et al*., 2020). Many agents against SCI showed strong anti-inflammatory activity (Esposito & Cuzzocrea, 2010; Ni *et al*., 2015; Wang *et al*., 2016; Chen *et al*., 2018). In the present study, LIN causes a significant reduction in the level of pro-inflammatory cytokines as compared to SCI rats. These results were found in agreement with a previous study where LIN showed a strong anti-inflammatory effect in RAW264.7 cells and lipopolysaccharide-induced lung injury model (Huo *et al*., 2013). The study was further extended to elucidate the mechanism behind the anti-inflammatory effect of LIN. The effect of LIN was investigated on the TLR4/NF-κB signaling pathway using western blot analysis. Toll-like receptor 4 (TLR4) is among the ten sub-types of Toll-like receptors involved in spinal inflammation. It mediates amyotrophic lateral sclerosis, ischemia-reperfusion injury, neuropathic pain, and trauma (Yao *et al*., 2013; Zhang *et al*., 2013; Cell Signalling Technology, 2014). The TLRs activate NF-κB that triggers the expression of transcription of various pro-inflammatory cytokines that are involved in the inflammatory response in SCI. Studies showed that TLR4/NF-κB pathway is found activated in nerve cells and microglia, and its blockade provides a protective effect against SCI (Fan *et al*., 2009; Ni *et*

al., 2015; Xu *et al*., 2016). In the present study, our data showed that LIN inhibited TLR4/NF-κB pathway proteins and therefore repressed the SCI-induced secondary injury.

CONCLUSION

In conclusion, the findings demonstrated that administration of Linalool alleviated spinal cord injury via anti-inflammatory and antioxidant activities in spinal cord tissues. These protective effects may be attributed to the inhibition of TLR4/NF-κB activation. Therefore, LIN could be a promising drug for the treatment of SCI patients in the future.

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