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Effect of Hesperetin on the Experimental (Crushed) Sciatic Nerve Injury in the Rat Models

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Abstract

Objectives: Peripheral nerve disorders are the most common neurologic complications in humans. Therefore, any effective intervention to treat or reduce the complications of these disorders can be helpful. This study aimed to evaluate the effect of hesperetin on the experimental (crushed) sciatic nerve injury in the rat models.

Materials and Methods: In this experimental study, 60 adult male rats were studied in five groups (n = 12/each). The sham group (without nerve injury and treatment), the control group (with untreated nerve injury), and three experimental groups with injured sciatic nerve received oral hesperetin (100, 200, and 400 mg/kg, respectively) by gavage. All rats were euthanized on second and fourth post-treatment weeks for histopathological assessment of the sciatic nerve.

Results: The results showed increased perineurium formation in the experimental group treated with 400 mg/kg hesperetin and a decrease in leukocyte infiltration in the experimental groups treated with 200 and 400 mg/kg hesperetin compared with the controls on second and fourth post-treatment weeks ($P < 0.05$). At the end of the second week, axon swelling significantly decreased in the group treated with 400 mg/kg hesperetin than the control group ($P < 0.05$). In addition, a decrease in the axonal count was observed in hesperetin-treated groups (200 and 400 mg/kg) after two weeks compared with the controls ($P < 0.05$).

Conclusions: The expression of the S100 gene in groups treated with 100 and 400 mg/kg hesperetin showed a significant decrease compared with the control group on days 14 and 28. Our findings indicated that hesperetin positively affects sciatic nerve repair in the rat model.

Keywords: Hesperetin, Rat, Sciatic nerve, Injury

Introduction

Peripheral nerve disorders are the most common neurologic complications that require immediate treatment (1). Despite advances in medical devices, the therapeutic strategy is still a preferred therapy for peripheral nerve injury. The prognosis and treatment in this type of patient also depend on the degree of nerve damage. Medical treatment is the most common strategy for peripheral nerve rehabilitation and surgery (2).

Hesperetin is a flavonoid found in some juices such as oranges and red fruits. Flavonoids cause the coloration in flowers, and sometimes leave. Also, due to being able to attract insects, they are effective in pollination and fertility of plants. Flavonoids may perform a functional role in increasing the plant resistance against pathogens and are also strong absorbers of ultraviolet rays (340-250 nm). Flavonoids positively affect the function of genes and enzymes in the structure of sexual organs and the growth of pollen grains. They also chelate some metal ions such as iron and copper and prevent their oxidation by inhibiting the catalyzed elements (3).

Considering the influential role of antioxidants to neutralize oxidative stress products and generate free radicals caused by peripheral nerve injury, it seems that using hesperetin helps repair the peripheral nerve damage. Hesperetin with a significant antioxidant property is of great importance in researches since it causes no side effects. Hesperetin is one of the synthetic drugs, which is a potential therapeutic agent with high antioxidant activity. Hesperetin (3, 5, 7 trihydroxy, 4 methoxy flavonoids) is one of the most abundant flavonoids in citrus with anti-inflammatory, anti-cancer, and antidepressant activities (4).

Since there are no reports on the effect of hesperetin on nerve repair in studies, this study aimed to evaluate the impact of hesperetin on the experimental (crushed) sciatic nerve injury in rat models.

One of the most critical body organs is the central nervous system and peripheral nerves. Any peripheral nerve disorders cause mild to severe abnormalities in patients. In addition to the damage to the central nervous system, many diseases and injuries are caused by peripheral nerve disorders. Despite advances in technology and laboratory equipment and numerous studies in nerve repair, the lack of answers to many questions and appropriate treatment in this regard is still a vital challenge. Hesperetin, with properties such as increasing the cell membrane stability, maintaining antioxidant activity in eliminating free radicals, reducing oxidative stress, and decreasing tissue damage, has a key role in the stages of damage and repair

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Key Messages

- Hesperetin, a flavanone in the peel of citrus fruits, possesses various biological capabilities that include antioxidant and anti-inflammatory actions.
- The present study focuses on the potential role of hesperetin in the experimental crush neuritis, particularly considering antioxidant and anti-inflammation.

of peripheral nerve lesions. The protein of S100 family is a low molecular weight and calcium-related protein, which is usually observed in neurons. One of the advantages of S100 protein is to identify degenerative changes in the nervous system, neoplastic disorders, and various inflammation treatments (5).

This study aimed to provide an appropriate therapeutic strategy to accelerate the healing process of peripheral nerve damage by using hesperetin to reduce inflammation and adverse effects of oxidative reactions and express this substance's positive role in maintaining the myelin sheath and finally have a healthy axon and cell structure.

Materials and Methods

Animals

In this experimental study, 60 healthy and adult male Wistar rats (weight rage: 300-350 g) were purchased from Pasteur Institute, Tehran, Iran. The animals were kept in a room with invariant temperature of 20 ± 1°C and relative humidity 42 ± 1% in a 12-hour light/dark cycle. Then, rats were randomly divided into 5 groups (n=12/each). Standard laboratory diets and drinking water were provided for the animals.

Moreover, the animals were adapted to the laboratory conditions one week before the experiment. Each rat was used only once and euthanized by the human method using thiopental sodium immediately after the investigation. Damaged (crushed) nerves treated with hesperetin for 2 and 4 weeks were sampled for pathological examination.

All experimental phases were performed according to the principles of laboratory animal care and ethical guidelines for investigation of experimental pain in conscious animals (6). Also, animal handling and experimental procedures were according to the National Institutes of Health (US) guide for the care and use of laboratory animals (NIH Journal No. 23-25, 1996 revision) and current Iranian government regulations. Hesperetin was purchased from Safir Azma (Sigma Chemical Co., China) (Purchase code: 69097990).

Experimental Protocol

In this experimental study, animals were studied in 5 groups (n=12/each). The sham group (without nerve injury and treatment), the control group (with untreated nerve injury), and three experimental groups with sciatic nerve injury received oral Hesperetin (100, 200, and 400 mg/kg, respectively) by gavage. All surgical procedures were undergoing anesthesia using intraperitoneal injection of ketamine hydrocholoride (60 mL/kg) and xylazine hydrocholoride (10 mL/kg) (7). After scrapping and initial preparing the animals' left leg, they were put on the right side, and an incision was made in the posterior-outer skin in the left femur. Muscles and fascia were pulled aside gently; once the sciatic nerve was observed, using a standard hemostat mosquito, it was pressed for 60 seconds (to put pressure on the nerve, the forceps were set to 1). In order to detect, the wound site was marked by stitching the nearest muscle onto the crushing site with a non-absorbable nylon (0-5) suture, then the muscles were put near each other and subcutaneous tissue and skin were stitched by using vicryl (0-4) and nylon (0-3) suture, respectively, in a simple all round and single way (8).

Histological Evaluation

Experimental groups were treated with hesperetin oral gavages and a similar amount of distilled water for 4 weeks (9). All rats were euthanized on second and fourth post-treatment weeks for pathological assessment of nerve repair with intravenous injection of thiopental sodium (10).

Histopathological Evaluation

For the histopathological evaluation of injured neural tissue, the distal part of the sciatic nerve was used and it was fixed in 10% formaldehyde buffer. Initially, paraffin was used for sciatic nerve fixation. The resulting tissue incision (5 µm) was deparaffinized and stained with hematoxylin & eosin (H&E) method. H&E staining was used to examine leukocyte infiltration, axon swelling, and axon loss score. Another tissue incision (5 µm) was provided, deparaffinized, and stained with Trichrome method. We used Trichrome staining method to study the production of connective tissue of perineurium and epineurium.

Immunohistochemical Method

To determine the expression of *S100* gene, another two parts of each 5 µm-thick block were prepared and stained using the immunohistochemistry kit instructions (S100 beta antibody; Catalog number: orb 27461) (11). Then, the sciatic nerve tissue was studied with a microscope (Optica-B-500Bi) and the following were studied:

- The nerve sheaths including the epineurium, perineurium, endoneurium, and nerve fibers.
- The neural cells including axon, Schwann cells, and fibroblasts
- Vascular condition
- Percentage of the *S100* gene expression

Tissue samples were then examined with an optical microscope. The production of epineurium and epineurium was graded according to scoring system 0-4: 0 (0%), 1 (less than 25%), 2 (25%-50%), 3 (50%-75%), and 4 (Complete). In addition, the presence of inflammatory

cells was rated by using scores 0 (high; >75%), 1 (Average upward; 50%-75%), 2 (Average downward; 25%-50%), 3 (low; <25%), and 4 (No inflammatory cells). Axon swelling was graded according 0 (Obvious; more than 75% axon diameter), 1 (Average upward; 50%-75% axon diameter), 2 (Average downward, 25%-50% axon diameter), 3 (low; <25% axon diameter), and 4 (no axon swelling). The numbers were rated as 0 (less than 25% natural nerve) 1 (25% natural nerve), 2(50% natural nerve), 3(75% natural nerve), and 4 (similar natural nerve) (12).

Statistical Analysis

All of the statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS, version 24.0 for Windows; SPSS Inc., Chicago, IL). The results were calculated as mean ± standard deviation (SD) and analyzed using Kruskal-Wallis test. *P* value less than 0.05 and 0.01 was considered statistically significant.

Results

Perineurium growth significantly decreased in the control group after two post-treatment weeks ($P<0.05$) (Figure 1). However, perineurium growth in the group treated with 400 mg/kg hesperetin significantly increased after two post-treatment weeks, compared with the controls ($P<0.05$) (Figure 2; c1). Inflammatory cells increased significantly in the control group after two post-treatment weeks ($P<0.05$) (Figure 1). However, inflammatory cells in the hesperetin-treated groups (400 mg/kg after 2 weeks and 200 and 400 mg/kg after 4 weeks) were significantly reduced with the control group ($P<0.05$) (Figure 2).

In addition, axon swelling in the hesperetin-treated group (400 mg/kg) was significantly reduced compared to the control group after two weeks ($P<0.05$). After four weeks, a significant increase ($P<0.05$) in the number of axons was observed in the group treated with hesperetin (200 and 400 mg/kg) compared to the control group (Figure 1).

Contrary to the factors expressing the improvement of the sciatic nerve, the expression of the *S100* gene at doses of 100 and 400 mg/kg decreased significantly on days 14 and 28, respectively, compared with the control group ($P<0.01$) (Figures 3-6).

Discussion

As far as we know, there are no reports, experimentally, on the effect of hesperetin on inflammatory nerve cells. In this experimental study, this parameter showed a

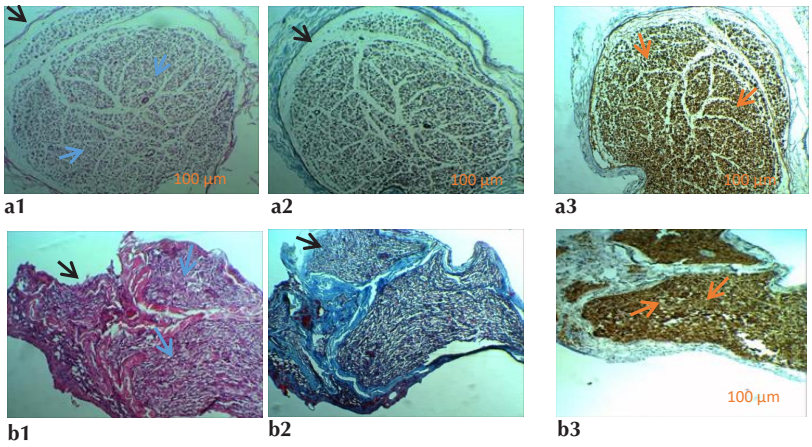

Figure 1. Histopathological Section From Crushed Nerve Fiber. Black arrows show the condition of myelin sheath in H&E staining (a_1) and Trichrome (a_2). Blue arrows express a population of neurons that won't return to normal condition. S100 (a_3) demonstrates the cells in which more than 90% of neurons was stained with strong positive intensity after 14 days (Orange arrows). Tissue structure changed and inflamed nerve cells were observed (Blue arrows). In S100 (b_3), above 80% of nerve cells were stained with strong positive intensity after 28 days (Orange arrows).

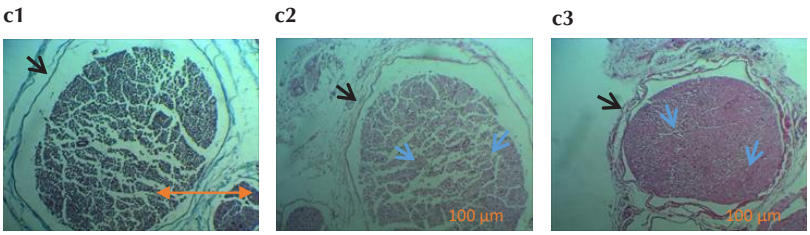

Figure 2. Histopathological Section From Crushed Nerve Fiber.. (c1) expresses Trichrome staining in the experimental group at dose of 400 mg/kg and the black arrows show the improvement of perineurium after 14 post-treatment days compared with the control group. (c2) shows H&E staining in the experimental group at dose of 400 mg/kg. Black arrows indicate the status of the myelin sheath, and blue arrows indicate the state of the nerve cells regeneration after 14 days.

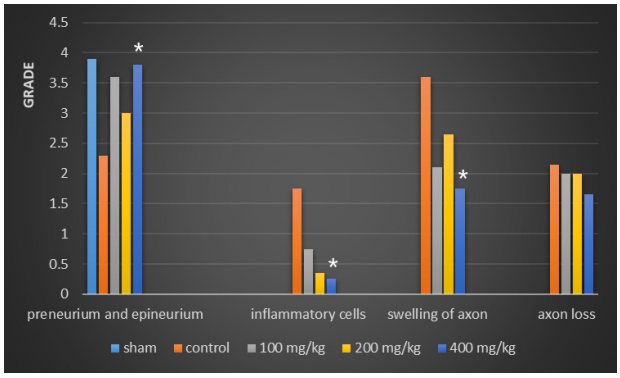

Figure 3. The Effect of 100, 200, and 400 mg/kg Hesperetin on Perineurium and Epineurium Formation, the Presence of Inflammatory Cells, Axon Swelling, and Axon Loss Score in Experimental Sciatic Nerve Injury in the Rat Models After Two Post-treated Weeks ($P<0.05$). * represents significant changes between the treated and the control groups ($P < 0.05$).

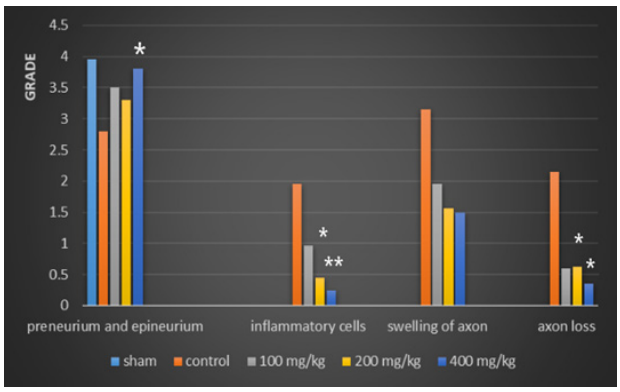

Figure 4. Effect of 10, 200, and 400 mg/kg Hesperetin on Perineurium and Epineurium Formation, Inflammatory Cells, Axon Swelling, and Axon Loss Score in the Experimental (Crushed) Sciatic Nerve Injury in the Rat Models After 4 Post-treatment Weeks. * and ** show significant changes between the treated and control groups, ($P<0.05$ and $P<0.01$, respectively).

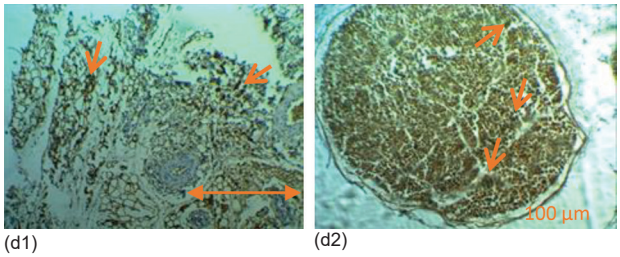

Figure 5. (d1) and (d2), show S100 staining (Immunohistochemical staining) in 100 mg/kg and 400 mg/kg treated groups, respectively. More than 25% of neurons with moderate positive intensity after 14 days and about 70% of nerve cells with strong positive intensity after 28 days were stained (Orange arrows).

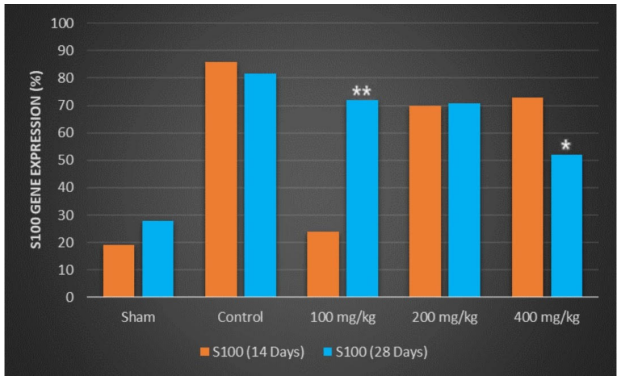

Figure 6. Effect of 100 and 400 mg/kg Hesperetin on the S100 Gene Expression After 14 and 28 Post-treatment Days, Respectively. Significant changes between the 14 and 28 days after crushing are shown. * $P<0.05$ and ** $P<0.01$

significant decrease in the groups treated with 200 and 400 mg/kg hesperetin. Furthermore, axon swelling in the groups treated with 400 mg/kg hesperetin after two post-treatment weeks and axon loss score in 200 and 400 mg/kg treated groups rats after four post-treatment weeks decreased.

Oxidative stress plays a key role in sciatic nerve injury pathogenesis. In nerve injury, oxidation affects the nerve due to reactive oxygen species (ROS) production (13). Overproduction of ROS is associated with lipids, proteins and nucleic acids that adversely affect the cell function and cell injury (14). High metabolism in the nerve increases ROS production and reduces the antioxidant capacity (15). Cell stability is maintained by antioxidant enzymes that eliminate ROS (16). Flavonoids as antioxidant compounds destroying free radicals are redoxed so that they may prevent neurological diseases (17,18). Hesperetin is a natural compound belonging to the class of flavonoids. Recently, several studies have been done on the protective properties of hesperetin against various oxidants, such as peroxynitrite and hydrogen peroxide (19-21). Although hesperetin has different pharmacological activities, the clinical use of this drug was limited because of its oral absorption and low biological distribution and bioavailability. Nano drug delivery systems are extensively used to increase the dispersion of hydrophobic drugs and phytochemicals and improve the biological distribution, uptake, and bioavailability of Hesperetin (22,23). One research showed that Hesperetin and nano-hesperetin significantly reduced stress oxidative in the brain and plasma inflammatory markers. Therefore, it can be concluded that nano-hesperetin has a protective effect on the nerves and can be helpful to treat neural diseases in the animal models, compared with hesperetin (24).

Due to the antioxidants, anti-inflammatory, and anti-apoptotic effects of flavonoids, they can be considered the most helpful factors for neurologic disorders (25,26). Hesperetin flavonoid (3-5-7 trihydroxy-4-methoxy flavanone) is a flavanone, a member of flavonoid subgroup found in citrus species (27).

Although hesperetin made from hesperidin, is a biologically active molecule in the body, only a few laboratory studies have evaluated hesperetin instead of hesperidin. These studies showed that hesperetin is a

strong free-radical scavenger that enhances cellular anti-inflammatory activity (28). Hesperetin has previously been identified as a neuroprotective agent in various models (29,30), but the protective role of hesperetin in AD models has not been investigated to date. A study showed that the neuroprotective effect of Hesperetin is a multipotent effect involving inhibition of oxidative stress, nervous inflammation, and cell death through apoptosis and cognitive stabilization. Hesperetin is more likely to be found as a promising therapeutic agent for neurological disorders, because of its antioxidant, anti-inflammatory, and anti-apoptotic properties against neurodegeneration and memory impairment (31).

Previous studies showed that hesperetin not only has antioxidant, anti-inflammatory, anti-apoptotic, and anti-tumor effects (32-34), but also it can inhibit inflammation in different types of cells by controlling the extracellular signal regulatory pathways (ERK) and mitogen-activated protein kinase (35).

Hesperetin has also been shown to have protective effects against diagnostic memory impairment in the presence of high oxidative stress in the mice model of (36). However, the role of Hesperetin in the regulation of microglia cells is unknown. In a study, the inflammatory neuroinflammatory effects of hesperetin on lipopolysaccharide (LPS)-induced BG-2 microglial cells were investigated. The results indicated that Hesperetin strongly inhibited nitric oxide production and the expression of inducible nitric oxide synthase in LPS-induced BV-2 microglial cells. Moreover, it reduced the secretion of inflammatory cytokines, including interleukin (IL)-1β and IL-6. In addition, Hesperetin with reducing the phosphorylation of extracellular signal regulated kinase (ERK) by 1.2 and p38 mitogen-activated protein kinase exerted its anti-inflammatory effects. Hesperetin suppressed the activation of astrocytes and microglias in the brains of LPS-challenged mice. This study concluded that Hesperetin inhibits microglia inflammation and could be a prophylactic treatment for neurodegenerative diseases (37). Lanza et al studied the effect of antioxidant compounds of flavonoids on neuromuscular reconstruction. Due to the limitation of the present study, there was no possibility of determining malondialdehyde, superoxide dismutase, and glutathione peroxidase levels in hesperetin-treated mice with nerve injury (38).

Considering the role of hesperetin in preventing oxidative stress reactions, it speeds up the improvement of nerve injury. In the present study, despite faster Sciatic nerve repair and improvement in the examined factors, the mild expression of *S100* gene in the group treated with 100 and 400 mg/kg hesperetin after 14 and 28 post-treatment weeks can prove this fact. Since the expression of this gene decreased in case of nerve damage and degeneration and it increases in case of nerve regeneration and improvement (9), we expected an increase in gene expression in the Hesperetin -treated groups. Considering the results (Figure 5), we concluded that *S100* gene is somewhat sensitive to nerve regeneration and healing. It is highly suggested that effect of the expressions of *NSE* and *GFAP* genes on the sciatic nerve repair by using Hesperetin is examined in the future.

The present study showed an increased perineurium formation, the number of axon, as well as inflammatory cells in hesperetin-treated rats. Finally, the results showed the positive effects of hesperetin on the sciatic nerve repair in the rats' models. Because there are no similar studies to compare the obtained results about hesperetin on the sciatic nerve repair, further studies are highly recommended to examine the direct cellular and molecular impacts of hesperetin on the sciatic nerve repair.

According to previous studies, hesperetin affects peripheral nerve regeneration by preventing oxidative stress reactions and increasing antioxidant factors. In the present study, hesperetin with a significant effect on the four investigated factors showed that it has antioxidant and healing properties for nerve damages.

Authors' Contribution

Conception and design of the study: AJ. Acquisition of data: NM. Analysis and interpretation of data: AA. Drafting of the manuscript: PM. Critical revision of the manuscript for important intellectual content: AJ, NM. Statistical analysis: AA.

Conflict of Interests

The authors declare that there is no conflict of interest.

Ethical Issues

The Ethics Committee of Islamic Azad University, Sciences and Research Branch, Tehran, Iran confirmed the study protocol (Code: IR.IAU.SRB.REC.1398.1096).

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