

# The Effect of Long Term Administration of Aspartame on the Sciatic nerve of adult male albino rats and the Possible Therapeutic Role of Ozone (Histological and Biochemical Study)

Original  
Article

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## ABSTRACT

**Introduction:** Aspartame is a widely used artificial non-nutritive sweetener, which replaced sucrose in foods for enhancing flavor while reducing calories. It was confirmed that aspartame was a multipotential carcinogenic agent increases the risk of lymphoma and neurological tumors.

**Aim of the work:** This work is aimed to evaluate the effect of aspartame on the structure of the sciatic nerve and the possible role of ozone therapy.

**Material and methods:** The present study was carried out on 30 adult male wistar albino rats divided into three groups: control (I), aspartame(II) and ozone and aspartame treated (III) groups. Group (II) received aspartame (250 mg/kg/d) dissolved in distilled water and administered by gastric tube for 3 months. Group (III) received aspartame as in Group II, then received an ozone dose of 1.1 mg/kg/d intraperitoneal (IP) for 30 days. Rats were anesthetized and sciatic nerves were processed for light, electron microscope, morphometric and Real time-PCR analysis.

**Results:** The sciatic nerve of aspartame group showed loss of regular architecture, separation of myelin lamellae with splitting of myelinated axons. Schwann cells appeared with vacuolated cytoplasm and collagen fibrils could be seen in the endoneurium. Immunohistochemically, there was decrease in expression of myelin basic protein (MBP) in myelin sheath and decrease in axon/fiber ratio (g ratio) statistically. Apparent structural and histomorphological improvement were noticed in ozone group.

**Conclusion:** Aspartame induced histopathological changes in sciatic nerve. Our data suggests that ozone therapy has an important role in sciatic nerve regeneration by increasing the expression of neurotrophic and angiogenic factors establishing more favorable environment for nerve regeneration.

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**Key Words:** Aspartame, neurotrophic factors, peripheral nerve, ozone.

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## INTRODUCTION

The neurons in peripheral nervous system (PNS) differ from those of central nervous system (CNS) as they have the potential ability for regeneration after injury<sup>[1]</sup>. The peripheral nerves repair continues to be a critical clinical challenge for plastic, reconstructive, orthopedic, and hand surgeons<sup>[2]</sup>. Peripheral nerves injury (PNI) is often accompanied by loss of sensations, apraxia (partial or complete), chronic pain, and occasionally the permanent disability<sup>[3]</sup>.

Aspartame is a widely used artificial non-nutritive sweetener, which replaced sucrose in foods for enhancing flavor while reducing calories. It is consumed by hundreds of millions of people worldwide. It is found in more than 6000 products, for example soft drinks, candies, tabletop sweeteners, breakfast cereals, chewing gum, puddings, yoghurt products and some pharmaceuticals such as vitamins and sugar-free cough drops<sup>[4,5]</sup>.

Several studies on laboratory animals have been carried out to verify its toxicity. It was confirmed that aspartame was a multipotential carcinogenic agent increases the risk of lymphoma, leukemia, urinary tract tumors, and neurological tumors, even at a daily dose (20 mg/kg) that is much less than the acceptable daily dose (40 mg/kg)<sup>[6,7]</sup>.

In addition, several studies have suggested an association between aspartame consumption and the risk of type 2 diabetes<sup>[8]</sup>, preterm delivery<sup>[9]</sup>, nephrotoxicity, hepatotoxicity<sup>[10]</sup>. With regard to neurotoxicity of aspartame, most of the previous studies were concerned about central nervous system toxicity. Aspartame affects the cerebral cortex<sup>[11]</sup> and cerebellar cortex<sup>[12]</sup>, affecting memory, learning, and behavior<sup>[13]</sup>.

Ozone is a powerful oxidant agent that enhances the oxygenation of tissues and triggers the secretion of cytokines. It has been used as a therapeutic agent for chronic and diabetic wounds<sup>[14]</sup>. Ozone exerts its beneficial effects

by suppression of infection, regulation of inflammation and increasing local oxygen tension at the site of injury<sup>[15]</sup>. Ozone is particularly useful for infected and necrotic wounds and poorly oxygenated tissues<sup>[16]</sup>.

Ozone (O<sub>3</sub>) is non-persistent with a half-life of approximately 20 min at normal temperatures. It decomposes and disperses in water easily<sup>[17]</sup>. O<sub>3</sub> can restrain inflammatory cell factors, activate cyclooxygenase, and decrease the stress reaction to histiocytic oxidation, augmenting the histiocytic ability of resisting oxidation and free radicals. It can serve as a painkiller and is anti-inflammatory<sup>[18]</sup>.

The aim of the present study was to evaluate the effect of aspartame on the structure of the sciatic nerve in adult male albino rats and the possible role of ozone therapy.

## **MATERIALS AND METHODS**

### **1- Animals:**

The present study was carried out on 30 adult male wistar albino rats, weighing 150–200 gm for each. They were obtained from the Animal House, Faculty of Medicine, Zagazig University, Egypt. Rats were housed in stainless steel cages with a 12-h light/dark cycle and allowed tap water ad libitum. All experimental procedures were approved and performed in accordance with the guidelines of the institutional animal care and committee of Faculty of Medicine, Zagazig University. They were allowed to acclimatize for 2 weeks before starting the experiment and observed for general health and suitability for induction in the experiment and included in the study.

### **2- Experimental design:**

The rats were divided into three equal groups.

**Group I (Control Group):** received ordinary diet and water.

**Group II (Aspartame Group):** received aspartame (250 mg/kg) once per day<sup>[19]</sup> dissolved in 2 cc of distilled water and administered by gastric tube for 3 months. This dose corresponded to the acceptable daily intake in humans (defined by the World Health Organization) of 40–50 mg/kg/d. Species correction required a five to six times higher dose in rats than humans, as rats metabolize aspartame faster than humans<sup>[20]</sup>.

**Group III (ozone and aspartame treated group):** received aspartame as in Group II, then received an ozone dose of 1.1 mg/kg once per day intraperitoneal (IP) for 30 days<sup>[18]</sup>.

### **Ozone application:**

We used the medical ozone Hyper Medozon generating device at Rheumatology Department, Faculty of Medicine, Zagazig University, Egypt. The ozone given to each animal was adjusted to a final dose of 1.1 mg/kg and was given IP once daily for 30 days.

At the time of sacrifice, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (200 mg/kg)<sup>[21]</sup>. A longitudinal skin incision was performed at the upper hind limb level with blunt separation of gluteal muscles to expose the sciatic nerve and 10 mm long nerve fragments were collected. Part of it was frozen at - 80°C until used for RNA extraction and real time PCR analysis. The other part processed for histological analysis.

### **• Histological study:**

- For light microscopy: specimens were fixed in 10% formalin solution and processed to prepare 5 m thick paraffin sections that were stained with hematoxylin and eosin<sup>[22]</sup>.

- For Immunohistochemistry: Sciatic nerves were fixed with 4 % paraformaldehyde in PBS (phosphate buffered saline) overnight, dehydrated and embedded in paraffin. Immunocytochemical labeling was performed by monoclonal anti-MBP antibody (Millipore Corporation Billerica, USA) on 5µm thick sciatic nerves sections. Briefly, deparaffinized sections were pre-incubated with citrate buffer and were rinsed with 0.01 mol/L PBS three times. Sections were treated with 0.3 % hydrogen peroxide. The sections then incubated with BSA 3 % at 37 C for 1 hour to block the non-specific binding. The slides were then incubated with Anti-Myelin Basic Protein Antibody (Millipore MAB382) with 1:100 concentration. After rinse with PBS, the sectioned tissue was incubated with the biotinylated secondary antibody for 10 min. After three times wash with PBS, the section was rinsed with Streptavidin HRP. Immunoreactivity was visualized using DAB for 30 min. Subsequently, the slides were counterstained with Mayer's hematoxylin and mounted with a cover slip. Photomicrographs were taken with a light microscope and were analyzed using Olysia Bio software<sup>[23]</sup>. Immunohistochemical stain for MBP (myelin basic protein) is used for detection of myelin sheath which appeared brown in color.

- For electron microscopy: Specimens were cut into 1 mm 3pieces and stored in the same fixative overnight at 4°C (2.5%glutaraldehyde buffered with 0.1 mol/L phosphate buffer at pH 7.4for 2 h). They were post fixed in 1% osmium tetroxide for 1 h, dehydrated through graded alcohol series, and embedded in epoxy resin. Ultrathin sections (50 nm thick) were collected on copper grids and stained with uranyl acetate and lead citrate<sup>[24]</sup>.

### **• RNA extraction and Real time-PCR analysis:**

Homogenization of nerves was done until tissue the clumps were no longer visible. Total RNA was isolated using RNA-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea) following the manufacturer instructions. The concentration and purity of RNA were estimated by a spectrophotometer (UNICO, UV2000, China). RNA was reverse transcribed using Power cDNA synthesis Kit (iNtRON Biotechnology)

according to the manufacturer protocol. Each real time PCR reaction was performed in a total volume of 20  $\mu$ L containing 5  $\mu$ L of the cDNA, 100 pmol/ $\mu$ L of each primer (0.5  $\mu$ L each)(Biolegio), 10  $\mu$ L of EvaGreen PCR Master mix (Jena Bioscience) and 4  $\mu$ L PCR-grade water. The sequence of the primers used is listed in Table 1<sup>[25-27]</sup>. PCR technique was done in Biochemistry Department, Faculty of Medicine Zagazig University.

**Table 1:** Primer sequences of studied genes

| Gene              | Primer sequence   |
|-------------------|---|
| Rat Netrin-1      | Forward 5'-CAATGCGACTGAAGGAGACGA-3'<br>Reverse 5'-ATCTCCAAACTGCCTCCTGCT-3'    |
| Rat Ninjurin-2    | Forward 5'-CCACCACCTGGTCTTCATA-3'<br>Reverse 5'-AGGCTGAAGTGGCTTTAG-3'         |
| Rat BDNF          | Forward 5'- CAAGCAACTTGGCCTACCC-3'<br>Reverse 5'- GAGCATCACCCGGAAGTGT-3'      |
| Rat GDNF          | Forward 5'-ATGAAGTTATGGGATGTCGTGGCT-3'<br>Reverse 5'-GGGTCAGATACATCCACACCG-3' |
| Rat VEGF          | Forward 5'-AGAGCAACATCACCATGCAG-3'<br>Reverse 5'-CAGTGAACGCTCCAGGATTT-3'      |
| Rat Angiopoitin-1 | Forward 5'-TTGTGATTCTGGTGATTGTGG-3'<br>Reverse 5'-CTTGTTTCGCTTATTTTGT-3'      |
| Rat B-actin       | Forward 5'-ACTATCGGCAATGAGCGGTTTC-3'<br>Reverse 5'-AGAGCCACCAATCCACACAGA-3'   |

Real-time PCR was performed according to the following protocol: initial denaturation and polymerase activation at 95°C for 2 min, then 40 cycles of 95°C for 15 sec; annealing at 55°C for 45 sec and elongation at 65°C for 45 sec. Expression of studied genes was measured and the level of B-actin was used as an internal control.  $\Delta$ Ct was the difference in the threshold cycles for target genes and  $\beta$ -actin.

#### • Morphometric study

The image analyzer computer system Leica Qwin 500 (Leica Ltd, Cambridge, UK) in the image analyzing unit of the Pathology department, Faculty of Dentistry, Cairo University, Cairo, Egypt, was used. The diameter of the regular myelinated fibers of the sciatic nerve and their axons in five non overlapping fields was measured manually, and then the axon/fiber ratio was estimated, which is known as the g-ratio<sup>[28]</sup>.

#### • Statistical analysis

The mean values of the data obtained from the image analyzer were calculated and statistically compared using Statistical Package for Social Sciences (SPSS), version 14 (Chicago, Illinois, USA). Differences between the studied groups were examined for statistical significance as regards the various parameters using the analysis of variance test. This test is used to find a significant difference between more than two groups. A P value of less than 0.05 was considered significant. Data were tabulated<sup>[29]</sup>.

## RESULTS

### *Histological results:*

Examination of H&E stained sections of adult control group showed sciatic nerve formed of bundles of nerve fibers, each bundle is invested by thin layer connective tissue perineurium (Fig 1A). In Aspartame group sciatic nerve showed loss of regular architecture and most of nerve fibers were separated from each other and from overlying perineurium (Fig.1B). Ozone and aspartame treated group showed apparently normal architecture of sciatic nerve. Nerve fascicles were surrounded by perineurium (Fig. 1C).

Higher magnification of H&E stained sections of adult control group showed sciatic nerve fascicle contained group of nerve fibers surrounded by perineurium. Myelinated nerve fibers are formed of axoplasm surrounded by unstained area of dissolved myelin. Schwann cells nuclei appeared in-between the nerve fibers. Unmyelinated nerve fibers were also seen (Fig. 2A). Aspartame group showed disorganization of nerve fascicles. Most of nerve fibers were widely separated from each other and from overlying perineurium with dilated blood vessels were also observed (Fig. 2B). Ozone and aspartame treated group showed apparently normal nerve fascicles formed mostly of myelinated nerve fibers and Schwann cells nuclei in endoneurial spaces. Nerve fibers were still widely separated (Fig. 2C).

Immunohistochemical study of MBP of the control group revealed strong positive staining for MBP in the myelin sheath (Fig.3A). While, aspartame group showed weak positive staining for MBP (Fig. 3B). In ozone and aspartame treated group myelin sheath showed moderate positive staining for MBP in comparison to aspartame group (Fig. 3C).

In toluidine blue stained sections of the control group showed a nerve fascicle of sciatic nerve surrounded by perineurium and containing transverse section of myelinated nerve fibers of various sizes with endoneurium in between. Myelin sheaths appeared as well preserved darkly stained structures; often rounded or elliptical, Schwann cells cytoplasm appeared paler than the myelin (Fig. 4A). In aspartame group revealed many nerve fibers with irregular thickening of myelin giving them distorted appearance. Wide separations in between the fibers were also seen (Fig. 4B). Ozone and aspartame treated group showed apparently normal myelinated nerve fibers formed of darkly stained myelin sheath surrounded by pale stained Schwann cells separated by endoneurium (Fig.4C).

Ultrathin sections of control adult group revealed multiple axons; some appeared myelinated while others were unmyelinated. Myelinated axons have compact regular myelin sheath. Schwann cells with their euchromatic

nuclei were seen surrounding the myelin sheath. Collagen fibrils were also seen in between nerve axons (Fig. 5A). Aspartame group showed separation of myelin lamellae with focal loss of compact lamellar structure (splitting of myeline sheath). Axons surrounded by Schwann cells with vacuolated cytoplasm. Collagen fibrils could be seen in the endoneurium Also, separation of myelin sheathes were seen from the axons at some sites. Collagen fibrils were seen in the wide spaces in between the nerve fibers (Fig. 5B and C). In Ozone and aspartame treated group, most of myelinated and unmyelinated nerve axons and Schwann cells were apparently normal (Fig. 5D).

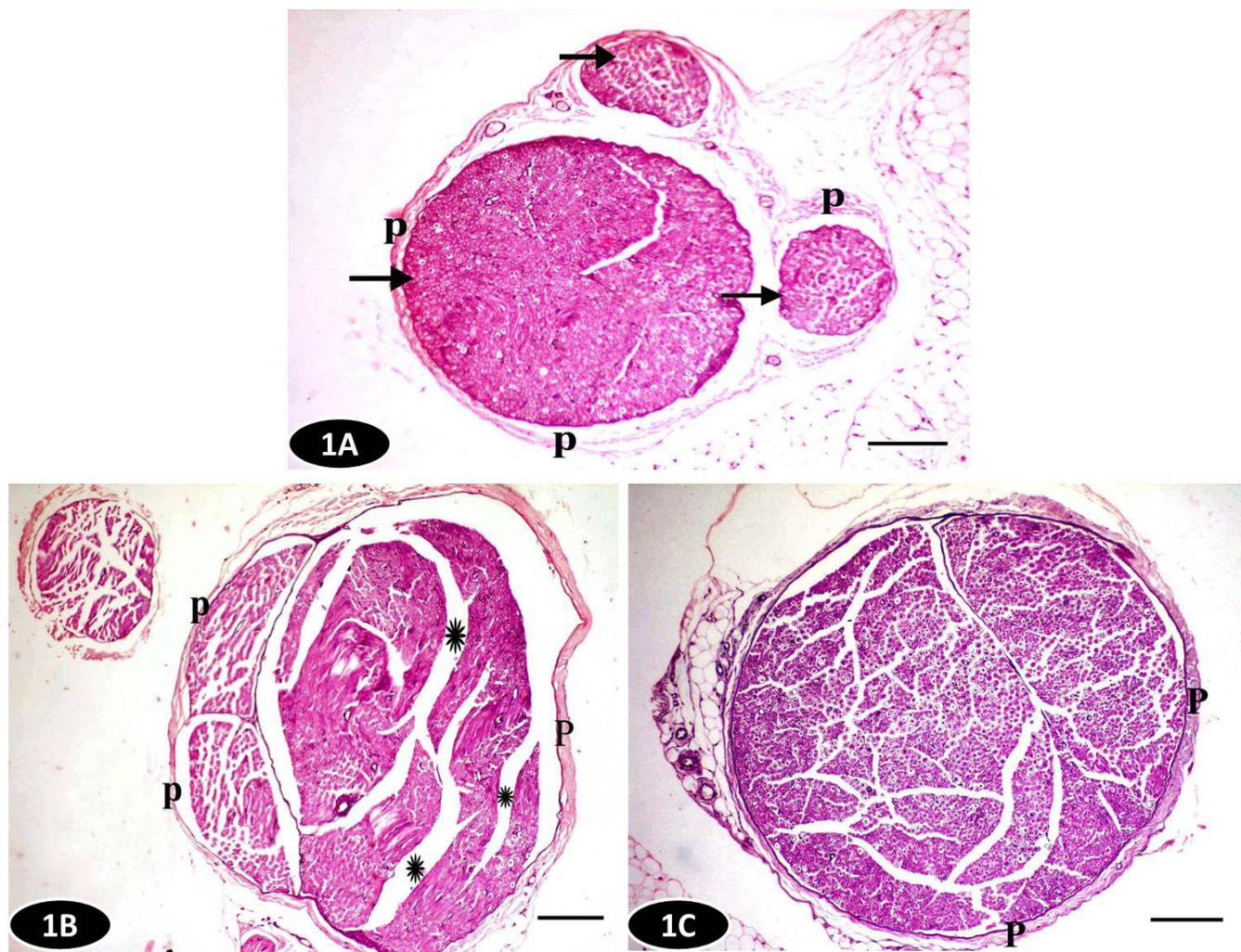
**Real time-PCR results**

There was a significant increase in netrin-1, ninjurin, brain derived neurotrophic factor (BDNF), glial cell line derived neurotrophic factor (GDNF), Vascular endothelial growth factor (VEGF) and angiopoitin-1 gene expression

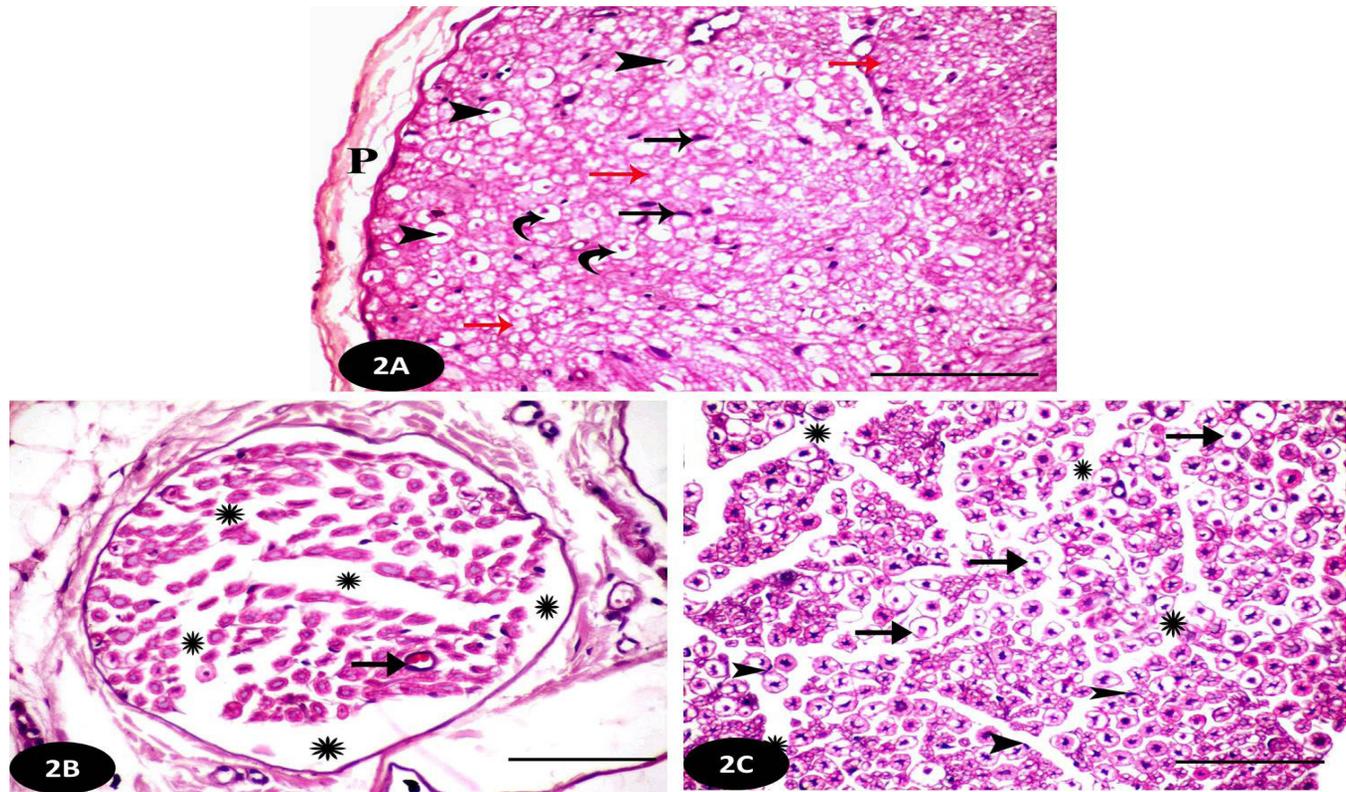
in ozone treated group than normal controls, aspartame group ( $p < 0.05$ ). (Table II).

**Histo-morphometric and statistical results**

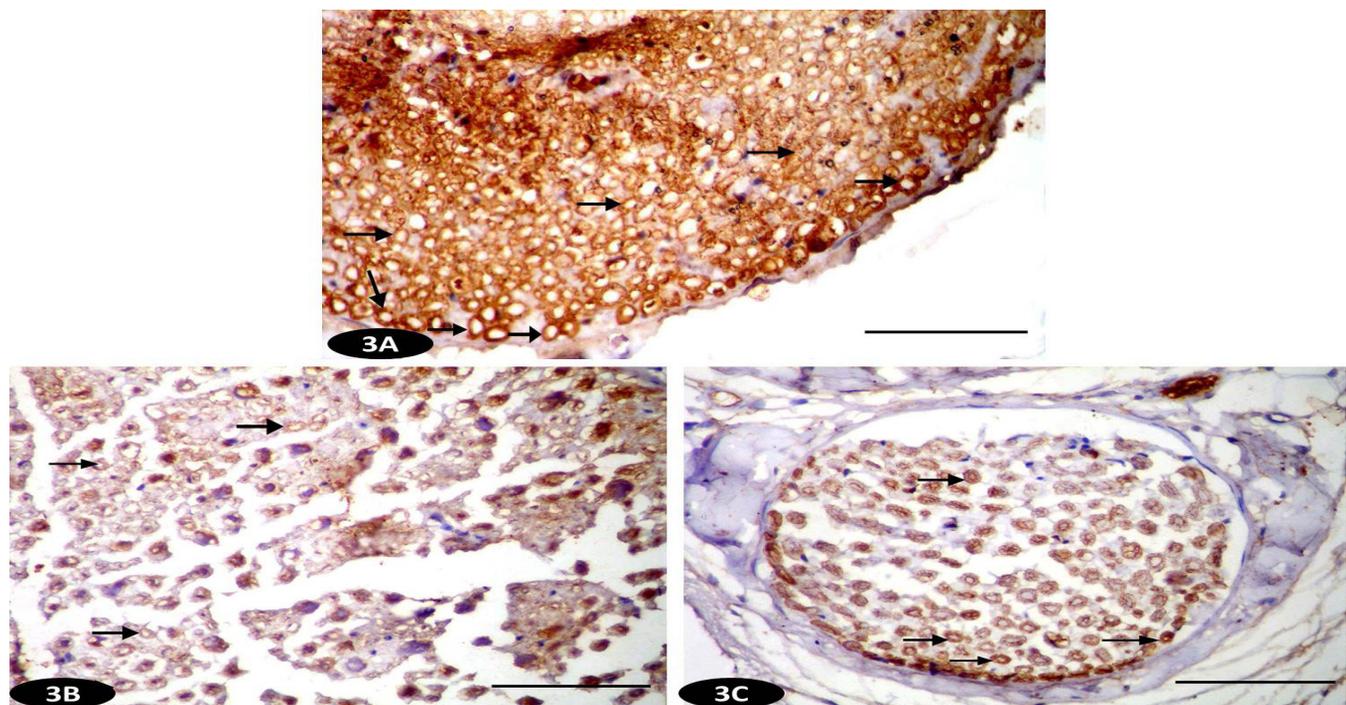
There was statistical significant difference between control and other groups regarding the axon/fiber (A/F) ratio in which the mean level of axon/fiber (A/F) ratio was significantly decreased in aspartame and aspartame with ozone groups than in control group ( $p < 0.05$ ). The comparison among different groups of the study by LSD test; showed that there was significant decrease in mean level of (A/F) ratio in aspartame group compared to control and aspartame with ozone groups ( $0.384 \pm 0.075$ ), ( $0.499 \pm 0.067$ ) and ( $0.470 \pm 0.064$ ) respectively ( $p < 0.05$ ). However there was no statistical significant difference in the mean level of A/F ratio between control and aspartame with ozone groups ( $0.499 \pm 0.067$ ) and ( $0.470 \pm 0.064$ ) respectively ( $p > 0.05$ ) (Table III).



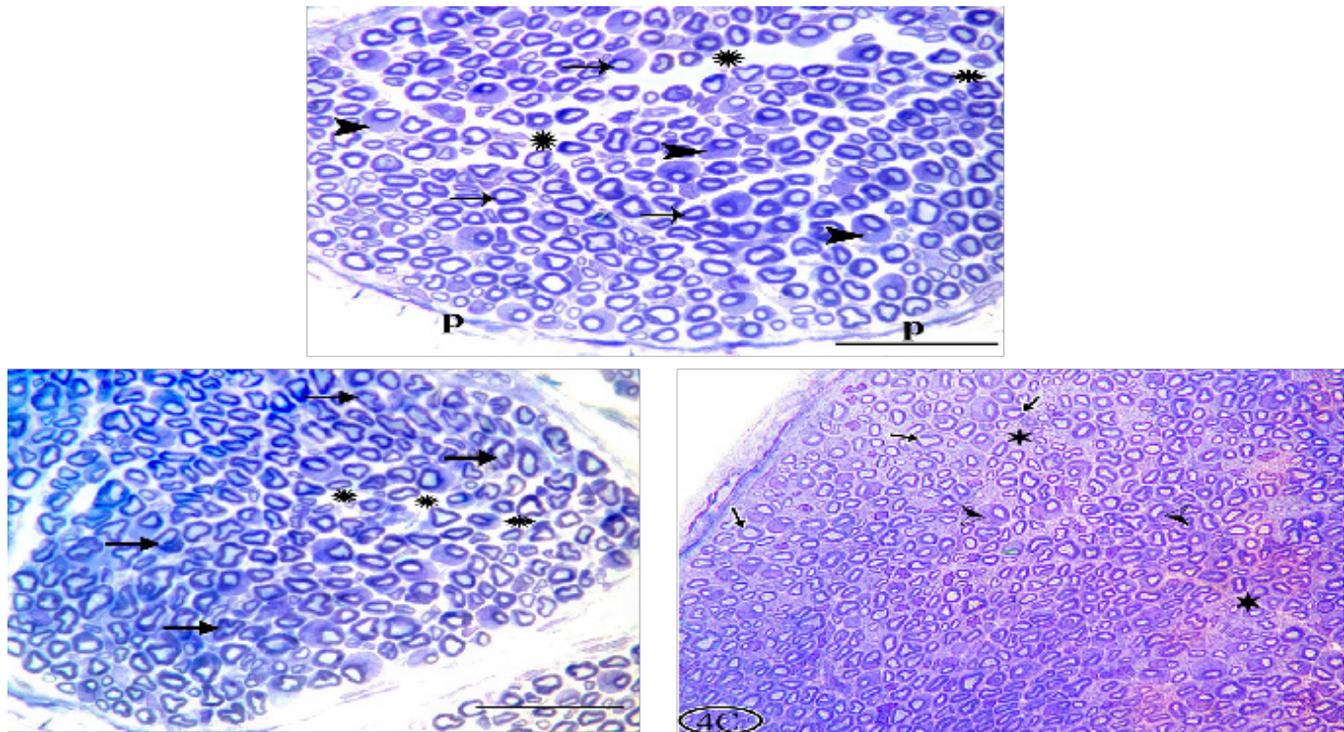
**Fig. 1:** A photomicrograph of transverse section of H&E-stained sciatic nerves of all studied groups showing in (A): Control adult male albino rat’s sciatic nerve showing bundles of nerve fibers (arrow), each bundle is invested by perineurium (P). (B): Aspartame group showing loss of regular architecture of sciatic nerve. Perineurium (P) is widely separated from underlying nerve fibers. Also, nerve fibers are separated from each other (asterisk). (C): Ozone and aspartame treated group showing apparently normal architecture of sciatic nerve. Nerve fascicles are surrounded by perineurium (P) (H&E×100)



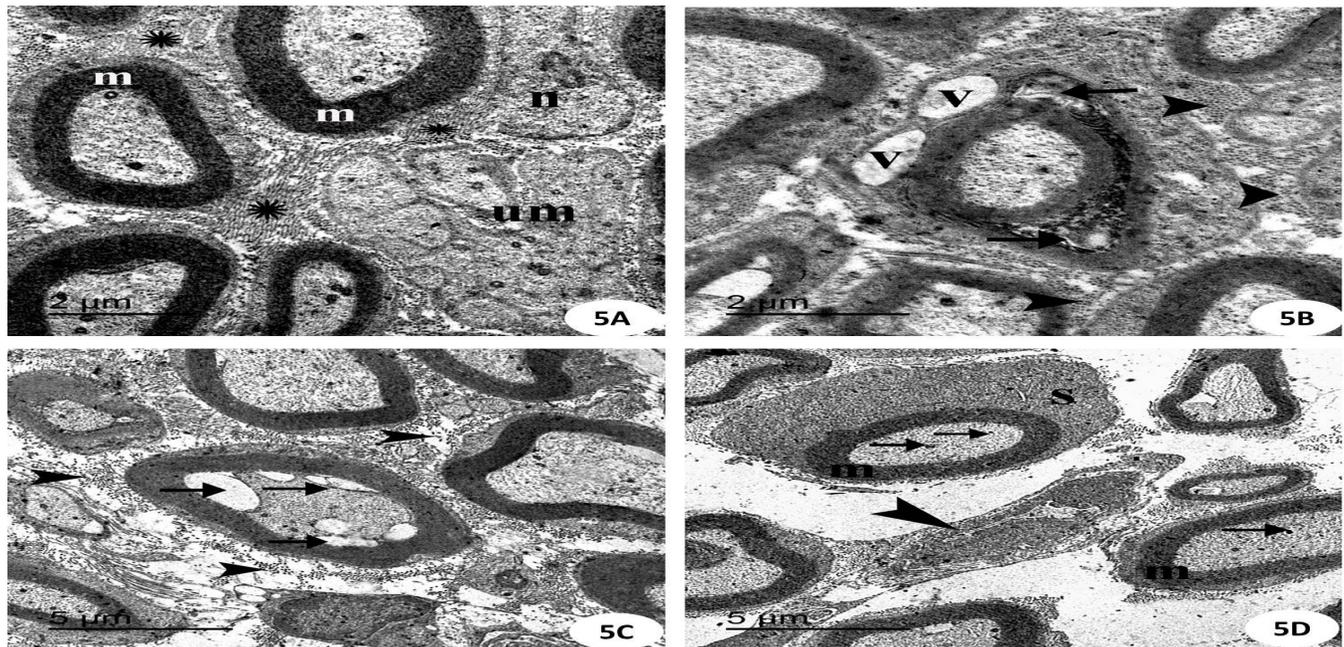
**Fig. 2:** A photomicrograph of H&E-stained sciatic nerves of all studied groups showing in (A): Control adult sciatic nerve fascicle containing group of nerve fibers surrounded by perineurium(p). Myelinated nerve fibers are formed of axoplasm (arrow head) surrounded by unstained area of dissolved myelin (curved arrow). Schwann cells nuclei (arrow) appear in-between the nerve fibers. Unmyelinated nerve fibers (red arrow) are also seen. (B): Aspartame group showing disorganization of nerve fascicles. Most of nerve fibers are widely separated from each other and from overlying perineurium (asterisk). Dilated blood vessel (arrow) is observed. (C): Ozone and aspartame treated group showing apparently normal nerve fascicle formed mostly of myelinated nerve fibers (arrow) and Schwann cells nuclei (arrow head) in endoneurial spaces. Nerve fibers are still widely separated (asterisk). (H&E×400).



**Fig. 3:** Immunohistochemical stained sections of sciatic nerves showing (A): Control group with strong positive staining for MBP in the myelin sheath (arrow). (B): Aspartame group showing weak positive reaction for MBP in the myelin sheath (arrow). While, (C): Ozone and aspartame treated group showing moderate positive reaction for MBP (arrow). (Immunohistochemical for MBP ×400)



**Fig. 4:** Toluidine blue stained sections of all studied groups showing (A): Control group nerve fascicle of sciatic nerve surrounded by connective tissue perineurium (P) and containing transverse section of myelinated nerve fibers of various sizes. Myelin sheaths appear as well preserved darkly stained structures, often rounded or elliptical (arrow) with endoneurium (asterisks) in between. Schwann cell cytoplasm (arrow head) looks paler than the myelin. (B): Aspartame Group showing irregular thickening of myelin (arrow) in many nerve fibers giving them distorted appearance. Wide separation (asterisk) in between the fibers is also seen. (C): Ozone and aspartame treated group showing nearly normal myelinated nerve fibers formed of darkly stained myelin sheath (arrow) surrounded by pale stained schwann cells (arrow head) separated by endoneurium (asterisks). (Toluidine blue  $\times$  400).



**Fig. 5:** Electron micrograph of sciatic nerve of all studied groups in (A): control group showing multiple axons; some appear myelinated while others appear unmyelinated. Myelinated axons have thick regular myelin sheath (m). Some unmyelinated axons (um) are seen surrounded by Schwann cell cytoplasm. Schwann cell euchromatic nucleus (n) and collagen fibrils (asterisk) in between nerve axons are also seen. (B): Aspartame group showing separation of myelin lamellae with focal loss of compact lamellar structure (splitting of myelin sheath) (arrow). Axons surrounded by a Schwann cell with vacuolated cytoplasm (V). Collagen fibrils (arrow head) could be seen in the endoneurium. (C): Aspartame group showing separation of myelin sheath (arrow) from the axon at some sites. Collagen fibrils (arrow head) could be seen in the endoneurium. (D): Ozone and aspartame treated group showing apparently normal structure of myelinated nerve fibers formed of compact myelin sheath (m) surrounded by Schwann cells cytoplasm (s). The axon displays neurofilaments (arrow) which have been cross-sectioned; giving the axon a stippled appearance. A blood capillary (arrow head) in endoneurial space is seen.

(TEM, A x 2000, B x 2500, Cx1000, Dx1000)

**Table 2:** Expression levels of studied genes in studied groups

| Groups<br>Parameter | Control<br>Mean±SD | Aspartame group<br>Mean±SD | Aspartame and Ozone Group<br>Mean±SD | F test | p         |
|---------------------|--------------------|----------------------------|--------------------------------------|--------|-----------|
| • Netrin-1          | 1.15±0.19          | 0.48±0.27 <sup>a</sup>     | 1.77±0.35 <sup>a,b</sup>             | 29.06  | <0.0001** |
| • Ninjurin-2        | 1.06±0.21          | 0.51±0.11 <sup>a</sup>     | 1.39±0.27 <sup>a,b</sup>             | 45.93  | <0.0001** |
| • BDNF              | 1.03±0.2           | 0.69±0.11                  | 1.97±0.51 <sup>a,b</sup>             | 42.24  | <0.0001** |
| • GDNF              | 1.13±0.21          | 0.78±0.17 <sup>a</sup>     | 1.86±0.39 <sup>a,b</sup>             | 40.47  | <0.0001** |
| • VEGF              | 1.18±0.57          | 1.09±0.24                  | 2.03±0.52 <sup>a,b</sup>             | 12.36  | <0.0002** |
| • Angipoin-1        | 1.3±0.16           | 0.71±0.08 <sup>a</sup>     | 2.11±0.32 <sup>a,b</sup>             | 110.27 | <0.0001** |

a: significant when compared with control group

b: significant when compared with aspartame group.

**Table 3:** Comparisons between mean values the axon/fiber (A/F) ratio (g-ratio) in the different studied group using ANOVA (analysis of variance) test.

| Groups<br>Parameter | Control     | Aspartame group | Aspartame and Ozone Group | F    | p      |
|---------------------|-------------|-----------------|---------------------------|------|--------|
| Mean±SD             | 0.499±0.067 | 0.384±0.075     | 0.470±0.064               | 7.55 | 0.002* |
| LSD test            |             | 0.002*          | 0.62                      |      |        |
| a=versus control    |             | a               | a                         |      |        |
| b=versus aspartame  |             | 0.02*           | b                         |      |        |

Number of sacrificed rats for each group was 10 rats.

SD: Standard Deviation.

\*\* : highly significant ( $p < 0.001$ ).

## DISCUSSION

The results obtained from the present study showed that the administration of aspartame at a dose 250 mg/kg/day produce histopathological changes of the sciatic nerve. In the present study, H&E stained sections showed loss of regular architecture of sciatic nerve with separation of nerve fibers from each others. Dilated blood vessels were also observed. Aspartame induced neurotoxic effects and ultrastructural changes in the sciatic nerve mainly affecting myelin sheath in the form of separation of myelin lamellae, focal loss of compact lamellar structure (splitting of myelinated sheath) and separation of myelin sheathes from the axons at some sites. These degenerative changes may be due to the direct toxic effect of aspartame on sciatic nerve. This was in agreement with many authors which described the effect of methanol which produce morphological changes of thyroid gland<sup>[30]</sup>. Disruption of myelin lamellae with separation of it from some sites of axons which was detected in aspartame group. This result attributed by Kaufmann *et al.*<sup>[31]</sup> to the injury of myelin-producing cells.

Mourad *et al.*<sup>[32]</sup> contributed the neurotoxic effects of aspartame to its triple toxins: aspartic acid, phenylalanine, and methanol which are formed after ingestion and metabolism of aspartame in the gastrointestinal tract. These metabolites undergo further breakdown producing formaldehyde and formic acid. Abhilash *et al.*<sup>[33]</sup> reported that a small amount of aspartame significantly increases plasma methanol, formaldehyde, and formate levels leading to the formation of superoxide anion and hydrogen peroxide which may contribute to increased production of free radicals<sup>[34]</sup>.

Ultrastructurally, Schwann cells appeared with vacuolated cytoplasm and collagen fibrils were seen in

the wide spaces in between the nerve fibers in aspartame group. Salzer<sup>[35]</sup> suggested that when Schwann cells are injured, swelling of their cytoplasmic processes and separation of myelin lamellae occurred. Andersson *et al.*<sup>[36]</sup> added that abnormal myelin loops is an indicator of axonal atrophy which represents an acute secondary response of functioning myelin sheaths to the decline in axonal size. It was reported that these vacuoles in Schwann cell cytoplasm might be electrolucent fat vacuoles which present as the cumulative effect of increased myelin degeneration and catabolism<sup>[37]</sup>. López-Lluch and Navas<sup>[38]</sup>; Rangaraju *et al.*<sup>[39]</sup> reported that Schwann cells are rich in polyunsaturated fatty acids, which serve as a substrate for reactive oxygen species mediated lipid peroxidation which could explain our finding. Since lipids are sensitive to free radical damage, myelinated nerves provide an optimal environment for lipid peroxidation mediated oxidative damage to organelles and cellular proteins with increased proteolytic susceptibility<sup>[40,41]</sup>.

In the present study, immunohistochemical examination of MBP in aspartame group showed decrease in its expression in myelinated sheath in comparison to control group. While ozone and aspartame treated group showed increase in its expression in the myelinated sheath of myelinated nerve fibers. This is in agreement of Sameni and Panahi<sup>[42]</sup> who reported that MBP is the major constituent protein of the nervous system that binds to the myelin lipids to maintain the myelin structure and promotes the myelination process. So it is used as an indicator of the degree of CNS and myelin damage. The g-ratio is an important indicator of myelinated fiber function. As the g-ratio is determined by the ratio of axon to fiber diameters. The present study showed that the g-ratio value measured in the adult rat sciatic nerve was about 0.499. The optimal action potential conduction in

myelinated fibers occurs when g-ratio values are close to 0.6<sup>[28]</sup>. In our study, morphometric analysis of the sciatic nerve of aspartame group revealed a significant reduction in the g-ratio (axon/fiber ratio). This reduction may be due to decreased axon diameter or an increase in fiber diameter. The increased fiber diameter is most probably caused by irregular thickening or splitting of myelin<sup>[43]</sup>. In this study, the decreased axon diameter might have been due to axonal atrophy. Hence, when the morphological changes improved in the ozone group, the g-ratio increased again.

The present work showed improvement in the sciatic nerve structure after ozone application. Immunohistochemically, strong positive reaction for MBP was detected in myeline sheath of myelinated nerve fibers. This is in accordance of Ozbay *et al.*<sup>[18]</sup> who reported that there was improvement in facial nerve function after ozone therapy. This regeneration was evaluated by assessment of the degree of macrovacuolization, vascular congestion, and myelin sheath thickness<sup>[44]</sup>. Valko *et al.*<sup>[45]</sup> suggested that Ozone may promote a moderate oxidative stress that in turn increases endogenous antioxidant systems leading to a decrease in free radicals.

Geremia *et al.*<sup>[46]</sup> reported that brain derived neurotrophic factor (BDNF) had an important role in response of the cell body in injured neurons. When exposed to BDNF, stem

cells differentiated into neuronal lineages in vitro. Höke *et al.*<sup>[47]</sup> added that glial cell line derived neurotrophic factor (GDNF) is the most essential factor for formation of both motor and sensory neurons during the regeneration process. Vascular endothelial growth factor (VEGF) and angiopoietin-1 also assists nerve regeneration, due to the close relationship existing between the nerve fibers and the blood vessels during this process<sup>[48]</sup>. In an experimental study, VEGF showed an effect on blood supply, with an increase of axonal regeneration and of Schwann cells, stimulating nerve regeneration<sup>[49]</sup>. Netrins as netrin-1 and ninjurin-2 promote outgrowth of neurites and have a possible role in neuronal response to injury<sup>[50]</sup>. In the current study, we found a higher expression of netrin-1, ninjurin, neurotrophic factors such as brain derived neurotrophic factor (BDNF), glial cell line derived neurotrophic factor (GDNF) in ozone treated group compared to aspartame and control groups. In addition, angiogenic factors such as vascular endothelial growth factor (VEGF) and angiopoietin-1, and neurite guidance proteins such as netrin and ninjurin-2 were also expressed highly in ozone treated group than aspartame and healthy rats. Taken together, highlighting the role of ozone in improvement of peripheral neuropathy caused by aspartame.

In conclusion, aspartame induced histopathological changes in sciatic nerve after its administration for 3 months. Our data suggests that ozone therapy has an important role in sciatic nerve regeneration, basically by increasing the expression of neurotrophic and angiogenic factors establishing more favorable environment for nerve

regeneration. We suggest that the use of aspartame for longer period will cause more changes in sciatic nerve. So, further studies are needed to confirm our findings.

#### CONFLICT OF INTEREST

There are no Conflicts of interest.

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## الملخص العربي

## تأثير تعاطي الأسبارتام على المدى الطويل على العصب الوركي في ذكور الجرذان البيضاء البالغة والدور العلاجي المحتمل للأوزون (دراسة هستولوجية وكيميائية حيوية)

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**المقدمة:** يعتبر الأسبارتام مادة تحلية صناعية غير غذائية تستخدم على نطاق واسع ، والتي حلت محل السكر في الأطعمة لتعزيز النكهة والتقليل من السعرات الحرارية. وقد تأكد أن الأسبارتام هو من العوامل المسببة للسرطان ويزيد من خطر حدوث أورام الغدد اللعابية والأورام العصبية.

**الهدف من العمل:** يهدف هذا العمل إلى تقييم تأثير الأسبارتام على تركيب العصب الوركي والدور المحتمل للعلاج بالأوزون.

**المواد وطرق البحث:** أجريت الدراسة الحالية على ٣٠ من ذكور الجرذان البيضاء البالغة مقسمة إلى ثلاث مجموعات هي: المجموعة الضابطة (١) ، المجموعة المعالجة بالأسبارتام (٢) والمجموعة المعالجة بالأوزون والاسبرتام (٣). تلقت المجموعة (٢) الأسبارتام (٢٥٠ مجم / كجم / يومياً) مذابة في الماء المقطر وتعطى بواسطة أنبوب المعدة لمدة 3 أشهر. تلقت المجموعة (٣) الأسبارتام كما في المجموعة الثانية ، ثم تلقت جرعة الأوزون من 1.1 مغ / كغ / يومياً داخل الغشاء البريتوني لمدة 30 يوماً. تم تخدير الجرذان وتمت تحضير الأعصاب الوركية من أجل فحصها بالمجهر الضوئي و الإلكتروني و التحليل المورفومتري وتفاعل البلزمة المتسلسل.

**النتائج:** أظهر العصب الوركي لمجموعة الأسبارتام فقدان التركيب المنتظم ، وفصل في صفائح المايلين مع تقسيم غمد المايلين. كما ظهرت خلايا شوان مع وجود فجوات في السيتوبلازم وتمكن من رؤية الياف الكولاجين في الغلاف العصبي الداخلي. وبالدراسة الهستوكيميائية مناعية وجد انخفاض في التعبير عن البروتين الأساسي في المايلين وانخفاض في نسبة المحور العصبي/الاياف إحصائياً. وقد لوحظ تحسن في التركيب الهيستولوجي في مجموعة الأوزون.

**الخلاصة:** استنتجنا ان الأسبارتام تسبب في تغيرات نسيجية في العصب الوركي وتشير بياناتنا إلى أن علاج الأوزون له دور مهم في تجديد العصب الوركي عن طريق زيادة التعبير عن العوامل العصبية والوعائية التي تنشئ بيئة أكثر ملاءمة لتجديد الأعصاب.