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Citicoline Administration Increases the *Brain-derived Neurotrophic Factor* (*Bdnf*) Expression in the Trigeminal Ganglion of Rats Post-mental Nerve Injury

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The number of neurons in the sensory ganglion decreases after a peripheral nerve injury (PNI) caused by oral trauma or maxillofacial surgery, resulting in an incomplete nerve regeneration process. Thus, there is an urgent need to reduce the risk for potential complications after PNI, including neuropathic/ectopic pain and allodynia. Citicoline administration reportedly can improve motor function and prevent neuropathic pain in a rat model of PNI. Therefore, the present study aimed to assess the effect of citicoline administration on *Brain-derived neurotrophic factor* (*Bdnf*) expression, which is an early indicator of an ongoing nerve regeneration process, and the number of trigeminal neurons at the chronic phase after a PNI in a rat model. The PNI model was established by clamping the mental nerve of Wistar rats with a non-serrated clamp for 30 s. The animals were divided into the following three groups: sham-operated; clamp-injured rats receiving saline as the controls; and clamp-injured rats receiving a daily dose citicoline 50 mg/100g body weight intraperitoneally immediately after surgery for 7 days. They were sacrificed on days 1,3, and 7 for the acute phase analysis to examine the changes in *Bdnf* expression using quantitative reverse transcription polymerase chain reaction. Subsequently, the chronic phase analysis was done by counting the neuron number in the trigeminal ganglion on day 28 post-injury using the stereological method. In the acute phase, citicoline administration increased the *Bdnf* expression by 2.19 times only on the third-day post-injury, indicating the start of an early regenerative process. However, in the chronic phase, the total number of neurons in the trigeminal ganglion remained similar in all groups, suggesting the possibility of inadequate injury level. In conclusion, although there was no neuronal loss after a mental nerve injury, citicoline administration increased the Bdnf expression at the trigeminal ganglion immediately after the nerve injury, and this may accelerate nerve regeneration.

Keywords: *Bdnf*; Citicoline; Peripheral Nerve Injury; Trigeminal Ganglion.

Peripheral nerve injury (PNI) is a potential complication occurring after oral trauma and maxillofacial surgery caused by surgical procedures, including odontectomy on impacted teeth, dentoalveolar surgery near the mental foramen and mandibular canal, orthognathic

surgery, jaw tumor removal, placement of internal fixation screws in fractured mandibular bones, and dental implant placement, which could lead to nerve compression[1]. The injured neurons in the peripheral nervous system (PNS), unlike those in the central nervous system (CNS), can regenerate spontaneously[2,3]. Although many cases of PNI are managed through observation over several weeks to months, incomplete nerve regeneration is frequently detected due to the lengthy and insufficient regeneration process[4]. Interestingly, after PNI, approximately 10%–30% of neurons in the sensory ganglion are likely to die primarily through apoptosis[5,6]. Thus, the prevention and treatment of PNI are essential to reduce the risk for complications, including neuropathic pain, allodynia, and ectopic pain, and ultimately for complete peripheral nerve regeneration[7].

Sensory neurons require neurotrophic factors, including Nerve Growth Factor (NGF), Brain-derived Neurotrophic Factor (BDNF), and Ciliary Neurotrophic factor (CNF), for survival, proliferation, and regeneration[6,8]. The endogenously secreted neurotrophic factors can enhance the survival of injured neurons and increase their expression during PNI[9]. However, their responses are slow[10–12]. BDNF induces an intrinsic neural growth program[13], prevents atrophy of neurons after axotomy, stimulates GAP-43 mRNA expression, and promotes axonal regeneration[14,15]. However, the direct administration of neurotrophic factors is expensive and its preparation is complicated, highlighting the urgent need for alternative treatments. One of the less costly substances, such as mecobalamin, has been shown to induce peripheral nerve regeneration via the upregulation of neurotrophic factors, including BDNF, in the sensory ganglion of a mouse model of PNI[16]. However, due to the nature of mecobalamin as a supplement that required high-dose administration, its clinical benefits are limited[16,17].

Interestingly, citicoline is another potential alternative for promoting peripheral nerve regeneration. Citicoline is an exogenous form of cytidine-5'-diphosphocholine (CDP-choline), an endogenous intermediate in the synthesis of phosphatidylcholine. The local synthesis of phosphatidylcholine in distal axons is critical for normal axon growth[18]. Citicoline administration induces phospholipid synthesis, maintains membrane integrity, and creates new membrane materials necessary for growing axons[18,19]. The antioxidant potential of citicoline in preventing free radical damage and fostering phosphatidylcholine synthesis in axons has also been reported[20]. In clinical practice, citicoline has been used to induce nerve regeneration in various CNS pathologies, including ischemic stroke, cognitive disorders, and glaucoma[21–23]. In a PNI rat model, citicoline administration can potentially improve motor function[24,25] and prevent post-injury neuropathic pain[26], in a dose-dependent manner[27]. As compared with *in situ* application, the systemic administration of citicoline has a similar effect, in terms of inducing axonal regeneration and motor function recovery[28] as well as preventing neuropathic pain[26]. However, whether citicoline affects peripheral nerve regeneration and prevents neuronal loss in the sensory ganglion is still unknown. Thus, based on the reported effect of citicoline on peripheral nerve regeneration, the present study aimed to assess the effect of systemic citicoline administration on *Bdnf* gene expression as an early indicator of nerve regeneration in the trigeminal ganglion at the acute phase and to estimate the number of neurons at the chronic phase of the rat PNI model.

MATERIALS AND METHODS

Animal model

The animal study was approved by the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (number KE/FK/829/EC/2015). Three to four-month-old male Wistar rats were used in this study. The experimental setup is illustrated in Figure 1. Hereto, the animals were divided into the sham-operated, saline-treated, and citicoline-treated groups. In the sham-operated group, an incision was made on the right jaw, which was directly sutured. The saline-treated group was clamp-injured and received saline by intraperitoneal injection, whereas the citicoline-treated group received a daily dose of citicoline of 50mg/100g body weight (BW) (Dexa Medica, Indonesia) intraperitoneally, starting at 5 min post-injury for 7 days.

On the day of surgery, intramuscular

anesthesia was administered using a mixture of 0.3-ml ketamine-HCl and 0.05-ml xylazine in 1-ml saline. PNI was established by exposing the right mental nerve to a clamp injury made from a 4-mm non-serrated clamp for 30 s. The clamp strength was 16 kg. Following the surgery, the animals received a daily oral dose of amoxicillin 50mg/100-g BW and 20 mg/100g BW ibuprofen for 3 days. The animals were sacrificed on days 1,3, and 7 post-injury for the acute phase analysis and on day 28 post-injury for the chronic phase analysis.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Three rats per group were sacrificed on days 1, 3, and 7 post-injury. The rats were decapitated, and the right trigeminal ganglion was dissected for RNA isolation using the RNeasy mini kit (Qiagen, Germany). Altogether, 2 µg RNA was reverse-transcribed using Transcriptor FirstStrand cDNA synthesis (Roche, Switzerland).

Quantitative RT-PCR specific primers were designed using Primer-BLAST from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Rat *Bdnf* expression was analyzed using a LightCycler FS DNA MasterPLUS SYBR Green (Roche, Switzerland) on a LightCycler Carousel (Roche, Switzerland) following manufacturer's protocol. The *Bdnf* expression is an early indicator of peripheral nerve regeneration after an injury[29]. Relative gene expression was calculated based on $2^{-(Cp\ Bdnf-Cp\ Gapdh)}$ equation normalized to the reference gene *Gapdh*.

Neuron counting

On day 28, six rats per group were transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. The right trigeminal ganglions were exposed and excised by cutting the trigeminal roots at their entrance in the brainstem, the mandibular branch 5 mm distally to the ganglion, and the maxillary branch at the level of the orbital fissure. The ganglion was kept in a fixative at 4°C until subsequent processing.

The ganglions were randomly rotated along their longitudinal axis and individually embedded in 20% agar to guarantee isotropy along the longitudinal axis of the ganglion. The ganglion-containing agar underwent dehydration and clearance before being processed for methyl methacrylate plastic embedding Technovit (EMS, USA). Before the final embedding step, each ganglion was rotated once more along its longitudinal axis. Vertical uniform random sections (VURSs) were used instead of the simpler Systematic Uniform Random Sampling (SURS), because the sampling was designed for another study. The VURSs were obtained serially on a rotary microtome at a thickness of 30 µm. Every other section (f_1 = ½) was mounted on object glasses and stained with toluidine blue. Every three sections (f_2 = ½) were observed using an Olympus microscope 60 x objective lens and a numerical aperture of 1.4. Counting frames were laid using the newCAST stereological software (Visiopharm, Denmark) to obtain the area sample fraction (f_3 = 1/24.2). The total fraction sampling was 1/2 x 1/3 x 1/24.2 = 6.89 x 10^{-3}. The neurons were counted with the nucleolus as the counting unit. The counted neurons (Q^-) were used to calculate the total number of neurons for each ganglion using the following formula: total number of neurons = Q^- x 1/f. The coefficient of error was calculated accordingly[30].

Data analysis

Prism 9 (GraphPad, USA) was used for preparing the graphs and statistical analyses. Neuronal counting data were examined by performing a one-way analysis of variance (ANOVA), as indicated in the figure legends. P-values < 0.05 were considered significant.

RESULTS AND DISCUSSION

PNI model in Wistar rats

Modeling PNI has been performed in *in vitro, in vivo* to *ex vivo* studies. Previous *in vivo* studies have employed different animal types, ranging from Drosophila, zebrafish, and rodents, to non-human primates, depending on the studied nerve types[31]. Moreover, the proportion of neuronal deaths varied in different injury models and within different time frames[31–34]. Therefore, in the present study, we used Wistar rats to model PNI with slight modifications from a former study[35]. The injury was done at the mental nerve, a general somatic afferent sensory nerve of the face. It is a branch of the posterior trunk of the inferior alveolar nerve, itself a branch of the mandibular nerve (CN V_3), itself a branch of the trigeminal nerve (CN V). The mental nerve emerges from the mandibular foramen mentalis and branches below the musculus

depressor anguli oris into the following three parts: one branch innervates the skin of the chin, and the other two innervates the skin and mucosa of the lower lip. The neuronal cell bodies are located in the ipsilateral trigeminal ganglion[36].

Mental nerve injury in rodents has been used as a model of PNI and regeneration models[35]. The injury was established by clamping the mental nerve using a non-serrated clamp (**see Materials and methods**). Immediately after surgery, the clamp-injured rats received treatment either with saline as the control group or with a daily dose citicoline for 7 days. In our study, all rats survived until the termination day and no unclosed wound was observed in the operated area. Moreover, daily observation post-surgery and during treatment showed that the eating behaviors of the animals did not change due to the clamping injury at the mental nerve. In our previous study, clamping injury at the mental nerve considerably reduced the diameter of the nerve fiber and axons, as shown by osmium tetroxide (OsO_4) staining. Subsequently, the administration of citicoline ameliorated the injury, as evidenced by the larger nerve fiber and axon diameters, suggesting a nerve regeneration process (published data)[37]. Together, our PNI model is shown to be a representative model for studying the effect of citicoline on peripheral nerve regeneration.

Citicoline treatment increases Bdnf expression in the trigeminal ganglion during the post-injury acute phase

First, to examine the effect of citicoline administration on the PNI model, we assessed the gene expression level of *Bdnf*, which is among the most important neurotrophic factors for survival, migration, and differentiation in nerve regeneration[15]. Previously, citicoline was shown to promote nerve regeneration in pathologic conditions, such as multiple sclerosis, (MS) by enhancing early remyelination in the rat model of de- and remyelination even at a low dose[38]. Moreover, citicoline also improves the functional recovery and regeneration of the sciatic nerve in rats, as shown by the lower density of connective tissue surrounding the nerve, higher axon counts and diameters, thin collagenous scar formation, lesser neuropathic pain intensity, and improved

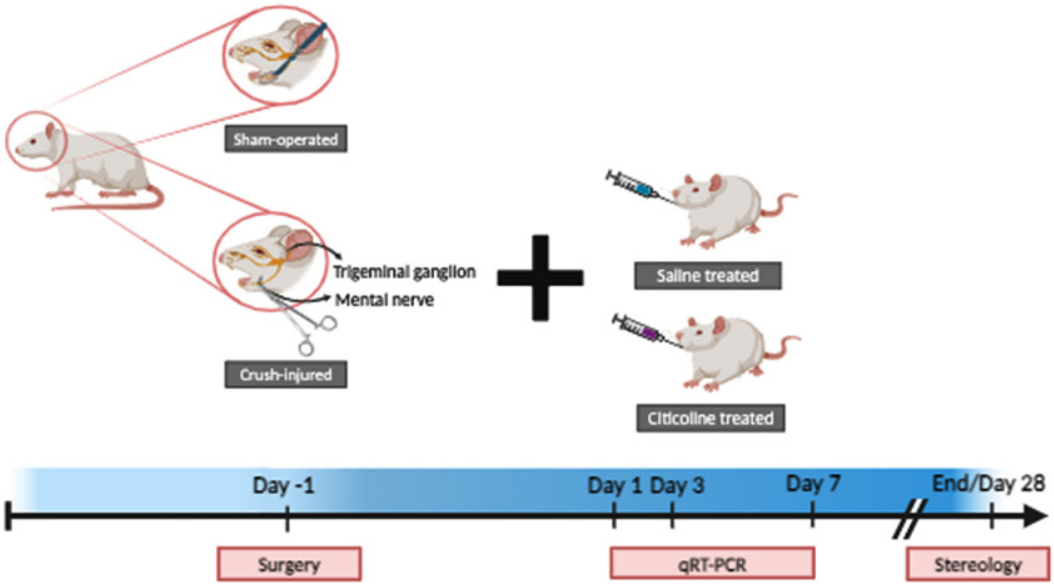

Fig. 1. Experimental setup to model PNI in Wistar rats. The mental nerve at trigeminal ganglion was clamp-injured using a non-serrated clamp at Surgery day (Day -1). Subsequently, the animals were treated using saline as the controls or a daily dose of citicoline (50mg/100g BW) for 7 days. The RNA extraction from trigeminal ganglion was done at the acute phase for analysis using qRT-PCR, while stereology analysis was performed at the chronic phase by counting the number of neurons. Image was created using BioRender

motor function[24,26]. The mechanisms by which citicoline exerts those effects are well described in the literature as the exogenous source of phosphatidylcholine, a type of phospholipid found in the neuronal membrane, which is critical for neurite growth and neuronal regeneration[39].

The effect of citicoline on *Bdnf* expression after PNI over time is shown in Figure 2. In our study, intraperitoneal citicoline administration for 7 days after mental nerve injury increased the level of *Bdnf* expression by approximately 1.5 times in the trigeminal ganglion of the saline-treated group, as compared with the sham-operated group, which may suggests the baseline expression of *Bdnf* after injury (Figure 2). Only on the third day (Day 3), citicoline administration increased the *Bdnf* expression up to 2.19 times in the trigeminal ganglion of the citicoline-treated

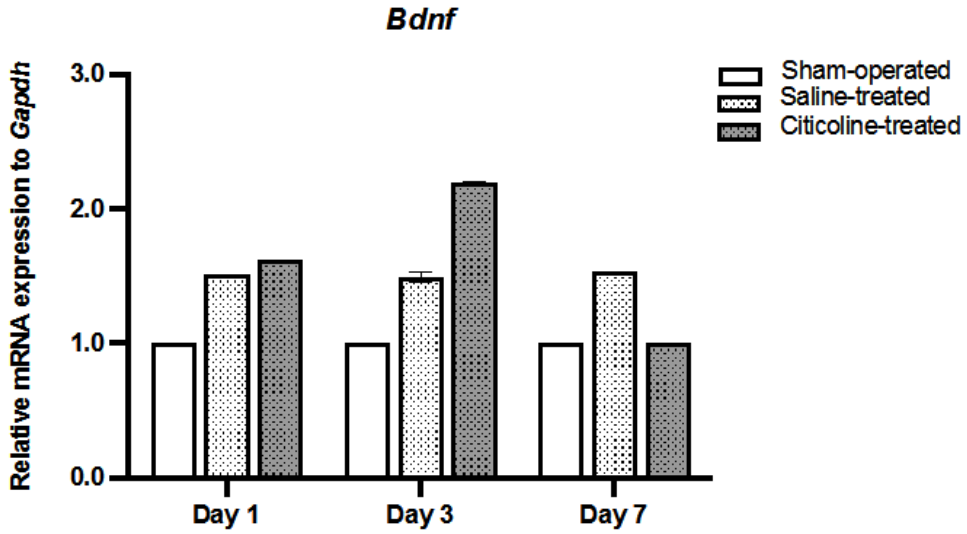

Fig. 2. The expression level of *Brain-derived neurotrophic factor* (*Bdnf*) in the trigeminal ganglion after ipsilateral mental nerve injury treated with saline or citicoline (50mg/100g body weigt) at the acute phase (days 1, 3, and 7) post-injury

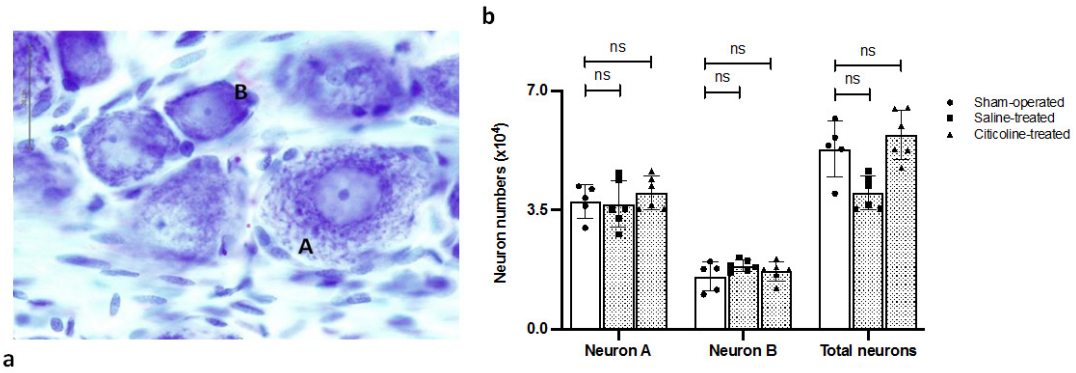

Fig. 3. (a) Representative microscopic image of neuronal types observed in trigeminal ganglion after ipsilateral mental nerve injury, stained using toluidine blue. Two types of neurons were determined based on their size, namely the larger Neuron type A (A) and the smaller Neuron type B (B). Scale bar indicates 35µm. (b) Total neuron numbers in trigeminal ganglion after ipsilateral mental nerve injury treated with saline and citicoline (50mg/100g BW) at chronic phase (28 days post-injury). Statistical analysis was done using one-way ANOVA with P value < 0.05.

group, as compared with the sham-operated group. However, after 7 days, the *Bdnf* RNA expression level decreased to a level similar to that of the sham-operated group, indicating that the citicoline effect on *Bdnf* expression only occurs shortly after an injury, as it is likely that BDNF is involved in the early response to nerve injury and accelerates the nerve regeneration process[40]. To the best of our knowledge, our data showed for the first time the in-time fluctuation of *Bdnf* expression during the nerve regeneration process after injury. Nevertheless, further studies that include replication samples for qRT-PCR analysis are required to confirm our study findings.

Our result is in line with the results of a previous study showing that citicoline treatment stimulated *Bdnf* expression in isolated hypothalamic neurons exposed to oxidative stress[41]. Additionally, assessing the level of *Bdnf* expression is essential as the serum level of BDNF reportedly can be employed as a predictor for the development of trigeminal neuralgia, a severe chronic neuropathic pain affecting the trigeminal nerve[42].

Mental nerve injury does not change the number of neurons in the trigeminal ganglion

Following PNI, most neurons die through apoptosis, which may lead to functional loss of the nerve[5,6]. To examine the effect of citicoline administration after PNI in rats, neuronal counting was performed in the trigeminal ganglion at the chronic phase (28 days post-injury) in toluidine blue-stained sections using a design-based unbiased stereology. On average, 7—15 VURSs were observed, and 228—448 neurons were counted. The coefficient of errors was between 0.019 and 0.038%. The coefficients of variance of the sham-operated, saline-treated, and citicoline-treated groups were 6.5%, 7.2%, and 7.9%, respectively.

While counting for the total number of neurons, the neuronal types were also determined based on the size of the neurons, namely, the larger neuron A and the smaller neuron B (Figure 3a). Our data showed that the number of neuronal type A and B and the total number of neurons in the trigeminal ganglion after mental nerve injury were not significantly different between the groups (Figure 3b). The mental nerve is a branch of the inferior alveolar nerve, which is a branch of the trigeminal nerve's mandibular division. Therefore, clamping the mental nerve may be inadequate to produce damage that induces considerable neuronal loss at the trigeminal ganglion.

CONCLUSION

Overall, our study data exhibited that citicoline administration after PNI is beneficial in promoting nerve regeneration, as demonstrated by increased *Bdnf* expression, suggesting that citicoline administration can indeed accelerate nerve regeneration immediately after injury. As the first study that examined the effect of citicoline administration on *Bdnf* expression after PNI, further studies using BDNF inhibitors are needed to confirm our findings. Whether prolonged BDNF secretion leads to improved functional and structural recovery remains to be investigated. In our study, neuronal loss was absent in the trigeminal ganglion at 28 days after the mental nerve clamping injury. Subsequently, more animals are required in future studies to validate and reproduce this finding, as citicoline has a high potential to be clinically used to prevent the development of complications after PNI and to enhance complete nerve regeneration.

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Conflict of Interest

The author(s) do not have any conflict of interest.

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Data Availability Statement

This statement does not apply to this article

Ethics Statement

The animal study was approved by the Medical and Health Research Ethics Committee

(MHREC) Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (number KE/FK/829/EC/2015).

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Authors' Contribution

Rina Susillowati: Conceptualization, Methodology, Data Collection, Visualization, Funding Acquisition, Supervision, Writing – Original Draft, Review & Editing; Inna Armandari: Data Collection, Methodology, Analysis, Project Administration, Writing – Review & Editing; Pingky Krisna Arindra: Methodology, Data Collection, Resources, Writing – Review & Editing; David Pakaya: Methodology, Data Collection; Jens Randel Nyengaard: Supervision, Methodology, Resources, Writing – Review & Editing.

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