in vivo *35*: 1537-1543 (2021)
doi:10.21873/invivo.12407

The Effect of Lutein on Ischemia-reperfusion-induced Vasculitic Neuropathic Pain and Neuropathy in Rats

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Abstract. *Background/Aim: Neuropathic pain and neuropathy is commonly seen after ischemia-reperfusion injuries. Our aim was to evaluate the effect of lutein on ischemia-reperfusion (I/R)-induced vasculitic neuropathic pain and neuropathy in rats. Materials and Methods: An hour before anesthesia, 6 Albino Wistar male rats with I/R were orally administered with 1 mg/kg lutein (LIR group). Two groups of 6 such rats who underwent surgery were provided with 0.5 ml distilled water (as solvent) either via oral administration (SIR group) or by gavage (sham group or SG). One hour following the administration, the later femoral arteries of the LIR and SIR rats were closed using a sterile silk thread and ischemia was induced in the sciatic nerve for 4 h, followed by reperfusion for 24 h. The femoral artery of the SG group was not closed with suture. Next, 1 mg/kg lutein was re-administered only to the LIR group for 1 h, followed by measurement of the paw pain thresholds by the Basile Algesimeter. The levels of malondialdehyde (MDA), total glutathione (tGSH), nuclear factor-kB (NF-κB), and tumor necrosis factor-alpha (TNF-α) in the sciatic nerve tissues were measured, and the tissues were histopathologically examined. Results: We found that the MDA, NF-κB, and TNF-α levels were higher and the tGSH level was lower in the SIR group relative to those in the LIR group, and the differences were statistically significant. Significant histopathological damage was noted in the SIR group, whereas the LIR group demonstrated protection from oxidative damage. Conclusion: Lutein is potentially useful in the treatment of I/R-related neuropathy and neuropathic pain.*

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Key Words: Sciatic nerve, lutein oxidative injury.

Ischemia-reperfusion (I/R) injury is a complex pathological process that begins with a decrease in the tissue oxygen levels, followed by the production of free oxygen radicals, resulting in an inflammatory response (1). The I/R event in the vascular and nervous systems have been documented with an increasing ischemic and vasculitic neuropathic pain that clinically resembles the complex regional pain syndrome (2). In isolated vasculitis, a form of vasculitic neuropathy of the peripheral nervous system, medium- and small-size vessels in peripheral nerves are usually affected (3). The incidence of vasculitic neuropathies is greater in the lower extremities and causes severe pain (4). Some reports assert that neuropathy may be the first or even the only indicator of vasculitis (5).

An I/R event is also known to induce significant damages to the nerve cells as well as to the internal organs (6). Reportedly, the I/R process induces microvascular changes, which lead to structural and functional disorders of nerve tissues (7). The pathophysiology of I/R injury consists of a complex event that involves the obstruction of the capillaries, immune cell activation, free oxygen radical (FOR) production, lipid peroxidation (LPO), and increased antioxidant expenditure (8, 9). The together, these data suggest that drugs with antioxidant and anti-inflammatory properties may be useful in the treatment of vasculitic neuropathic pain.

Lutein ($C_{40}H_{56}O_2$) is a known antioxidant and anti-inflammatory tetraterpenoid (10). Lutein can inhibit the antioxidant activity inside cells by inhibiting lipid peroxidation and preventing reduction of the Glutathione (GSH) levels (11). Lutein has also been reported to possess anti-inflammatory properties (12). It has been demonstrated to protect tissues from damages caused by excessive production of nuclear factor-kB (NF-κB) and tumor necrosis factor-alpha (TNF-α) (13). Despite this, the effects of lutein on I/R-induced vasculitic neuropathy and neuropathic pain have not been investigated. I/R in the rat femoral artery has, however, been proposed as an experimental model of

vasculitic peripheral neuropathy with clinically observed neuropathic pain and peripheral nerve injury (9).

In this study, we investigated the effect of lutein on I/R-induced peripheral (sciatic nerve) vasculitic neuropathy and neuropathic pain in rats using biochemical and histopathological analysis.

Materials and Methods

A total of 18 Albino Wistar male rats (weight=275-288 g) were obtained from the Atatürk University Medical Experimental Application and Research Center (Erzurum, Turkey). All animals were housed and fed at the normal room temperature (22˚C) for 1 week prior to the experiment. The protocols and procedures of the animal study were approved by the local Animal Experimentation Ethics Committee (No: 77040475-641.04-E.1900086740).

Ketamin used in the experiment was obtained from Pfizer (Istanbul, Turkey), and lutein was obtained from Solgar (Leonia, NJ, USA). The rats were divided into 3 groups as follows: i) LIR group composed of I/R-induced rats treated with 1 mg/kg lutein, ii) SIR group composed of I/R-induced rats without lutein treatment, and iii) Sham group (SG) composed of rats with no I/R or lutein treatment.

Experimental procedure. The surgical procedures on the rats were performed in an appropriate laboratory environment and under sterile conditions. Anesthesia was induced with 60 mg/kg ketamine hydrochloride. An hour before anesthesia, 1 mg/kg lutein was orally administered to the LIR (n=6) group, and 0.5 ml distilled water (as solvent) was orally administered to the SIR (n=6) and *via* gavage to the SG (n=6) group. An hour after the administration of the drug and distilled water, all rats were fixed to the operating table in the supine position. The femoral and inguinal regions of the rats were shaved and sterilized with povidone-iodine solution. The right femoral arteries of the LIR, SIR, and SG rats were exposed with a skin incision made in the inguinal region. In the SG group, sutures were placed around the femoral artery of the rats without being tightened. In the LIR and SIR groups, ischemia was induced by occluding the femoral artery for 4 h using a silk suture (6-0) (Ethicon, New Brunswick, NJ, USA) and a slip-knot technique. At the end of this period, re-perfusion of the peripheral nerve tissues was achieved for 24 h. The area that had opened during the reperfusion was sutured and closed using a sterile surgical thread. At the 24th hour of reperfusion, 1 mg/kg lutein was re-administered to the LIR group, and the same volume of distilled water was administered to the SIR and SG groups. After 1 h, the paw pain thresholds of the rats were estimated by using the Basile Algesimeter (14). Immediately after the measurement, the rats were sacrificed by administering high-dose ketamine (120 mg/kg) anesthesia, and their sciatic nerve tissues were removed, for the determination of malondialdehyde (MDA), total glutathione (tGSH), nuclear factor-kB (NF-κB), and tumor necrosis factor-alpha (TNF-α) levels as well as for histopathological examination.

MDA analysis. The MDA assessment method was based on the one proposed by Ohkawa *et al*. (15). This method involves spectrophotometric assessment of the absorption of the pink complex created by a reaction between thiobarbituric acid (TBA) and MDA at high temperature (95˚C) and 532-nm wavelength. The sciatic nerve tissue homogenates were centrifuged for 20 min at 5,000 g, and the supernatants were applied for the determination of the amount of MDA. Next, 250 μl of the homogenates were added to a mix of 100 μl of 8% sodium dodecyl sulfate (SDS), 750 μL of 20% acetic acid, 750 μl of 0.08% TBA, and 150 μl of distilled water, and were mixed into capped test tubes by pipetting. The mixture was then incubated for 60 min at 100˚C, after which 2.5 ml of n-butanol was added to it and the mixture was subjected to spectrophotometric analysis. The amounts of the resultant red color were measured using 3-ml cuvettes at 532 nm wavelength. The MDA amounts in the samples were determined with reference to the standard graphics prepared by the analysis of the MDA stock solution that was previously prepared considering the dilution coefficients.

tGSH analysis. The 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) compound in the assessment environment is a disulfide chromogen and easily reduced by the sulfhydryl group compounds. The resultant yellow color after the reaction was spectrophotometrically assessed at 412 nm (16). The sciatic nerve tissue homogenates were centrifuged for 10 min at 12,000 g, and the supernatants were used for the determination of the MDA level. Next, 250 μl of a measuring buffer (200 mM Tris-HCl, pH=8.2 containing 0.2 mM EDTA), 500 μl of the supernatant, 100 μl of DTNB, and 790 μl of methanol were mixed into the capped test tubes by pipetting. The mixture was then incubated for 30 min at 37˚C and subjected to spectrophotometric analysis. The amount of the resultant yellow color was determined in 3-ml quartz cuvettes at 412-nm wavelength. The GSH amounts in the samples were determined with reference to the standard graphics using the GSH stock solution previously prepared considering the dilution coefficients.

TNF-α and NF-κB analysis. The tissue-homogenate levels of NF-κB and TNF-α were determined using the rat-specific sandwich enzyme-linked immunosorbent assay, Rat Nuclear Factor-Kappa B ELISA immunoassay kit (Cat. No: 201-11-0288; Shanghai Sunred Biological Technology Co. Ltd., Shanghai, PR China) and Rat Tumor Necrosis Factor α ELISA kits (Cat no: YHB1098Ra; Shanghai Sunred Biological Technology Co. Ltd., Shanghai, PR China). The analyses were performed according to the manufacturers' instructions. Briefly, monoclonal antibodies specific for either rat NF-κB or rat TNF-α were coated onto the wells of micro plates. The tissue homogenate, standards, and biotinylated specific monoclonal antibody and streptavidin-HRP were pipetted into these wells and then incubated at 37˚C for 60 min. After washing, the chromogen reagent A and chromogen reagent B were added to the wells and incubated at 37˚C for 10 min. These reagents produce a color after reacting with the bound enzyme. The reaction is then stopped by adding a stop solution. The intensity of the colored product produced is directly proportional to the concentration of the NF-κB and TNF-α present in the original specimen obtained from the rats. At the end of the experiment, the wells contents were read at 450 nm.

Histopathological examination. All the tissue samples were first fixed in a 10% formaldehyde solution and then assessed by light microscopy. Following the identification process, the tissue samples were washed under tap water in cassettes for 24 h. The samples were then treated with a conventional grade of alcohol (70%, 80%,

90%, and 100%) to remove the water contained within the tissues. Next, the tissues were passed through xylol, embedded in paraffin, cut into 4-5 μ-thick sections, and stained with hematoxylin–eosin. Their images were captured following assessment by the Olympus DP2-SAL firmware program (Olympus® Inc. Tokyo, Japan). Histopathological analyses were performed by a pathologist blinded to the study groups.

Statistical analyses. All data were subjected to the Kruskal-Wallis test using the SPSS version 18.0 software (IBM Corporation, Armonk, NY, USA). The differences among the groups were obtained using Wilcoxon rank-sum tests with Bonferroni corrections. Statistical significance was set at p-Value=0.05. The results were expressed as the mean±standard error of the mean (SEM).

Results

The sciatic nerve tissues showed statistically significant decrease in the pain threshold of the rat paw in the LIR group in comparison to the SIR group ($p<0.0001$; Table I).

The amount of MDA increased in the sciatic nerve tissues of the rats in the SIR group (Figure 1), while lutein could significantly suppress the MDA level increase in the sciatic nerve tissues after the SIR procedure ($p<0.0001$). Conversely, the amount of tGSH decreased in the sciatic nerve tissues of the rats in the SIR group, while lutein could significantly prevent I/R-induced tGSH decrease in the sciatic nerve tissues ($p<0.0001$).

The discontinuation of the blood flow from the femoral artery to the sciatic nerve tissues increased both TNF-α and NF-κB levels in the nerve tissues; however, lutein could significantly inhibit the increase of TNF-α and NF-κB levels in the nerve tissues after SIR ($p<0.0001$; Figure 2).

Histopathological findings. Histological examination of the SG sciatic nerve tissues revealed that the nerve structure was normal, the axons were surrounded by myelin sheaths and were located centrally, and the Schwann cell nucleuses were normal in shape. The middle layer of the connective tissue investments, the perineurium, properly covered each bundle of nerve fiber fascicles and its thickness was also normal (Figure 3). In the SIR group, the myelinated nerve fibers were swollen, and the myelin sheath surrounding the axons lost their central position. Dislocations and irregularities were noted in the nevre fascicules. The degenerated and distorted myelin sheaths attracted attention. The Schwann cells generally demonstrated hypertrophy and hyperplasia. The blood vessels were mostly congested. The perineurium was quite thick and revealed an irregular structure (Figures 4 and 5). In the LIR group, the myelinated nerve fibers were generally normal in sight and axons located centrally. The Schwann cells were normal in shape, the degeneration of the myelin sheats had vanished, and the blood vessels also appeared normal. In addition, all connective tissue structuring was normal (Figure 6).

Table I. *The effect of lutein on ischemia-reperfusion (I/R) induced vasculitic neuropathic pain.*

Group	Pain threshold (gr) 1st hour	Analgesia activity (%) 1st hour
SIR	5.7±0.5	
LIR	19.5±1.2	70.8
SG	29±1.2	80.4

LIR: Lutein ischemia-reperfusion; SIR: ischemia-reperfusion with no lutein; SG: sham group.

Discussion

In this study, the effect of lutein on I/R-induced sciatic nerve neuropathic pain and vasculitic neuropathy were examined in rats by biochemical and histopathological examinations. Our results revealed that the application of the LIR procedure to the sciatic nerve tissues significantly reduced the pain threshold in the paw of the experimental rats. These findings suggest that hyperalgesia develops in the region innervated by the sciatic nerve. Moreover, it has previously been emphasized that the application of vascular occlusion or an I/R event to nerve tissues results in vasculitic neuropathic pain, which is clinically similar to the regional pain syndrome (2).

Chung *et al*., have employed the same approach as ours to evaluate the peripheral neuropathic pain and found that neuropathic pain and an experimental model of vasculitic peripheral neuropathy both develop after the discontinuation of femoral arterial blood flow (9, 17). Neuropathic pain manifests as spontaneous pain, such as i) hyperalgia, ii) allodynia, iii) pain independent of stimulus, or iv) pain induced by stimulus (pain hypersensitivity) after the peripheral nerve damage. Vasculitic peripheral neuropathic pain manifests as an acute pain arising from ischemic injury of the peripheral nerves (9, 18). Our experimental results together with the literature data indicate that the application of the I/R procedure to sciatic nerve tissues may result in neuropathy and neuropathic pain.

In the literature, the pathophysiology of peripheral nerve I/R tissue damage has been associated with increased FOR production (19). In fact, the application of the reperfusion procedure to the ischemic tissues is considered as a pathophysiological event separate from ischemic injury. It has been long argued that the reasons that initiate reperfusion injury include superoxide radicals, hydrogen peroxide, and hydroxyl radicals (20). In our study, an increase in the MDA level and a decrease in the tGSH level were recorded in the sciatic nerve tissues of rats treated with I/R; these results are consistent with those reported in the literature. It has been reported that the MDA level increases and the GSH level

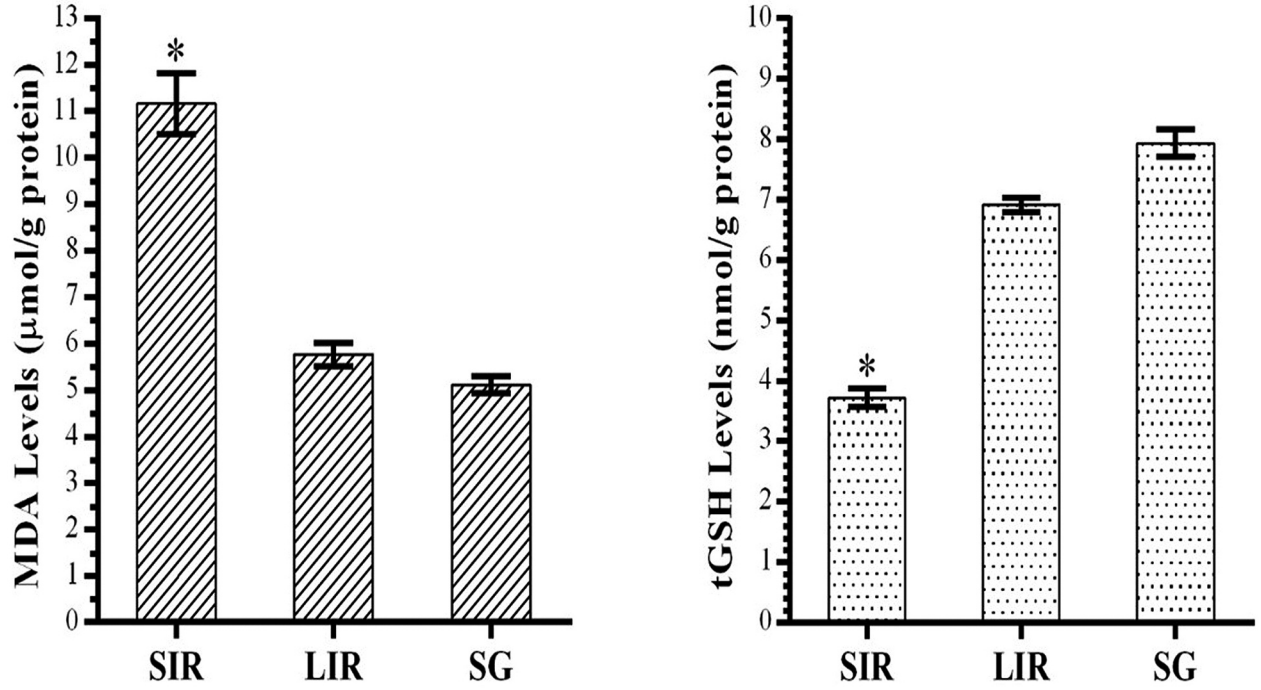

Figure 1. *Malondialdehyde (MDA) and total glutathione (tGSH) levels of SIR, LIR and SG groups. *p<0.0001, according to LIR and SG groups (n=6). LIR: Lutein ischemia-reperfusion; SIR: ischemia-reperfusion with no lutein; SG: sham group.*

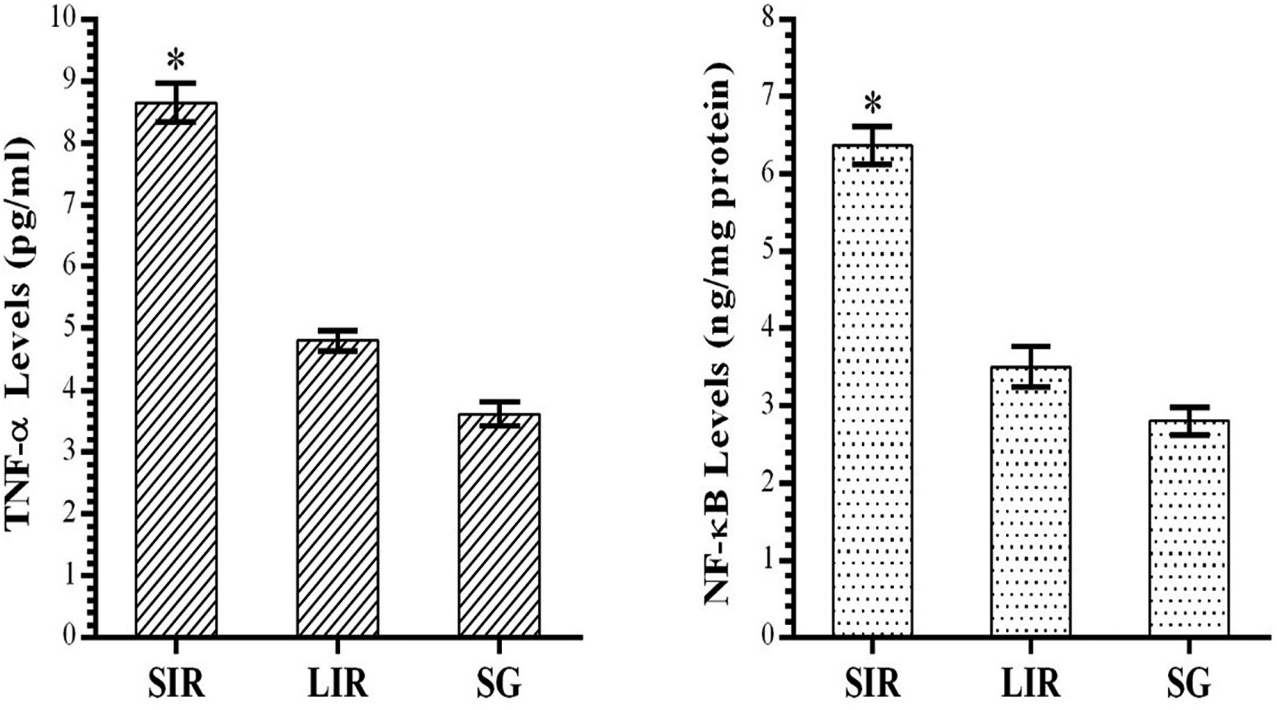

Figure 2. *Nuclear factor-kB (NF-κB) and tumor necrosis factor-alpha TNF-α levels of SIR, LIR and SG groups. *p<0.0001, according to LIR and SG groups (n=6). LIR: Lutein ischemia-reperfusion; SIR: ischemia-reperfusion with no lutein; SG: sham group.*

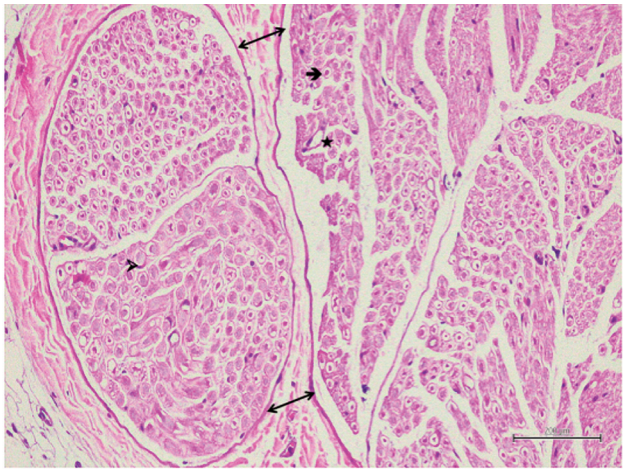

Figure 3. *Hematoxylin–eosin staining in sciatic nerve tissue in the control group. →: Myelinated axon, ➢: Schwann cell nucleus, ★: blood vessel, ↔: perineurium, Magnification: ×200.*

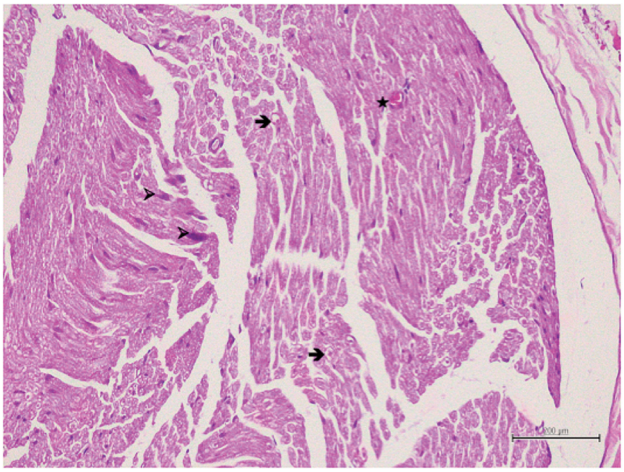

Figure 4. *Hematoxylin–eosin staining in sciatic nerve tissue in the ischemia/reperfusion group. →: Degenerated myelinated axon, ➢: hypertrophic and hyperplasic Schwann cell nucleus, ★: dilated and congested blood vessel, Magnification: ×200.*

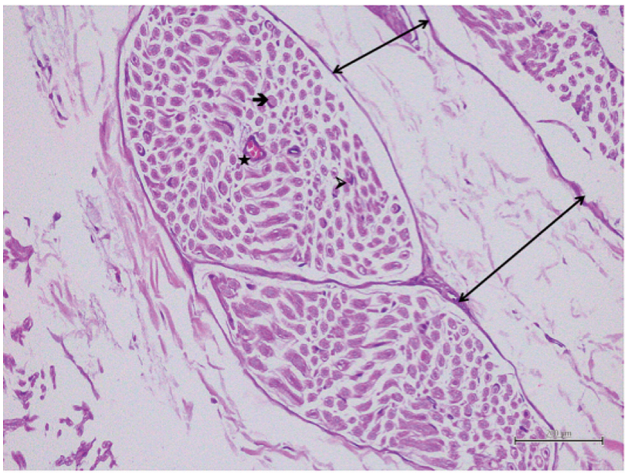

Figure 5. *Hematoxylin–eosin staining in sciatic nerve tissue in the ischemia/reperfusion group. →: Myelinated axon, ➢: hypertrophic and hyperplasic Schwann cell nucleus, ★: blood vessel, ↔: perineurium, Magnification: ×200.*

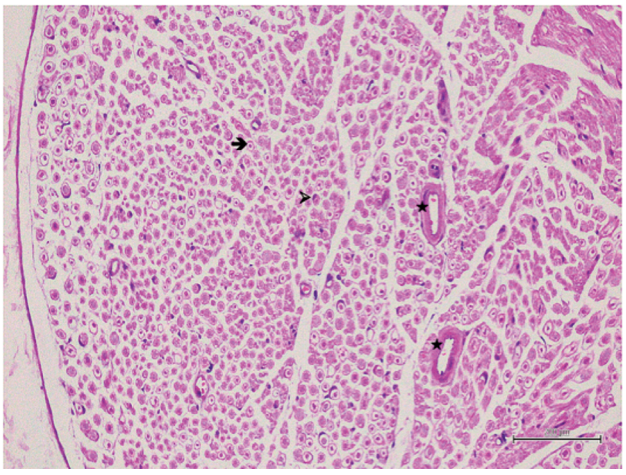

Figure 6. *Hematoxylin–eosin staining in sciatic nerve tissue in the ischemia/reperfusion + lutein group. →: Myelinated axon, ➢: normal Schwann cell nucleus, ★: normal blood vessel, Magnification: ×200.*

decreases under oxidative stress in parallel with an increase in FOR production (21). It has also been documented that FORs cause the formation of toxic products, such as MDA, by oxidizing lipids found in the cell membrane (22). MDA is an oxidant product that increases and exacerbates oxidative damage (23). We attributed the reason for decreasing tGSH to FOR expenditure due to the overproduction of FORs. Furthermore, we noted that if the antioxidants are insufficient in neutralizing the oxidants, the oxidant/antioxidant balance gets disrupted in favor of the oxidants; this inference based on our experimental results is consistent with the literature (21).

The application of I/R as found to increase the production of proinflammatory cytokines, such as TNF-α, as well as transcription factors regulating proinflammatory signaling, such as NF-κB, in the sciatic nerve tissues. The inhibition of TNF-α, NF-κB, and other proinflammatory cytokine-related pathways is accepted as a possible target in the treatment of

inflammation and neuropathic pain (9). During neuronal inflammation, the activation of NF-κB regulates the expression of various proinflammatory mediators, resulting in nerve damage (24). Past studies have demonstrated that the NF-κB expression in the nerve tissues during the femoral artery-associated sciatic nerve I/R event induces a neuroinflammatory response. In addition, an I/R event can lead to vasculitic peripheral neuropathy, and the expression of TNF-α and NF-κB are involved in vasculitic neuropathic pain (9). In agreement with the literature, our results indicate that the I/R procedure induces proinflammatory signaling production in sciatic nerve tissues.

Lutein was examined for its effect on neuropathic pain caused by sciatic nerve I/R and was found to significantly prevent the pain threshold reduction in the rat paws. There is no report in the literature on the effect of lutein on the sciatic nerve vasculitic damage and in the induction of neuropathic pain. However, some past studies suggest that lutein can prevent neuropathic pain. For instance, Tan *et al*., have reported that lutein can avoid brain injury caused by cytokine-induced inflammation and FOR-induced oxidative stress (25). Furthermore, it has been reported that lutein protects the optic nerve tissues from damage from ethambutol and isoniazid *via* the inhibition of the MDA, TNF-α production and tGSH consumption (26). Our results are consistent with reports of the protective effect of lutein in tissues damaged from the overproduction of NF-κB and TNF-α (13). Therefore, we believe that the analgesic effect of lutein can be attributed to FOR production and proinflammatory cytokine inhibition.

In our study, the findings of the biochemical examinations were perfectly consistent with those of histopathological examinations. In the sciatic nerve tissues of the SIR group, the MDA, NF-κB, and TNF-α levels were high and the tGSH level was low, while significant severe pathological findings were recorded in the myelinated nerve fibers, myelin sheath, nerve fascicles, Schwann cells, perineurium, and blood vessels. As per past reports, the histopathological damages occurred during the I/R process, such as neuronal edema and nerve fiber degeneration, are associated with FORs (7, 19). Some past reports state that ischemia develops after blood vessel occlusion due to vasculitis, and histopathological changes occur in the nerve tissues, which support our results (18, 27). Our histopathological findings of increased FOR and proinflammatory mediator production conforms to those reported elsewhere (28, 29). In addition, a study by Karakurt *et al*., have also demonstrated the antioxidant activity of lutein and its inhibitory effect on proinflammatory cytokine production (30).

In conclusion, our biochemical and histopathological results demonstrate that significant damage develops in the sciatic nerve tissues following a temporary occlusion of the femoral arteries. This sciatic nerve I/R injury caused regional hyperalgesia. Lutein could significantly reduce the pain resultant from the sciatic nerve I/R injury, prevent the increase in the levels of oxidative and proinflammatory markers. The results of our experimental study suggest that lutein may be useful in the treatment of I/R sciatic nerve injury.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

SY was responsible for study design was done by SY, data collection by SY, RM, and HS; experimental procedures by RM and HS; data analysis by GNY and SO; histological examination by GNY; statistical analyses by HS; writing manuscript by SY and SO; final revisions by SO.

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Received January 12, 2021
Revised February 7, 2021
Accepted February 11, 2021