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Original Article

Zerumbone Neuro-modulates Spinal Motor and Sensory Neurons of Rats: a c-Fos study

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ABSTRACT

Background: *A natural sesquiterpene Zerumbone (ZER) is abundant in rhizomes of Zingiber zerumbet (L) Smith, well-known for its beneficial effects. Very few reported studies of Zerumbone (ZER) as well as its bioactive compounds focusing on its neuromodulatory activities based on it functional neuro-anatomy.* **Objective:** *The aim of this study was to investigate the neuroanatomical functional site of the zerumbone in rat spinal motor and sensory neurons by means of c-Fos immunohistochemistry.* **Results:** *Our present study demonstrated that i.p., administration of zerumbone (100 mg/kg) significantly ($p<0.01$) increase in the number of c-Fos immunoreactive neurons in the dorsal and ventral nuclei of the rats.* **Conclusion:** *The result of the present study strongly indicates that zerumbone has neuromodulatory effects on sensory and motor neurons. To the best of our knowledge, it is the first report which indicates that zerumbone might play crucial roles in motor and sensory neuromodulation. Our findings warrant further study to reveal the underlying molecular mechanisms of zerumbone induced sensory and motor neuromodulation. We suggest the c-Fos immunohistochemical functional mapping of zerumbone in central nervous system of model animals to reveal its therapeutics potential and mechanisms.*

Keywords: *Zerumbone, c-Fos, Central nervous system, immuno-histochemistry, rats, spinal neurons.*

INTRODUCTION

Zerumbone is an active compound isolated from the wild ginger plant, *Zingiber zerumbet* (L.) Smith. is a member of the ginger family (Zingiberaceae). *Zingiber zerumbet* is locally known as lempoyang, This wild ginger primarily grows in tropical and subtropical regions. This plant is native to Southeast Asia. In traditional Asian folk medicine, the rhizome of this plant is reported for their vast medicinal properties. A wide range of studied documented the possible pain relief effects of zerumbone and strongly attributed that it effectively inhibited pain in models of nociception and inflammation including stomach ache, tooth ache. In a mice and rat model of neuropathic pain and nociception, zerumbone attenuated allodynia and hyperalgesia. The beneficial effect of zerumbone in rats with streptozotocin-induced diabetic nephropathy (DN) has been documented.

Reports further demonstrated that zerumbone exhibited neuroprotective effects in the transgenic mouse model, ameliorating neuroinflammation and restored non-cognitive and cognitive behavioral impairments. Studied showed that the neuroprotective effect of zerumbone resulted from its anti-neuroinflammatory activity. Recent studies indicated that zerumbone has several pharmacological effects, namely, anti-cancer, chemo-preventive, and anti-inflammatory effects. Zerumbone has been shown to reduce the expression of inflammatory cytokines and molecules in different inflammatory-induced cell cultures (kitayama et al., 1999, Matsuo et al., 2014; Koga et al., 2016; Burkill et al., 1966; Baby, 2009; Yob et al., 2011; Tzeng et al., 2013 ; Somchit et al., 2012; Chien et al., 2016; Butt and Sultan, et al., 2011; Sahebkar et al., 2011 ; Kim et al., 2019; Sultana et al., 2010; et al., 2001 ; Sulaiman et al., 2009; Perimal et al., 2010; Sulaiman et al., 2010; Zulazmi et al., 2015; Zulazmi et al., 2017; chai et al., 2016; Gopalsamy et al., 2017).

Although there have been quite a number of studies conducted to understand the effectiveness of *Z. zerumbet* in a wide range of biological activities, there has been very few reported studies of Z. zerumbet as well as its bioactive compounds focusing on neuromodulation based on it functional neuro-anatomy. To the best of our knowledge, there are no studies that have focused on the underlying molecular mechanism of action of zerumbone in anti-nocipection, therefore the neuro modulatory pain pathways of zerumbone has not been investigated. Thus although numerous ethno-medicinal studies have reported the therapeutic properties of zerumbone on pain modulation, the mode of action of its anti-nocicepetive activity has not been elucidated.

Spinal cord dorsal horn nucleus is a critical area in relaying and modulating pain. Corticotrigeminal pathway demonstrated more than a century ago that the nociception inhibitory influences occurred through both presynaptic and postsynaptic mechanisms. Recent study in a neuropathic pain model demonstrated that suppressing the elevated activity of corticotrigeminal neurons suppressed this pain behaviors by means of reduced cFos expression in spinal nucleus (Wang et al., 20015, Melzack et al., 1965; LeBars et al., 1979; Willis et al., 1991; Sluka et al., 1993; Lin et al., 1994; Stifani et al., 2014).

Understanding the underlying neuromodulatory functional mechanisms of zerumbone on spinal sensory and motor neurons is among the fundamental steps required to elaborate the exact therapeutic potential of zerumbone in pain pathways as well as other effects observed elsewhere. Most importantly, the functional neuroanatomical map of zerumbone in spinal cord nuclei is not available.

The immediate early genes (IEGs) encoded protein c-Fos is considered to be a transcription factor. c-Fos is expressed at low or undetectable levels in most cell types, but can be rapidly and transiently induced by many types of stimuli, including tissue trauma. C-Fos is a functional anatomical marker of activated neurones within the central nervous system. Monitoring the nuclear expression of c-Fos, the protein product of the c-*fos* gene, is an established reliable anatomical technique for the functional mapping of the neuronal activity and can be helpful to examine the ability of neurons to react with changes in gene expression to external stimulation under physiological, pathological and pharmacological challenges (Vivacqua et al., 2012; Sheng et al., 1990; Morgan et al., 1991; Herdegen et al., 1998; Kazi et al., 2002; Kazi et al., 2007Li et al., 2020).

Investigating the molecular mechanisms of zerumbone induced nuromodulation in sensory and in the motor nuclei might open up new research avenue for zerumbone and its therapeutics potential in pain and motor disorder system. Therefore, this study we investigated the neuroanatomical functional site of the zerumbone in rat spinal nuerons by means of c-Fos immunohistochemistry.

METHODS

We obtained approval from the Institutional Animal Care and Use Committee for this study. All experimental procedures were carried out in accordance with CARE Universiti Teknologi MARA (UiTM), guidelines.

Male Sprague−Dawley rats (180−200 g), 5−6 per group, were used in all experiments. Animals were housed in groups of two, and had free access to food and water at all times. Animals were on a fixed 12 h light−dark cycle. In order to minimize stress, all animals were brought to the laboratory and acclimatized once daily for at least three days by exposure to the general handling and anaesthesia procedures. The surgical procedure was based on that described previously (Kazi et al., 2002). Briefly, rats were anaesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg) (CEVA Sante Animale, France). Zerumbone (Zer, > 98% of Purity) and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (Munich, Germany). Zerumbone was dissolved in DMSO to produce a stock solution. Same volume or amount of DMSO and/or PBS was applied as vehicles. A single intraperitoneal injection (1ml i.p.) of zerumbone (100 mg/kg) was given (Kazi et al., 2007; Li et al., 2020; Zulazmi et al., 2015). In control group (intact) i.p. normal saline (saline treated) was administered. In all cases the dosing volume was 1 ml. Rats of all groups were kept under deep anesthesia until sacrifice to minimize the pain perception. At 90 minutes after zerumbone / saline administration the deeply anesthetized rats were perfused transcardially with heparinized physiological saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, containing 0.2% picric acid for 20 min. The cervical part of the spinal cords were removed and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 4 hrs at 4℃. The spinal cords were cryoprotected in 25% sucrose phosphate-buffered saline (PBS). Serial 40 μm thick sections were cut with a freezing microtome and immersed in PBS. The immunocytochemical detection of c-Fos protein was performed using the peroxidase-antiperoxidase (PAP) detection protocol (Kazi et al., 2002 ; Kazi, and Gee, et al., 2007). To insure penetration of antibodies, the sections were pre-incubated in a solution containing 0.3% Triton X-100 in PBS after blocking endogenous peroxidase. Free-floating sections were then incubated for 3 days at 4℃ with rabbit antibody to c-Fos protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:4,000 in PBS containing 0.3% Triton X-100. The sections were incubated in a 1:2,000 dilution of goat anti-rabbit IgG (E-Y Laboratories Inc., San Mateo, CA) for 1 day at 4℃ and followed by the tertiary antibody, peroxidase-antiperoxidase (PAP) in a 1: 2,000 dilution and incubated for 1 day at 4℃. The primary, secondary and tertiary antibodies were diluted to appropriate concentrations in 0.3% bovine serum albumin and 1% normal goat serum and 0.05% sodium azide. Between the incubation steps, sections were thoroughly washed with PBS. The DAB method for visualization of peroxidase was used. The sections were treated for 20 min. at room temperature with 0.02% diaminobenzidine in 0.003% hydrogen peroxide. After the final rinses in 0.05 M Tris-HCl- buffer, pH 7.6, the sections were mounted onto gelatin-chrome alum-coated glass slides, air dried, dehydrated with ethanol, cleared in xylene, and coverslipped. The specificity of the immune reaction was assessed by omitting the primary antibody in which no specific immunostaining was detected.

The sections were analyzed under a microscope and the labeled c-Fos positive neurons per section were counted. Counts were obtained from a 1 in 2 series of sections and 6 sections per animal were used. Then, the mean number of c-Fos positive neurons (± standard deviation: S. D.) for each time point was calculated. The statistical analysis was performed using a paired *t* test and differences at $p<0.001$ were considered significant.

RESULTS

In this study at 90 minutes after the zerumbone administration (i.p.), a robust increase in Fos expression was found bilaterally in the dorsal (Fig.1 A & B) and ventral nuclei (Fig.2, B & C) of the spinal cord.

As shown in Fig. 1, compare to intact the higher numbers of densely labelled c-Fos positive neurons were observed bilaterally in the dorsal nucleus at 90 minutes after zerumbone administration (100 mg/kg, i.p.). The semi-quantitative analysis of the number of the c-Fos-positive nerve somata per section revealed 2.1± 0.5 in the right side and 2±.06 in the left side of the dorsal nucleus in the intact rats. Compared with the intact rats ($p<0.001$), the number of c-Fos immunoreactive cell bodies was 56 ± 3.5 per section in the left side and 58 ± 3.1 per section in the right one in the dorsal nucleus zerumbone administration increased Fos expression by 18 folds (Fig. 1C).

In the ventral nucleus, compared with the intact rats ($p<0.001$), the number of c-Fos immunoreactive cell bodies was 27.1 ± 2.2 per section in the left side and 29.0 ± 2.4 per section in the right side. In the ventral nucleus zerumbone administration increased Fos expression by 14 folds (Fig. 2D). These results suggested zerumbone pre-treatment induced activation of ventral nucleus of the spinal cord.

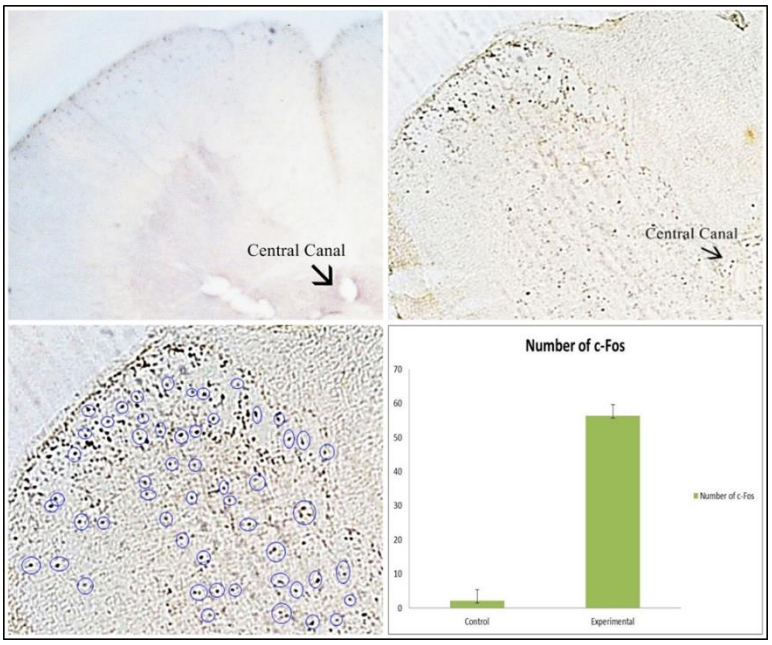

Figure 1: c-Fos expression in dorsal nucleus A Control; B experimental; C Higher magnificant of (B); D semi-quantitative analysis of c-Fos positive neurons.

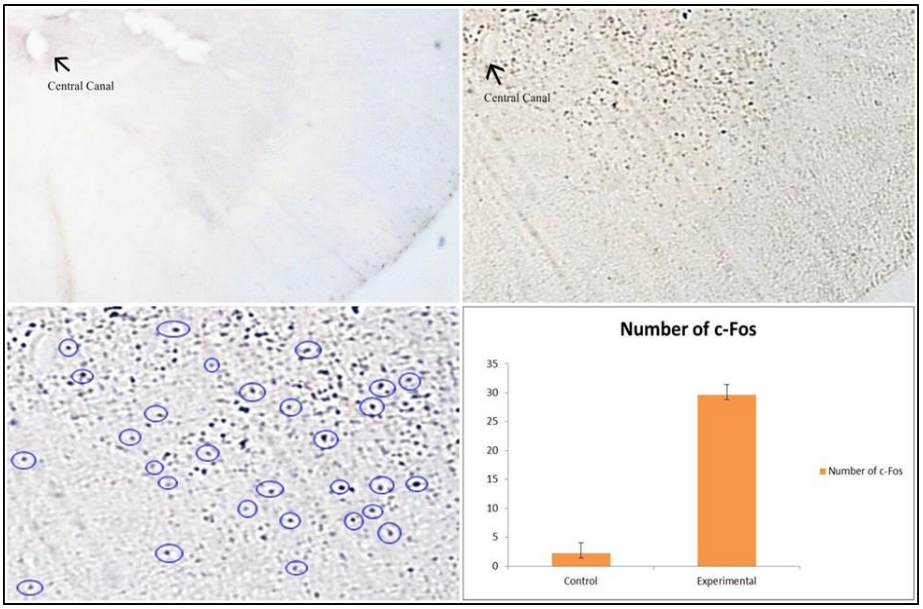

Figure 2: c-Fos expression in ventral nucleus A Control; B experimental; C Higher magnificant of (B); D semi-quantitative analysis of c-Fos positive neurons.

DISCUSSION

The result of this study demonstrated that zerumbone administration (100 mg/kg) induced increase in c-Fos expression in the dorsal and ventral nucleus of the spinal cord (Fig.1 & 2).

The central pain processing circuits for nociceptive information begin at the level of the spinal cord dorsal horn. Interneuronal networks in the dorsal horn are responsible not only for the transmission of nociceptive information but also help modulate that information and pass it on to other spinal cord neurons, therefore, certain patterns of stimulation can lead to increase nociceptive transmission or inputs result in the inhibition of projection neurons. The balance of these excitatory and inhibitory processes is the mechanism of modulation of pain in the spinal cord dorsal horn. The excitatory neurotransmitters in the dorsal horn include amino acids, particularly glutamate, as well as neuropeptides, such as SP, CGRP, VIP, somatostatin, and others. For example, peripheral nerve damage, can lead to an up regulation or down regulation of these. Another example is the increased stores of glutamate in the dorsal horn that occurs after the development of experimental arthritis.

The excitatory neurotransmitters in the dorsal horn are mediated by a number of neurotransmitters, including inhibitory amino acids, such as GABA and glycine, as well as neuropeptides, such as encephalin (Melzack et al., 1965; LeBars et al., 1979; Willis et al., 1991; Sluka et al., 1993; lin et al., 1994; Stifani et al., 2014). In the light of these studies, the increased in c-Fos expression in present study suggest that zerumbone might have neuromodulatory function on spinal dorsal neurons.

Motor neurons are located in the ventral horn of the spinal cord and control effectors muscles in the periphery. To ensure fine coordination, motor neurons acquire and retain the identity of muscles they innervate and integrate the functional neuronal circuitry of movements. Disorders in the spinal motor neurons lead to atrophy and/or spasticity of the associated musculatures. For example, the motor symptoms of the Parkinson disease are involved in spinal motor neurons (Stifani et al., 2014; Vivacqua et al., 2012). Therefore, the zerumbone induced increased in c-Fos expression in our study strongly indicated that understanding the underlying neuromodulatory functional mechanisms of zerumbone on spinal motor and sensory neurons is among the fundamental steps required to elaborate the exact therapeutic potential of zerumbone.

Primary afferents of trigeminal neurons express receptors for a variety of neurotransmitters and neuroactive substances. Primary trigeminal afferents carry somatosensory information from mechanoreceptors, thermoreceptors and nociceptors in the face, the oral and nasal cavities. Most importantly, the axons of the trigeminocervical nucleus are densely connected with axons of spinal ganglia of C_1–C_3 spinal nerves via interneurons of the trigeminocervical nucleus. Pain originating from the teeth, jaws or TMJ project into the cervical area and vice versa. Therefore, nociception from the C_1–C_3 innervation area also project to the orofacial region. This relation represents the fundamental neuroanatomical basis of cervicogenic orofacial pain (COP). This pattern is known as trigeminocervical functional convergence and studies documented that its linked with many clinical interconnecting syndromes, e.g., atypical facial pain, cervical spondylosis, occipital neuralgia, migrane, TMJ pain. Increased in c-Fos expression in this study suggest that further study may reveal the possible therapeutics implication of zerumbone in cervicogenic orofacial pain (Lazarov et a., 2007; Bogduk et al., 2009).

Previous study reported that zerumbone increased cAMP levels(Kim et al., 2018). cAMP is one of the most common and universal second messengers. The cAMP regulate highly diverse physiologic processes, metabolism, secretion, calcium homeostasis, muscle contraction, cell fate, and gene transcription, thereby to permit delicate adaptations of biologic responses (Beavo et al., 2002; Siddiq et al., 2015). Report demonstrated that drug compound altered specific splicing patterns of gene (survival motor neuron gene, *SMN2* gene lacks exon 7) which represented a new approach to modification of gene expression in disease treatment. Previous study demonstrated that c-*fos* expression levels in alpha motoneurons were associated with the activity levels of their corresponding muscle (Andreassi et al., 2001; Omori et al., 2005).

cAMP-responsive element-binding protein (CREB) phosphorylation mediatesd *c-fos* expression in response to agents that increase intracellular concentrations of cAMP or Ca^{2+}. Similarly, nerve growth factor appeared to stimulate *c-fos* transcription via phosphorylation of CREB. CREB-binding sites have been found in the promoter regions of immediate-early genes such as *c-fos.* The c-AMP-responsive element binding protein (CREB) and its phosphorylated product (P-CREB) are nuclear proteins expressed after stimulation of pain-producing areas of the spinal cord (Ginty et al., 1994; Sassone et al., 1988; Andreassi et al., 2001). Therefore our present result strongly suggests further study to investigate the zerumbone regulated neuromodulation in a spinal model of inflammation, hyperalgesia sensory and neuronal spasticity.

Therefore, our present result indicate that in order to reveal the exact functional role of zerumbone in spinal motor and sensory nuclei further study is required to identify the nature of these c-Fos expression (e.g., neurotransmitter, receptors e.t.c.) observed in this study (Kawaguchi et al., 199; Liu et al., 2007).

Recent study observed that zerumbone modulate spinal receptors in neuropathic pain model. Taken together, these findings along with our present results suggest that further study is required to reveal the underlying neuromodulatory mechanism of zerumbone on spinal motor and sensory neurons. In the light of the above mentioned reports our present results highlight the fact that further studies are clearly needed to elucidate the underlying functional neuromodulatory mechanisms that played by zerumbone to induce c-Fos expression in the spinal sensory and motor neurons.

To our knowledge, the present study demonstrated for the first time that spinal sensory and motor neurons of rat CNS are possibly the neroanatomical functional sites of zerumbone neuromodulation. Further studies are necessary to substantiate this observation, as spinal sensory and motor neurons contains a variety of peptides often different biochemical and functional nature and not all activated neurons express c-Fos (Kawaguchi 1997; Kawaguchi et al., 1995). Previous study indicated that c-Fos expression in the brain was induced by transsynaptic stimulation (hirakawa and Kawata 1993). Therefore, our present result indicate that in order to reveal the exact functional role of zerumbone in spinal motor and sensory nuclei further study is required to identify the nature of these c-Fos expression (e.g., neurotransmitter, receptors e.t.c.) observed in this study.

A previous report demonstrated that c-*fos* expression and fMRI both methods detect similar brain nuclei in response to same stimulus pressure in anaesthetized rats (Curran et al., 1996). More specifically, functional *c-fos* imaging has become available *in vivo*. Single-stranded phosphorothioate-modified oligonucleotides with sequences complementary to *c-fos* (sODNs) were coupled to superparamagnetic iron oxide nanoparticles (SPIONs) and injected intracerebroventricularly. These SPION-cfos constructs after hybridising to stimulus-induced *c-fos* mRNA produced a magnetic resonance signal that can be detected in living animals by MRI (liu et al., 2007, Lazovic et al., 2005). These reports strongly support that c-Fos has become a valuable translational tool to bridge clinical and preclinical research in animal or in animal models. In the light of the above studies our present results indicate that EL might have wide range of unknown therapeutic functions.

CONCLUSION

Our present findings demonstrated for the first time that zerumbone may modulate the c-Fos expression in the rat spinal sensory and motor neurons. Furthermore, zerumbone might have wide range of unknown therapeutic potentials in orofacial sensory and motor neuromodulatory pathways and indicate for further study of possible potential effects of zerumbone in trigeminocervical pathways as well. The results obtained here will be useful for further studies to gain more insights into the zerumbone induced underlying neuromodulatory mechanisms in spinal sensory and motor neurons of rat CNS.

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