



Olive Polyphenol as Neuroprotective in Chronic Cervical Myelopathy Rabbit Model

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Abstract

BACKGROUND: Olive polyphenols are known to be an anti-oxidants and anti-inflammatory agents.

AIM: The purpose of this study was to determine the potential neuroprotective effect on chronic cervical myelopathy rabbit model.

METHODS: This study was divided into six groups; control negative (Sham-Operated) group, control positive 1 and 2, treatment groups 1, 2, and 3. Olive leaf extract (OLE) gives 350 mg/kg BW and spinal cord sample was taken at the compression level C5. Histopathological assessment and immunohistochemistry of neurofilaments (NF), S-100, brain derived neurotrophic factor (BDNF), and evaluation of functional motoric outcome were done before animals were terminated.

RESULTS: Chronic cervical myelopathy in rabbit model causes decreased expression of NF, S-100, BDNF, and decreased motor function. Oral administration of OLE increased the expression of these biomarkers and improved motor function outcomes.

DISCUSSION: These findings indicate that OLE may be effective in protecting chronic cervical myelopathy in rabbit model.

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Introduction

Cervical myelopathy is more common in men and also tends to present earlier in men than in women. Radiologically, spondylosis is present in 13% of men in the third decade and almost 100% of men over the age of 70. In women, the disease presents later, with 5% showing radiographic changes in the fourth decade, increasing to 96% in women over the age of 70. Changes are more common in patients with rheumatoid arthritis, where 85% of those with moderate-to-severe disease will have radiological changes [1]. Chronic myelopathy is the most common and serious neurological disorder in the elderly population caused by chronic progressive compression or irritation of the spinal cord in the neck. The clinical features of chronic myelopathy include localized neck pain and functional impairment of

motor function in the arms, fingers, and hands. If left untreated, this can lead to significant and permanent nerve damage including paralysis and death. Despite recent advancements in understanding the chronic myelopathy pathology, prognosis remains poor and little is known about the molecular mechanisms underlying its pathogenesis [2]. The natural history of cervical myelopathy involves both static and dynamic factors that trigger a cascade of reactions due to compression and ischemic spinal cord dysfunction. Myelopathy or spinal cord dysfunction can also be caused by congenital stenosis, degenerative changes, rheumatoid arthritis, and traumatic compression. Conservative management for mild cervical myelopathy is an option with close clinical and radiological follow-up [3].

If cervical canal stenosis is present, when extremes flexion or extension occurs that there is increased strain and shear pathological forces applied

on the spinal cord, which can potentially cause localized and widespread axonal injury [4], [5]. The vascular spinal cord plays an important role in the pathophysiology of cervical myelopathy. Spinal cord compression causes impaired perfusion to the spinal cord that underlies major underlying pathological events contributing to myelopathy. Anterior compression compromises perfusion through the transverse arterioles arising from the anterior sulcal arteries, while posterior cord compression compromises perfusion through the intramedullary branches of the central gray matter [6]. Oligodendrocytes, cells responsible for myelination of axons, respond poorly to ischemia and this may explain the demyelination that occurs with chronic cervical myelopathy [7], [8], [9].

Prognosis of chronic cervical myelopathy is closely related to the extent of the “secondary injury” mediated by a series of cellular, molecular, and biochemical cascades. As the secondary injury appears to be susceptible to pharmacological interventions including the use of antioxidant and anti-inflammatory agents [10]. Operative management is still the standard therapy for severe cervical myelopathy. A randomized, double-blind, placebo controlled, and multi-center study, assessing Riluzol, has any benefit for cervical myelopathy [3]. A 10-year prospective and randomized study found that there was no significant difference in outcomes or survival between a conservative and an operative treatment in patients with mild and moderate chronic cervical myelopathy [2]. There are about 30 phenolic compounds in olive plants, including oleuropein, olecanthal, tyrosol, and hydroxytyrosol [10], [11], [12], [13], [14], [15], [16]. Main pharmacologic effect of olive polyphenols is anti-inflammatory and anti-oxidant [17]. Olive phenols have been shown to be some of protective effects against brain hypoxia–reoxygenation, cerebral ischemia, brain damage after hypoxia – reoxygenation in diabetic rats, and aging [18]. In a mouse model of median cerebral artery occlusion, the administration of olive polyphenols reduced infarcts, edema, improved BBB, and improved neurological function [12], [19]. In a mouse model of spinal cord injury (SCI), administration of olive polyphenols reduced inflammatory biomarkers (TNF- α , IL6, IL1, iNOS, COX2, NFkB, NO, MDA, and GSH) and improved neurologic function [10].

In spite of some experimental evidence for the neuroprotective effects of olive phenolics in brain damages, acute SCI, however, no study has been performed to evaluate whether these constituents have protective effects on chronic cervical myelopathy. In the present study, we investigated the potential neuroprotective effect of olive polyphenol, a dietary antioxidant-anti-inflammatory of olive, in experimental rabbit chronic cervical myelopathy model with determination of neurofilaments (NF), S-100, brain derived neurotrophic factor (BDNF), and motor functional outcome. We modeled a mild myelopathy in

hope that conservative therapy without decompression could be performed. For the method of making animal models in this study, the authors have conducted two previous preliminary studies, the results are in accordance with myelopathy [20], [21].

Methods

Experimental animal

New Zealand white rabbits 12 weeks of age, weight: 2.6–3.0 kg (average: 2.9 kg), males were used in this study. Animals are given diet and water in the conventional laboratory. The room temperature is around 16–20°C with a light-dark cycle of 12 h. This study has obtained permission from the Ethical Committee of the Medical Faculty of Universitas Sumatera Utara, Medan, Indonesia.

Olive leaf extract (OLE)

This study uses OLE produced by Shaanxi Yongyuan BioTech Co., Ltd. Extract contains 40% Oleuropein, the extract dose is given at 350 mg/kg BW or equivalent to 140 mg Oleuropein, suspended in distilled water and administered to animals through oral gavage in 4 cc solution, OLE was administered in the morning. The doses given are in accordance with the previous studies [10], [12], [22], [23], converted from rat to rabbit doses [24].

Experimental group

This study uses 30 rabbits which are divided into six groups. First group (n = 5) control negative (sham) group, performed a skin incision, paraspinal muscle dissection, and lamina hole drill and no laminar screw was installed. The second group (n = 5) positive control-1 performed spinal cord compression with a screw and terminated on day 14. The third group (n = 5) positive control-2 performed spinal cord compression with a screw and terminated on day 21. Fourth group (n = 5) treatment-1 performed spinal cord compression with a screw together with the administration of OLE and terminated on day 14. The fifth group (n = 5) treatment-2 performed spinal cord compression with a screw, after 14 days given OLE, termination on day 21. Sixth group (n = 5) treatment-3, OLE was given 7 days before spinal cord compression with screws, terminated on day 21.

Surgical procedure

The rabbit was anesthetized using 50 mg/kg of Ketamine hydrochloride (Pfizer) and 10 mg/kg of Xylazine (Bayer), prophylactic antibiotic

Cefazolin 50 mg/kg [25]. Rabbit in prone position shaved in the posterior cervical area, disinfected with 10% betadine, sterilized with cloth cover, C4–C6 midline posterior cervical skin incision, small retractors was used, palpation of spinous processes, C5 paraspinal muscle dissection, and identification of lamina. One hole is made in the lamina C5 midline position using a high speed diamond drill bur (3 mm in diameter), until it penetrates the lamina (2 mm thick lamina), the burr hole is tapered at 4 mm, then the lamina hole is inserted into a screw (stainless steel) with a diameter of 4 mm and a length of 10 mm, until the entire thickness of the lamina, on the 1st day, the compression is given 0.5 mm (by turning the screw 180°), on the 7th day, the screw is turned 180° again (total compression is 1 mm), on the 14th day the screw is rotated another 180° (the total compression is 1.5 mm), after the installation of screw the skin was sutured. The position of the screw head is 0.5 cm below the skin, easily felt, so that in the 2nd and 3rd procedures, it is enough to open one skin suture and the screw is turned, the repeated procedure is carried out by sterilization and the same anesthetics method.

Motor function evaluation

Motor function was evaluated using the modification of Tarlov's classification [25] (Table 1). Evaluation was made before and immediately after the surgery and before animal termination.

Table 1: Modification of Tarlov's classification

Grade	Motor characteristics
0	Unable to have voluntary movements
1	Perceptible movements at joint, the hindlimbs follow
2	Good movements at joints, but unable to stand up
3	Can stand up and walk, but unable to start running quickly
4	Normal

Tissue evaluation and immunochemistry

The tissue of the C5 spinal cord area was taken and fixed with a buffer solution of 10% formalin. After that, dehydration was carried out using graded alcohol (30%, 50%, 70%, 80%, 96%, and absolute) for 60 min each. Clearing was used with xylol 2 times for 60 min each. Then, the soft paraffin embedding was carried out for 60 min at a temperature of 48°C. Furthermore, the paraffin is allowed to stand for 1 day until it becomes a hard block. The next day, it was attached to the holder and a 4 µm thick cut was made with a rotary microtome. Followed by the deparaffinization process, the glass object resulted from the paraffin block was immersed in xylol 2 times for 5 min each. After that, rehydration using serial alcohol (absolute, 96%, 80%, 70%, 50%, and 30%) for 5 min each. Then, rinsed in H₂O for 5 min. Then, the process of staining the slide was washed with phosphate buffer saline (PBS) pH 7.4 for 5 min. Then, stained with hematoxylin for 10 min. After that, soak it in tap water for 10 min. Then, rinsed with dH₂O. Dehydrated with alcohol 30% and 50%, respectively,

for 5 min. Then, stained with Eosin solution for 3 min. Then, rinsed with 30% alcohol. Washed with H₂O for 5 min and then dried. Then, do the mounting with a stick and cover with a glass cover.

Immunochemistry protocol

The distribution of microglia expressing NF, S100, and BDNF was observed by immunohistochemical techniques. Paraffin block containing spinal tissue was cut to a thickness of 4 µm using a microtome, then deparaffinized with xylol. Subsequently, rehydration was carried out with a decreased concentration of ethanol, followed by rinsing with PBS for 3 × 5 min. The tissue preparations were then incubated in DAKO[®] Buffer Antigen Retrieval in a microwave at a temperature of 94°C for 20 min and followed by cooling at room temperature for 20 min. The next step, the preparation was washed with PBS for 3 × 5 min and incubated in a peroxidase block (Novocast[®]) for 20 min. Furthermore, the preparation was washed again with PBS for 3 × 5 min and incubated in Protein Block for 20 min. After that, it was washed again with PBS for 3 × 5 min and incubated overnight (12–18 h) with primary antibody: Specific antibody anti-NF (RNF402) cat# sc-32729, specific antibody anti-S-100 (S1-61) cat# sc-53438, anti-p-Tau (PHE-13) cat# sc:32275: Specific antibody anti-BDNF (N-20) cat# sc-546, for 1 h at room temperature, then washed with PBS pH 7.2 for 3 × 5 min and incubated with a solution post-primary antibody for 45 min and followed by incubation with Novolink[®] Horse Radish Peroxidase for 60 min at room temperature. After incubation, the preparations were washed with PBS pH 7.2 for 3 × 5 min. Then, diamio benzidine was applied for 10 min and the preparation was washed with PBS pH 7.2 for 3 × 5 min, then counterstain with hematoxylin (Novocastra). Furthermore, dehydration was carried out using increased concentrations of ethanol. The next process is to do the purification with xylol, then do the mounting.

Immunohistology evaluation

Calculation of immunohistochemical results using techniques such as those in other study modified for spinal tissue [26], [27]. Examination of the number of brown cells in the nucleus or cytoplasm per 20 fields of view in the anterior horn compression area (C5) and cell counts was carried out separately between the two examiners (double blind). Examination and cell counts were performed on each slide in the field of view in the cortex of the spinal cord with ×400 and ×1000 magnification, for 20 fields of view, respectively.

Statistical analysis

Statistical analysis was performed using SPSS version 21 for Windows (SPSS Inc., Chicago, IL, USA).

To test the significance of differences of the variable expression between the two experimental groups, we performed analysis of variance (ANOVA) tests. The significance level was defined as $p < 0.05$.

Results

Evaluation of animal

Homogeneity test performed using one-way ANOVA showed that there was no significant difference in body weight between body weight before the treatment and body weight after the treatment ($p > 0.05$). This shows that the animal body weight data have a homogeneous variation. Thus, body weight is not a confounding variable that can affect the dependent variable in this study.



Figure 1: (a) C1-C7 rabbit cervical spine tissue with screw in midline lamina C5, (b) Spinal cord tissue at C4-C6 level, looks concave at screw compression area, (c) C5 with spinal cord compression screw day 14 (1 mm), (d) C5 with spinal cord compression screw day 21 (1.5 mm), (e) Axial Section Spinal cord sample from negative control, (f) spinal cord sample from positive control-2, axial section in compressed area C5, and (g) Spinal cord sample from negative control, red circle dotted line is area examined by IHC and cell count

Clinical assessment of experimental animals after compression showed no signs and symptoms of acute SCI. The motor function level of experimental animals given spinal cord compression decreased slowly until day 21. The motor function of the animal was assessed before being sacrificed, it seemed homogeneous in each group; on control negative group “4,” control positive-1 group “3,” control positive-2 group “2,” treatment-1 group “4,” treatment-2 group “2,” and

Table 2: Comparison of NF levels between groups

Group	NF	
	IHC	p-value
Control negative	7.80 ± 1.92	< 0.001
Control positive-1	4.60 ± 1.67	
Control positive-2	4.60 ± 1.81	
Treatment-1	11.80 ± 2.16	
Treatment-2	6.60 ± 2.07	
Treatment-3	11.80 ± 1.78	

*One-way ANOVA test, p value is significant if $p < 0.05$.

treatment-3 group “3.” One point improvement in motor function was found in the treatment-1 and 3 groups, while in treatment-2, there was no improvement in motor function.

Evaluation of spinal cord specimen

In the area of compression, the spinal cord was seen flattened in the anterior-posterior direction indicating chronic compression. No signs of acute trauma were seen in the spinal cord tissue such as intraspinal cord hemorrhage or contusion (Figure 1).

NF

In Figures 2 and 3, the number of neurons in the spinal cord tissue that expresses NF in various groups is shown.

Using the one-way ANOVA test, it can be seen that all groups have a significant value for each other group with p-value (< 0.001). Based on the *post hoc* test in Tables 2 and 3, only control positive-1 versus treatment-1 and control positive-2 versus treatment-3 have significant value.

Astroglial calcium-binding protein (S100-β)

In Figures 4 and 5, the number of neurons in the spinal cord tissue that expresses S100β in various groups is shown.

Using the one-way ANOVA test, it can be seen that all groups have a significant value for each other group with p-value (0.000). Based on the *post hoc* test in Tables 4 and 5, based on the IHC, all groups have significant value, except for control positive-2 versus treatment-2 group.

Table 3: Post hoc analysis of NF levels

Group	NF	
	IHC Δ	p-value
CN versus C+1	3.20	0.125
CN versus C+2	3.20	0.125
C+1 versus T1	-7.20	0.000
C+2 versus T2	-2.00	0.575
C+2 versus T3	-7.20	0.000

CN: Control negative, C+1: Control positive-1, C+2: Control positive-2, T-1: Treatment-1, T-2: Treatment-2, T-3: Treatment-3, Δ: Mean difference.

BDNF

Figures 6 and 7 show the number of neurons in spinal cord tissue that express BDNF in various groups.

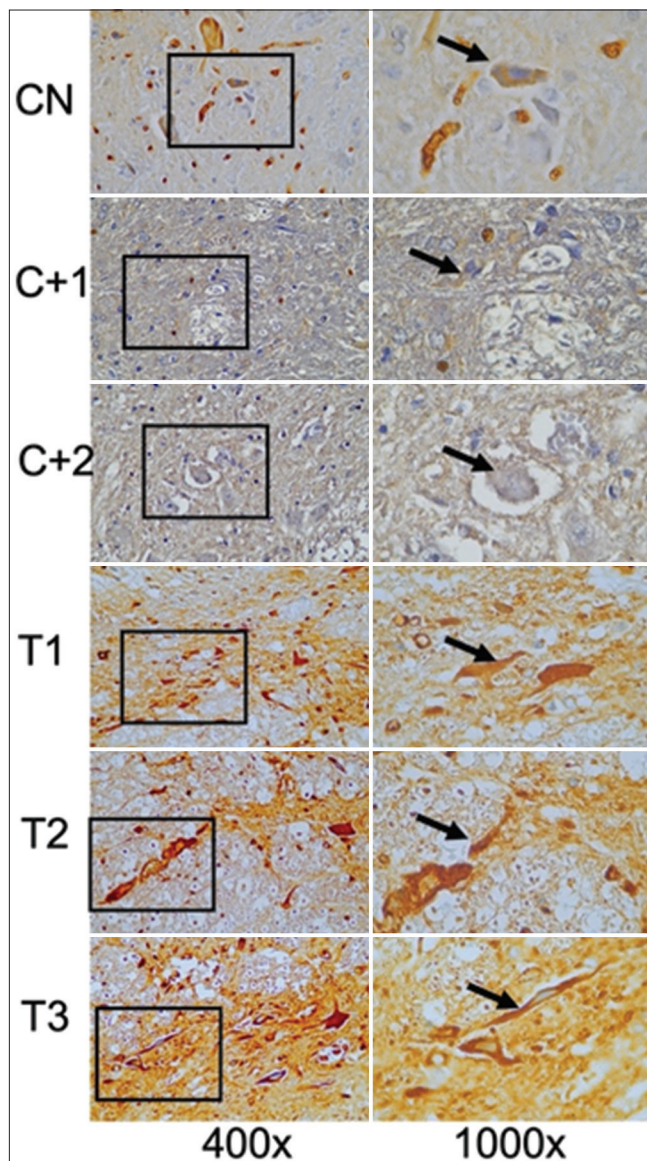


Figure 2: Immunohistochemical staining with neurofilaments (NF) markers, by counting the number of spinal cord tissue neuron cells expressing NF (brown color in the cell cytoplasm, arrows in the image). There was a decrease in NF in spinal cord tissue neuron cells after receiving compression treatment (Control positive 1 and 2) compared to the group without compression (Control negative) and NF in neuronal cells had an increase in the group given Olive leaf extract (OLE) (Treatment 1, 2, and 3) compared to the group without OLE (Control positive 1 and 2)

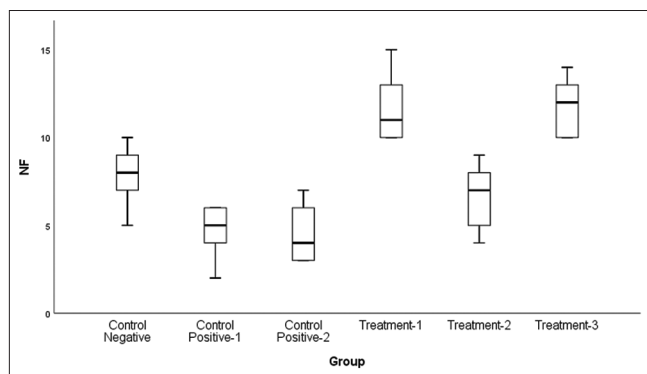


Figure 3: Graph of neurofilaments-expressing cells in various groups

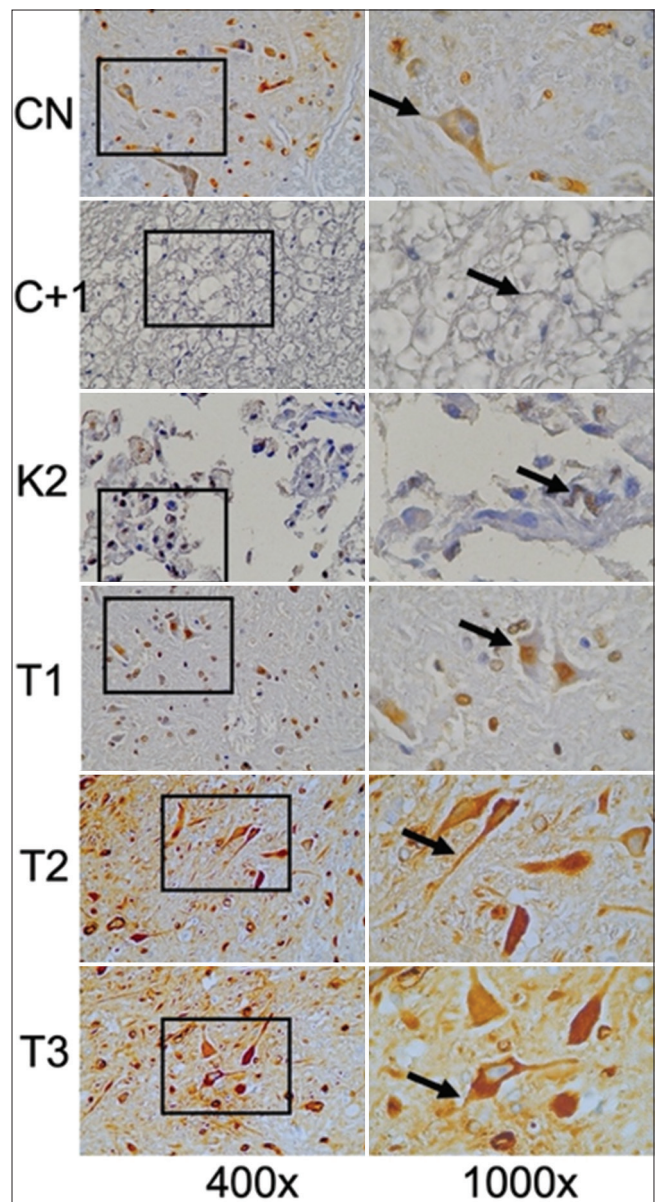


Figure 4: Immunohistochemical staining with the S-100β marker, by counting the number of spinal cord tissue neurons expressing S-100 (brown colour in the cytoplasm of cells, arrows in the figure). A decrease in S-100β was seen in spinal cord tissue neuron cells after getting compression treatment (Control positive 1 and 2) compared to the group without compression (Control Negative) and S-100 in neuronal cells experienced an increase in the group given Olive leaf extract (OLE) (Treatment 1, 2, and 3) compared to the group without OLE (Control positive 1 and 2)

Using the one-way ANOVA test (Table 6), it can be seen that all groups have a significant value for each other group with p-value (<0.001). Based on the *post hoc* test in Table 7, based on the IHC, all groups have a significant value for each group.

Discussion

S100-β is a calcium-binding protein found in glial cells and has previously been established as

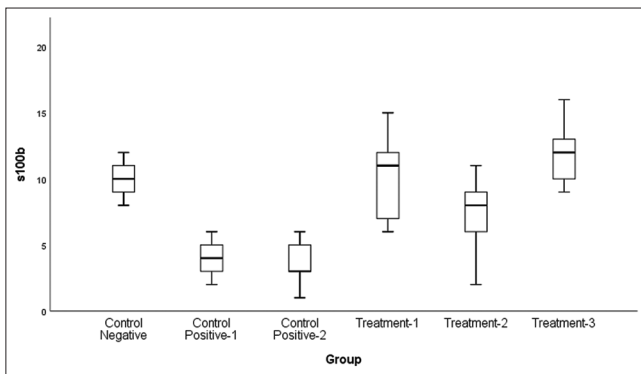


Figure 5: Graph of S100β expressing cells in various groups

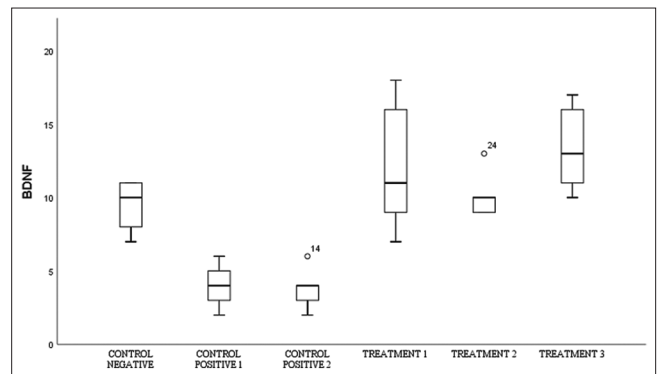


Figure 7: Graph of brain derived neurotrophic factor-expressing cells in various groups

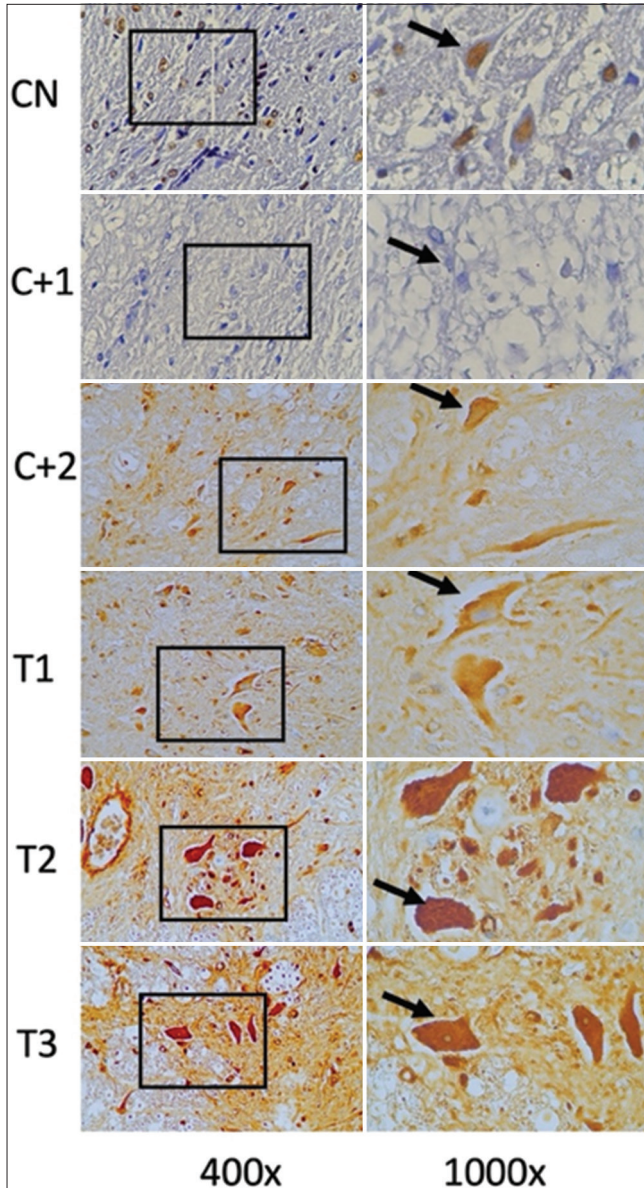


Figure 6: Immunohistochemical staining with brain derived neurotrophic factor (BDNF) markers, by counting the number of spinal cord tissue neuron cells expressing BDNF (brown color in the cell cytoplasm, arrows in the image). A decrease in BDNF was seen in spinal cord tissue neuron cells after receiving compression treatment (Control positive 1 and 2) compared to the group without compression (Control negative) and BDNF in neuronal cells had an increase in the group given Olive leaf extract (OLE) (Treatment 1, 2, and 3) compared to the group without OLE (Control positive 1 and 2)

a marker of brain injury, S100-β has a wide variety of homeostatic activities including regulation of calcium flow, cell proliferation and differentiation, enzymatic/metabolic activity, and MAPK stabilization. S100-β is a structural marker that has been reported to potentially predict chronic cervical myelopathy recovery in various studies.

Table 4: Comparison of NF levels between groups

Group	S-100β	
	IHC	p-value
Control negative	10.00 ± 1.58	0.000
Control positive-1	4.00 ± 1.58	
Control positive-2	3.60 ± 1.94	
Treatment-1	10.20 ± 3.70	
Treatment-2	7.20 ± 3.42	
Treatment-3	12.00 ± 2.73	

*One-way ANOVA test, p value is significant if p < 0.05.

Research conducted by [28] using ELISA revealed increased serum S100-β levels 6 h after injury in 30 mice that underwent chronic cervical myelopathy when compared to a control group receiving laminectomy alone. However, 24 h after injury, there was no significant difference in S100-β concentration between sham and injured mice. In another study conducted, the expression of S100-β reached a peak 24 h after SCI and thereafter continued to decline. Animal studies found S100-β expression peaks at 24–72 h as a manifestation of acute injury. With the chronic cervical myelopathy model, which the researchers developed, it appears that – the expression of S100-β decreased significantly in chronic cervical myelopathy phase in a rabbit model of spinal cord compression with a laminar screw. This means that the expression of S100-β cannot serve as a marker of chronic cervical myelopathy in the chronic phase.

Table 5: Post hoc analysis of S100 levels

Group	S-100β	
	IHC Δ	p-value
CN versus C+1	6.00	0.016
CN versus C+2	6.40	0.009
C+1 versus T1	-6.20	0.012
C+2 versus T2	-3.60	0.293
C+2 versus T3	-8.40	0.000

CN: Control negative, C+1: Control positive-1, C+2: Control positive-2, T-1: Treatment-1, T-2: Treatment-2, T-3: Treatment-3, Δ: Mean Difference.

To support the hypotheses that have been built, in relation to the chronic cervical myelopathy model, this study also analyzes the presence of NF. NF is a

cytoskeletal protein that is abundantly and uniquely expressed in the cytoplasm of axonal fibers in the CNS. NF regulates signaling and transport systems on axons and has become a focus in neurological disorders due to extracellular accumulation of NF. There are three types of NF: NF-light (NF-L), medium (NF-M), and heavy (NF-H) chain [29], [30]. NF-H will be released from the cytoplasm of damaged neurons in traumatic SCI. In addition, in the development of secondary injury, when apoptosis and inflammation of the nerves peak, NF is thought to escape extracellularly along with other cytoplasmic components. Therefore, NF loss is hypothesized to potentially indicate the severity of nerve cell loss in SCI as well as the degree of damage at the secondary stage.

Table 6: Comparison of BDNF levels between groups

Group	BDNF	
	IHC	p-value
Control negative	9.40 ± 1.81	< 0.001
Control positive-1	4.00 ± 1.58	
Control positive-2	3.80 ± 1.48	
Treatment-1	10.20 ± 1.64	
Treatment-2	12.20 ± 4.65	
Treatment-3	13.40 ± 3.04	

*One-way ANOVA test, p value is significant if $p < 0.05$.

In this study, it was found that the NF expression decreased on the 14th day of spinal cord compression and continued to decrease on the 21st day, this result is in accordance with a study conducted in rabbits, they found only 6.6% chronic cervical myelopathy model specimens expressing NF while non-chronic cervical myelopathy (control) 93.3%). Another study in humans with SCI found that NF expression at 12 h after complete injury continued to decrease and was still detectable until day 21, according to this study, it was still found until 21 day compression of spinal cord [30]. In the previous studies, we found significantly lower NF expression in myelopathy compared to normal spinal cord [29]. The stability of neurons, cytoskeleton, and its components (actin filaments, NFs, and microtubules) is maintained by NF. NFs are important structures of white matter and represent a very important group of protein structures that support the axon architecture of the central nervous system and peripheral nerves [31]. Human studies have found that CSF NF-L levels are positively correlated with motor function in cervical spondylotic myelopathy [32]. In this study, we found that the expression of NF decreased as an indication of degenerative axons, this is also often found in human chronic cervical myelopathy.

Table 7: Post hoc analysis of BDNF levels

Group	BDNF	
	IHC Δ	p-value
CN versus C+1	5.40	0.052
CN versus C+2	5.60	0.039
C+1 versus T1	-6.20	0.016
C+2 versus T2	-8.40	0.001
C+2 versus T3	-9.60	< 0.001

CN: Control negative, C+1: Control positive-1, C+2: Control positive-2, T-1: Treatment-1, T-2: Treatment-2, T-3: Treatment-3, Δ: Mean Difference.

BDNF has receptor which is tropomyosin kinase B and its activity is through 3 pathways; the first through the PI3K/Akt pathway which mediates cell survival, the second through the phospholipase C pathway which causes activation of protein kinase C and CAMK functions as neuroplasticity and the third pathway through Ras

which activates extracellular signal-regulated kinase which functions plasticity, survival, and growth [33]. In the SCI model study, the administration of oleuropein increased neurotrophic factor GDNF [34]. Human studies have shown that olive polyphenols increase BDNF levels and improve memory function [35]. In rat model, olive oil-enriched diet consumption during pregnancy and lactation significantly increased BDNF in brain [36]. In this study, chronic compression of the spinal cord caused a decrease in the expression of NF, S-100, and BDNF. The administration of OLE increased the levels of NF, S-100, and BDNF as well as improved functional motor outcomes in the chronic cervical myelopathy model, especially early treatment.

Conclusion

Chronic spinal cord compression elevating serum levels of IL-6 and TNF-alpha and lowering BDNF, according to this study. Oral olive leaf extract (OLE) diet administration decreasing serum levels of IL-6 and TNF-alpha, increased serum levels of BDNF, and improved functional motor outcome, particularly in early treatment (prophylactic and concomitant).

Ethical Approval

Approval has been given by Ethical Committee of Universitas Sumatera Utara.

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