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Original Article

Oleuropein Reduces Secondary Injury Pathways After Experimental Spinal Cord Injury Via HMGB1-Associated Signaling

Deneysel Spinal Kord Yaralanması Sonrasında Oleuropeinin HMGB1 ile İlişkili Sinyal Yolakları Üzerinden İkincil Hasar Mekanizmalarını Azaltıcı Etkisi

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ABSTRACT

Objective: Spinal cord injury (SCI) can cause profound disability and substantial morbidity and mortality. Oleuropein (OLE) is a phenolic compound with anti-inflammatory and antioxidant effects. High mobility group box 1 (HMGB1), released extracellularly after tissue injury, can amplify inflammatory signaling. This study investigated the effects of OLE administration following experimental SCI.

Methods: Twenty-four female rats were randomly assigned to sham, SCI, and SCI+OLE groups (n=8 each). The sham group underwent T9-T10 laminectomy without SCI. In the SCI and SCI+OLE groups, SCI was induced by 30-second extradural compression with a temporary aneurysm clip. OLE was administered intraperitoneally immediately after injury and again 1 h later. Animals were euthanized 24 h after surgery. Levels of HMGB-1, NF-κB, RAGE, TLR4, and Nrf2 were measured in spinal cord tissue samples by immunohistochemistry and Western blotting. Apoptosis-related protein levels were detected by Western blot. The TUNEL assay was used to quantify apoptotic cells. Oxidative status was assessed using the total oxidant status, the total antioxidant status, and the oxidative stress index.

Results: Levels of HMGB1, RAGE, TLR4, and NF-κB increased after SCI, whereas OLE reduced their levels. Nrf2 expression increased after SCI and

ÖZ

Amaç: Spinal kord yaralanması (SKY) ciddi sakatlıklara ve hatta ölüme yol açabilir. Oleuropein (OLE), antienflamatuvar ve antioksidan özelliklere sahip fenolik bir bileşiktir. Doku hasarı sonrası hücre dışına salınan high mobility group box 1 (HMGB1), enflamatuvar sinyalleri güçlendirebilir. Bu çalışmada deneysel SKY sonrasında OLE uygulanmasının etkileri araştırıldı.

Yöntemler: Yirmi dört dişi sıçan rastgele sham, SKY ve SKY+OLE gruplarına ayrıldı (her grupta n=8). Sham grubuna omurilik yaralanması olmaksızın yalnızca T9-T10 laminektomi uygulandı. SKY ve SKY+OLE gruplarında, 30 saniye süreyle geçici anevrizma klipsiyle ekstradural kompresyon uygulanarak SKY oluşturuldu. OLE, yaralanmadan hemen sonra ve 1 saat sonra intraperitoneal olarak verildi. Hayvanlar cerrahiden 24 saat sonra sakrifiye edildi. Omurilik doku örneklerinde HMGB1, NF-κB, RAGE, TLR4 ve Nrf2 düzeyleri immünohistokimya ve Western blot yöntemleriyle ölçüldü. Apoptozla ilişkili protein düzeyleri Western blot ile tespit edildi. Apoptotik hücrelerin nicelendirilmesi için TUNEL testi kullanıldı. Oksidatif durum, toplam oksidan durum, toplam antioksidan durum ve oksidatif stres indeksi kullanılarak değerlendirildi.

Bulgular: SKY sonrasında HMGB1, RAGE, TLR4 ve NF-κB düzeyleri artarken, OLE bu düzeyleri azalttı. Nrf2 ekspresyonu SKY sonrasında arttı ve OLE ile daha da yükseldi. Ayrıca OLE, apoptozla ilişkili protein

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was further upregulated by OLE. OLE also significantly reduced apoptosis, as evidenced by changes in apoptosis-related protein expression.

Conclusions: OLE exerted anti-apoptotic, anti-inflammatory, and antioxidant effects in this experimental SCI model, as demonstrated by analyses of spinal cord tissue and serum.

Keywords: Spinal cord injury, oleuropein, HMGB1, Nrf2, apoptosis

ekspresyonundaki değişikliklerle uyumlu olarak apoptozu anlamlı derecede azalttı.

Sonuçlar: OLE, deneysel SKY modelinde omurilik dokusu ve serum analizleriyle gösterildiği üzere antiapoptotik, antienflamatuvar ve antioksidan etkiler göstermiştir.

Anahtar kelimeler: Spinal kord yaralanması, oleuropein, HMGB1, Nrf2, apoptoz

INTRODUCTION

Spinal cord injury (SCI) is most commonly discussed in the context of trauma, where an initial primary mechanical insult is followed by a progressive secondary injury cascade that contributes substantially to neurological damage[1,2]. Secondary injury involves a tightly time-dependent interplay of neuroinflammation, oxidative stress, and programmed cell death; importantly, the inflammatory response is biologically complex and can be both detrimental and reparative depending on timing and cellular context[3,4]. A key mechanistic link between tissue damage and sterile inflammation is the release of damage-associated molecular patterns (DAMPs). High mobility group box 1 (HMGB1), also known as HMGB1, is a non-histone protein primarily found in the nucleus that normally participates in chromatin organization, but after injury it can be released passively during necrosis and secreted by immune cells, functioning extracellularly as a potent pro-inflammatory mediator[5,6]. Extracellular HMGB1 signals through receptors such as RAGE and TLR4, promoting downstream triggering of inflammatory cascades such as NF-κB, and has been implicated in neuroinflammation and cell death after SCI[7-9].

The early phase of secondary injury following spinal cord trauma is characterized by a rapid and self-propagating cascade involving sterile inflammation, oxidative stress, and apoptotic signaling, which evolves within hours and contributes significantly to lesion expansion[3,4]. Importantly, targeting upstream mediators that initiate and amplify this cascade may offer greater therapeutic benefit compared with interventions aimed at downstream effectors. Therefore, identifying key regulatory molecules that bridge tissue damage and inflammatory signaling is of considerable translational importance.

Experimental studies demonstrate that HMGB1 is increased after spinal cord trauma and is linked to the activation of inflammatory pathways, such as TLR4/NF-κB. Importantly, HMGB1 neutralization (e.g., anti-HMGB1 antibody) or inhibition (e.g., glycyrrhizin/GA) attenuates secondary injury and can improve functional outcomes, supporting HMGB1 as a mechanistic driver and potential therapeutic target in SCI[10-12].

Olive-derived products (Olea europaea L.) are rich in phenolic compounds, most notably oleuropein (OLE) and the related phenolics hydroxytyrosol (HT) and tyrosol. Olea europaea has been used in traditional practices for centuries, and its bioactivity has been largely attributed to these phenolic constituents[13]. Among olive phenolics, OLE is widely recognized as a major compound, particularly abundant in olive leaves and present throughout olive tissues. Importantly, OLE can undergo hydrolysis and yield HT (and related derivatives), which are considered key contributors to olive's biological effects[14]. Experimental and translational studies have reported that OLE and HT exhibit numerous pharmacological effects, including but not limited to anti-inflammatory, antioxidant, hypolipidemic, hypoglycemic, and cardioprotective properties[14,15].

Importantly, increasing evidence suggests that DAMPs, particularly HMGB1, serve a crucial function in mediating post-injury inflammation through activation of receptors such as TLR4 and RAGE[7,8]. Experimental studies have demonstrated that suppression of HMGB1 release or interference with its downstream signaling pathways can attenuate inflammatory responses and reduce tissue damage following SCI[10,12]. Given that OLE has been suggested to modulate oxidative stress and inflammation-related signaling cascades, it is biologically plausible that OLE may, at least in part, exert its protective effects through modulation of HMGB1-associated signaling pathways. However, this potential interaction has not been clearly elucidated in the context of SCI.

In the present study, we investigate whether OLE administration modulates oxidative stress, inflammation, and apoptosis in a preclinical SCI model, with a particular focus on HMGB1-associated inflammatory signaling and the Nrf2 antioxidant pathway. By targeting both upstream danger-associated signaling and downstream oxidative responses, this study aimed to elucidate the potential neuroprotective mechanisms of OLE in the early stages of spinal cord trauma.

MATERIALS and METHODS

Experimental Design and Animal Model

Twenty-four healthy female Sprague-Dawley rats (275±25 g; 9±1 weeks old) were allocated randomly to three groups (n=8 each): sham, SCI, and SCI+OLE. This study's methods and protocols were authorized by the Bezmialem Vakıf University Animal Experiments Local Ethics Committee (approval no: 2015/63, date of approval: 27 February 2015). Animals were housed individually under controlled laboratory conditions with a 12-hour photoperiod, and had ad libitum access to food and water.

Before surgery, rats were anesthetized by intramuscular injection of ketamine hydrochloride (60 mg/kg; Parke-Davis&Co, İstanbul, Türkiye) and xylazine hydrochloride (5 mg/kg; Rompun 2% solution, Bayer-Türk Ltd., İstanbul, Türkiye) into the hind limb while breathing spontaneously. After adequate anesthesia was achieved, the dorsal region was shaved, and the animals were positioned in the prone position on a firm sponge support.

Experimental SCI was induced using the Kerr-Lougheed aneurysm clip compression model[16]. The surgical field was disinfected over the T8-T11 region. In the sham-operated rats, only a T9-T10 laminectomy was performed. In the SCI and SCI+OLE groups, SCI was induced by 30-second extradural compression with a Yasargil temporary aneurysm clip [FT262T, Aesculap, Tuttlingen, Germany, closing force 90 g (0.88 N)] following a T9-T10 laminectomy.

In the SCI+OLE group, OLE (20 mg/kg) was administered intraperitoneally immediately after injury and was repeated 1 h post-injury in 5 mL/kg of 0.9% saline solution. In the sham and SCI groups, 0.9% saline solution was injected immediately after injury and 1 hour post-injury via the intraperitoneal route (5 mL/kg). All animals received antibiotic prophylaxis for one day after surgery. Bladder expression was performed twice daily to assist urination.

At 24 h postoperatively, all animals were anesthetized and adequate depth of anesthesia was confirmed. Intracardiac blood samples were obtained; 10 mL of blood was collected from each rat into dry, gel-free biochemical tubes. After blood sampling, the animals were euthanized by decapitation. Immediately after euthanization, laminectomy area was expanded 2 more levels cranially and caudally and tissue samples were taken carefully. The collected tissue samples were placed in tubes containing 10% formaldehyde.

Protein expression levels of HMGB1, NF-κB, RAGE, TLR4, and Nrf2 in spinal cord tissue samples were quantified by Western blot analysis and immunohistochemistry. Apoptosis-related proteins (pro- and anti-apoptotic markers) were evaluated by Western blotting. The number of apoptotic cells was determined using the TUNEL assay. Oxidative stress and antioxidant status were biochemically determined using total oxidant status (TOS) and total antioxidant status (TAS) kits, and the oxidative stress index (OSI) was calculated for both spinal cord tissue and serum samples.

Immunohistochemistry

Immunohistochemical staining for HMGB1, NF-κB, TLR4, RAGE, and Nrf2 was performed on formalin-fixed, paraffin-embedded spinal cord sections using the streptavidin-biotin complex (StrepABC) method. Positively charged slides containing 4-µm sections were deparaffinized and rehydrated, followed by antigen retrieval, endogenous peroxidase blocking, and protein blocking. Sections were incubated with the following primary antibodies: HMGB1 (Novus, NB100-2322; 1:100; 1 h; room temperature), TLR4 (Novus, NB100-56566; 1:100; overnight at 4 °C), RAGE (Novus, NBP2-03950; 1:50; overnight at 4 °C), NF-κB (Santa Cruz, sc-61876; 1:25; overnight at 4 °C), and Nrf2 (Santa Cruz, sc-722; 1:100; 1 h; room temperature). After incubation, sections were treated with a commercial secondary antibody kit (Thermo Scientific), visualized using a 3-amino-9-ethylcarbazole substrate kit, and then counterstained with Mayer's hematoxylin. Negative controls were prepared by omitting the primary antibody and replacing it with distilled water. Antigen retrieval was performed in citrate buffer (pH 6.0)[17].

TUNEL Assay for Apoptosis

We assessed apoptosis using a commercial TUNEL kit (ApopTag® plus peroxidase *in situ* apoptosis detection kit; Cat. No. S7101; Millipore, USA) following the manufacturer's protocol. We mounted 4-µm paraffin-embedded spinal cord sections on positively charged slides and incubated them overnight at 37 °C. Xylene was used to deparaffinize the tissue sections; they were then rehydrated and treated with proteinase K (20 µg/mL, 15-20 minutes). We inactivated endogenous peroxidase activity with 3% H_2O_2 in PBS and then incubated the sections in equilibration buffer. Next, terminal deoxynucleotidyl transferase (TdT) was applied to the slides, which were then incubated for 1.5 hours at 37 °C. Anti-digoxigenin peroxidase was then added; subsequently, we incubated the sections for 30-45 minutes at room temperature (22 °C). We developed

the chromogenic signal using 3,3′-diaminobenzidine substrate-chromogen solution and then counterstained them with methyl green. For negative controls, TdT was omitted and distilled water was substituted for the enzyme solution[17,18].

H-score and Apoptotic Index

Immunohistochemical staining for HMGB1, TLR4, RAGE, NF-κB, and Nrf2 was quantified using a histological scoring system (H-score)[19,20]. Briefly, we inspected the slides under a Leica microscope equipped with an ocular grid. Two blinded observers evaluated seven randomly selected fields from each section at ×400 magnification. Graded staining intensity in positively stained cells was classified as follows: 0 (no staining), 1 (weak), 2 (distinct), and 3 (intense). We used the following formula to assess apoptosis:

H-score = $\Sigma Pi(i+1)$, where i represents the intensity score and Pi is percentage of cells at that intensity.

Two blinded observers independently assessed the apoptotic index by counting TUNEL-positive cells in seven randomly selected fields at ×400 magnification. We expressed the results as the mean number of TUNEL-positive cells per microscopic field[21].

Oxidant-Antioxidant Assay

We measured TOS and TAS in serum samples and spinal cord tissue homogenates using commercially available kits (Rel Assay Diagnostics, Türkiye), using the method described by Erel[22,23].

For tissue analyses, spinal cord samples were mechanically disrupted using a bead-assisted homogenization system (MP Biomedicals, FastPrep-24™, USA). We transferred the tissue specimens to lysing-matrix tubes filled with ceramic beads and homogenized them in ice-cold PBS (pH 7.4) at 6.0 m/s in two 40-second cycles, with a 5-minute cooling interval on ice between cycles to minimize thermal degradation. Following homogenization, the samples were centrifuged at 10,000×g for 15 min at 4 °C. The resulting supernatants were carefully collected and subsequently used for all biochemical analyses.

Protein concentrations in tissue supernatants were quantified using the Bradford assay, and TAS and TOS results from tissue samples were normalized to total protein content.

Spectrophotometric analysis was carried out using a microplate reader (Thermo Scientific Multiskan FC, USA). TAS was measured by the reduction of the ABTS radical cation and reported as mmol Trolox equivalents/L using Trolox as the reference standard. TOS was determined by oxidation reactions calibrated against hydrogen peroxide and reported as μmol H_2O_2 equivalents per liter (μmol H_2O_2 equiv/L).

To provide an integrated indicator of oxidative balance, the OSI was calculated as the ratio of TOS to TAS according to Erel's approach, using the following formula: OSI (arbitrary units) = [TOS (μmol H_2O_2 equivalents/L)/ TAS (mmol Trolox equivalents/L)]×100. OSI values were expressed in arbitrary units.

Western Blot Analysis

Western blotting was performed to assess the protein expression levels of HMGB1, TLR4, RAGE, Nrf2, Bax, Bcl-2, caspase-3, and p53 in spinal cord tissue samples. For protein extraction, spinal cord tissues were homogenized with a bead-based homogenization system (MP Biomedicals, FastPrep-24™, USA) in ice-cold RIPA lysis buffer containing a protease inhibitor cocktail. We processed the tissue samples , under standardized conditions, in lysing matrix tubes containing ceramic beads to ensure efficient disruption. We subsequently centrifuged the homogenates at 12,000×g for 15 minutes at 4 °C and collected the supernatants as total protein extracts. We then measured protein concentrations using the Bradford assay and prepared equal amounts of protein (40 μg per lane) in Laemmli sample buffer. Samples were denatured at 95 °C for 5 minutes prior to electrophoresis and were separated on 10% SDS-polyacrylamide gels. Following separation, we transferred the proteins onto polyvinylidene fluoride membranes using an electroblotting system.

To minimize non-specific protein binding, we incubated the membranes in 5% non-fat dry milk prepared in tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The membranes were subsequently incubated with antibodies against HMGB1 (Novus, NB100-2322), TLR4 (Novus, NB100-56566), RAGE (Novus, NBP2-03950), Nrf2 (Santa Cruz, sc-722), Bax (Santa Cruz, sc-493), Bcl-2 (Santa Cruz, sc-492), caspase-3 (Santa Cruz, H-277), p53 (Santa Cruz, sc-126), and β-actin (1:1000).

After washing the membranes with TBST, we incubated them with horseradish peroxidase-conjugated secondary antibodies (1:5,000) for 2 h at room temperature. We detected the protein bands using an enhanced chemiluminescence detection system and quantified band intensities by densitometric analysis with Image Lab software and a Fusion FX5 imaging system (Vilber Lourmat, France).

Band intensities were normalized to β-actin, and relative protein expression levels were calculated with respect to the control group.

Statistical Analysis

Data were expressed as mean ± standard error of the mean. We compared the experimental groups using a one-way analysis of variance followed by Tukey's post-hoc multiple-comparisons test. Statistical analyses were conducted using GraphPad Prism (version 5; GraphPad Software, San Diego, CA, USA). A p-value <0.05 was considered statistically significant.

RESULTS

HMGB1 Immunohistochemical Expression

HMGB1 immunoreactivity was significantly increased in the SCI group relative to the sham-operated group (p<0.001). In the SCI+OLE group, OLE administration significantly reduced HMGB1 expression relative to the SCI group (p<0.001) (Figures 1, 2, and Table 1).

TLR4, RAGE, and NF-κB Immunoreactivity

Expression levels of TLR4, RAGE, and NF-κB were significantly elevated in the SCI group compared with the sham group (p<0.001). In the SCI+OLE group, administration of OLE significantly reduced TLR4 and RAGE expression relative to the SCI group (p<0.001), whereas NF-κB immunoreactivity decreased, but the reduction did not reach statistical significance (p>0.05) (Figures 1 and 2, and Table 1).

Nrf2 Levels

Nrf2 expression was significantly elevated in the SCI group compared with the sham-operated group (p<0.01). OLE administration further increased Nrf2 levels in the SCI+OLE group relative to the SCI group (p<0.001) (Figures 3, 4, and Table 1).

Apoptosis Detection by TUNEL Assay (DNA Fragmentation)

We used the TUNEL assay to detect apoptotic cells in spinal cord tissue. Relative to the sham-operated group, the SCI group exhibited a significantly higher number of TUNEL-positive cells (p<0.001). In the SCI+OLE group,

Table 1. Mean ± standard error values of HMGB1, TLR4, RAGE, NF-κB, and Nrf2 H-scores and TUNEL-positive cell counts in spinal cord tissue samples

Parameters	Sham	SCI	SCI-OLE
HMGB1 (H-score)	171.0±4.5	197.3±3.5	176.2±3.7
TLR4 (H-score)	219.6±4.1	308.3±4.3	238.7±5.0
RAGE (H-score)	213.1±4.4	276.7±6.2	233.1±4.2
NF-κB (H-score)	217.1±6.3	277.5±8.3	254.1±6.4
Nrf2 (H-score)	101.6±4.2	117.9±3.7	167.1±2.8
TUNEL-positive cell count (mean/area)	10.7±0.7	21.8±0.9	12.3±0.4

SCI: Spinal cord injury, OLE: Oleuropein

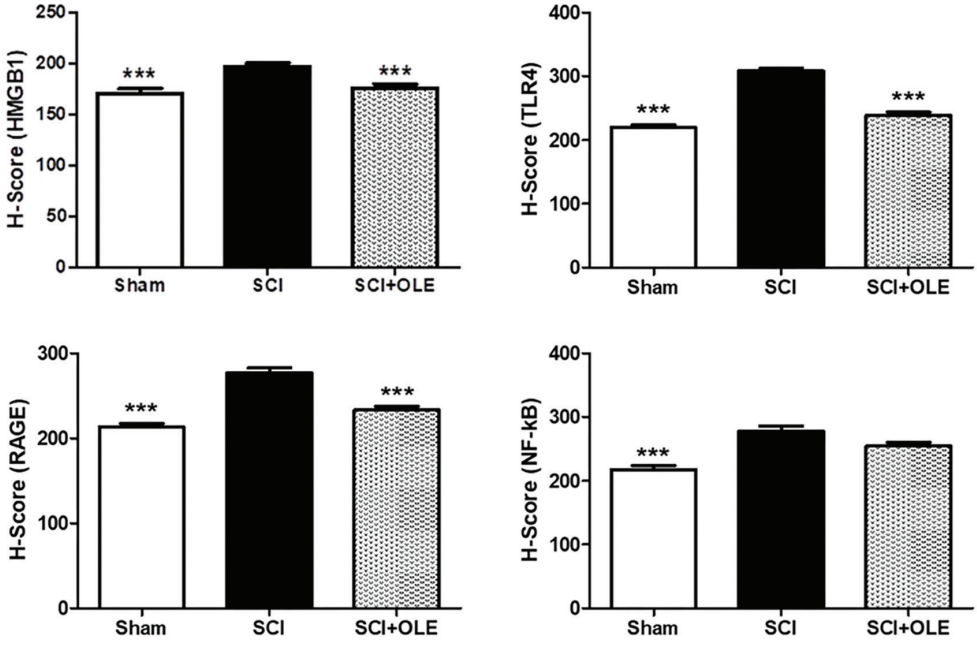

Figure 1. Immunohistochemical staining (H-score) of HMGB1, TLR4, RAGE, and NF-κB across the study groups

***: p<0.001 vs. SCI group, SCI: Spinal cord injury, OLE: Oleuropein

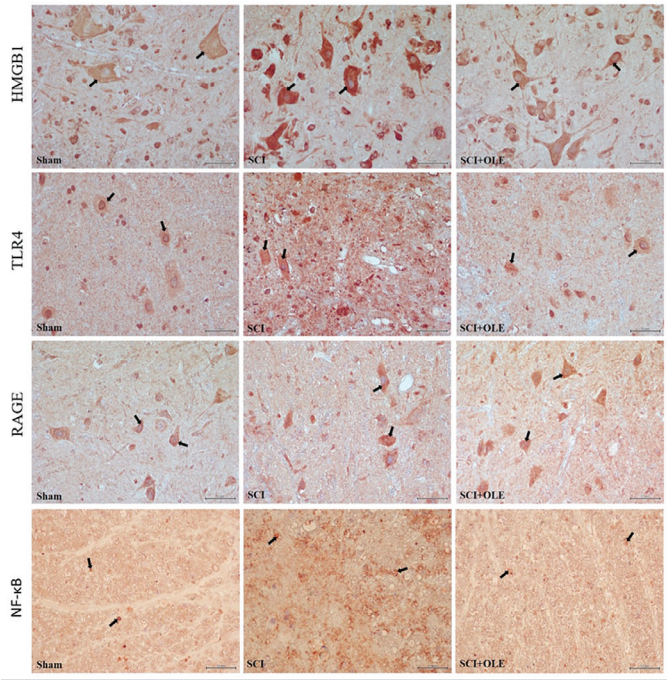

Figure 2. Immunohistochemical images showing HMGB1, TLR4, RAGE, and NF-κB expression across the study groups. Scale bar: 50 µm

SCI: Spinal cord injury, OLE: Oleuropein

OLE treatment significantly decreased the number of apoptotic cells compared with the SCI group (p<0.001) (Figures 5 and 6, Table 1).

Western Blot Results

HMGB1, TLR4, and RAGE Levels

Compared with the sham group, the SCI group exhibited significantly higher protein levels of HMGB1, TLR4, and RAGE, as determined by Western blot analysis. In the SCI+OLE group, OLE administration significantly reduced levels of HMGB1, TLR4, and RAGE compared with the SCI group (p<0.001) (Figure 7, Table 2).

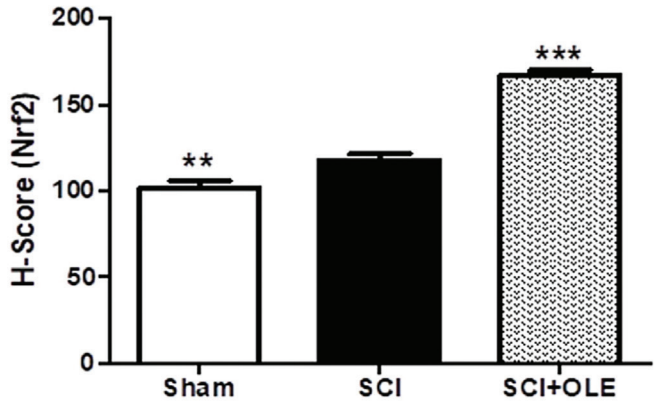

Figure 3. Immunohistochemical staining (H-score) of Nrf2 across the study groups

**: p<0.01, ***: p<0.001 vs. SCI group, SCI: Spinal cord injury, OLE: Oleuropein

Nrf2 Levels

Compared with the sham group, the SCI group exhibited significantly higher Nrf2 protein levels, as determined by Western blot analysis. (p<0.001). OLE administration further increased Nrf2 levels in the SCI+OLE group relative to the SCI group (p<0.001) (Figure 8, Table 2).

Bax, Bcl-2, Caspase-3, and p53 Levels

Bax protein expression was significantly elevated in the SCI group compared with the sham-operated group. (p<0.001). In the SCI+OLE group, OLE administration significantly reduced Bax levels compared with the SCI group (p<0.001) (Figure 9, Table 2).

Bcl-2 protein levels were significantly decreased in the SCI group compared with the sham-operated group (p<0.001). OLE treatment significantly increased Bcl-2 levels in the SCI+OLE group relative to the SCI group (p<0.001) (Figure 9, Table 2).

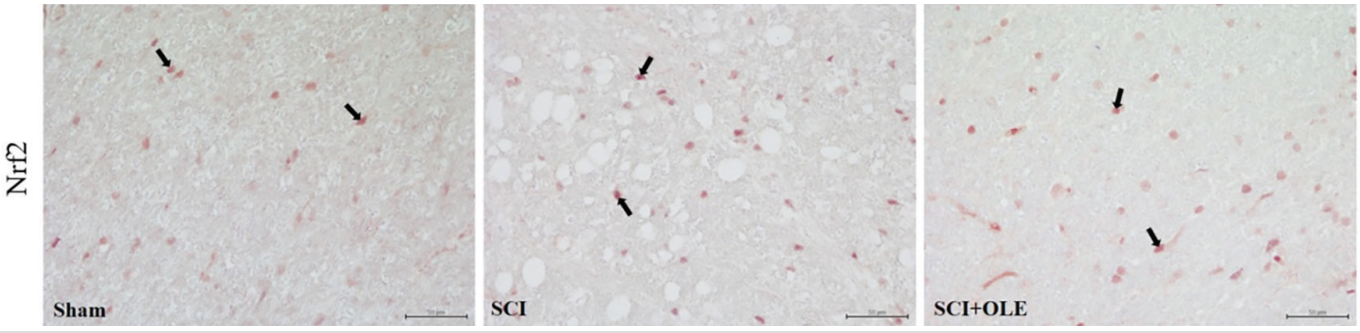

Figure 4. Representative immunohistochemical images showing Nrf2 expression across the study groups. Scale bar: 50 µm

SCI: Spinal cord injury, OLE: Oleuropein

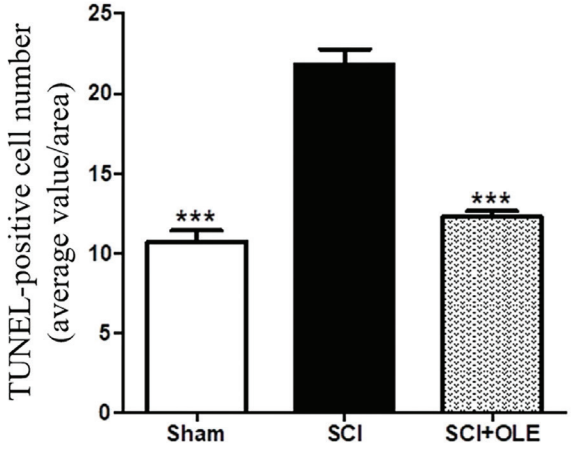

Figure 5. TUNEL assay results across the study groups

***: p<0.001 vs. SCI group, SCI: Spinal cord injury, OLE: Oleuropein

Caspase-3 protein levels were significantly increased in the SCI group compared with the sham group (p<0.001). OLE administration significantly reduced caspase-3 levels in the SCI+OLE group relative to the SCI group (p<0.001) (Figure 9, Table 2).

Similarly, p53 protein expression was significantly upregulated in the SCI group relative to the sham-operated group (p<0.001) and was significantly decreased following OLE treatment compared with the SCI group (p<0.001) (Figure 9, Table 2).

TAS, TOS, and OSI Results

TAS, TOS, and OSI were evaluated in both spinal cord tissue and serum samples. Compared with the sham-operated group, TAS values in both tissue and serum were significantly decreased in the SCI group (p<0.05). OLE administration significantly increased TAS levels compared with those in the SCI group (p<0.001; Figure 10).

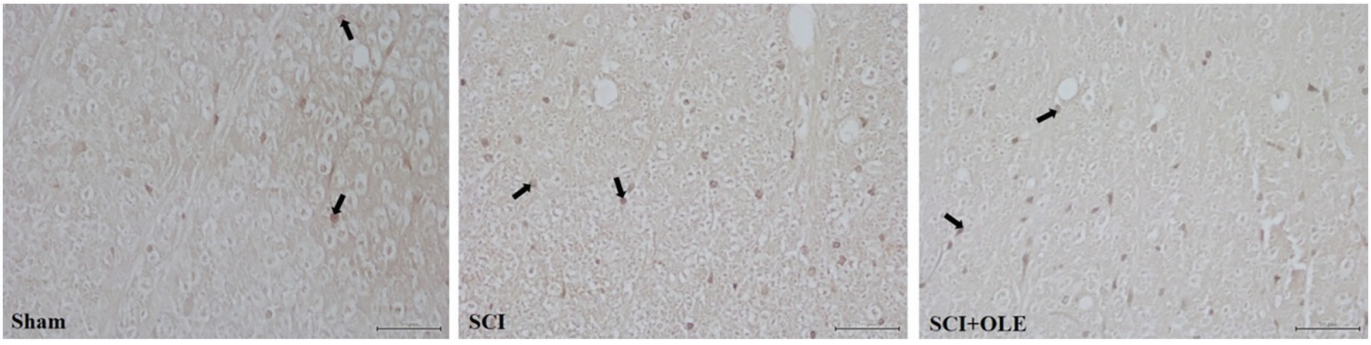

Figure 6. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling in sham, SCI and SCI+OLE groups. Scale bars: 50 μm

SCI: Spinal cord injury, OLE: Oleuropein

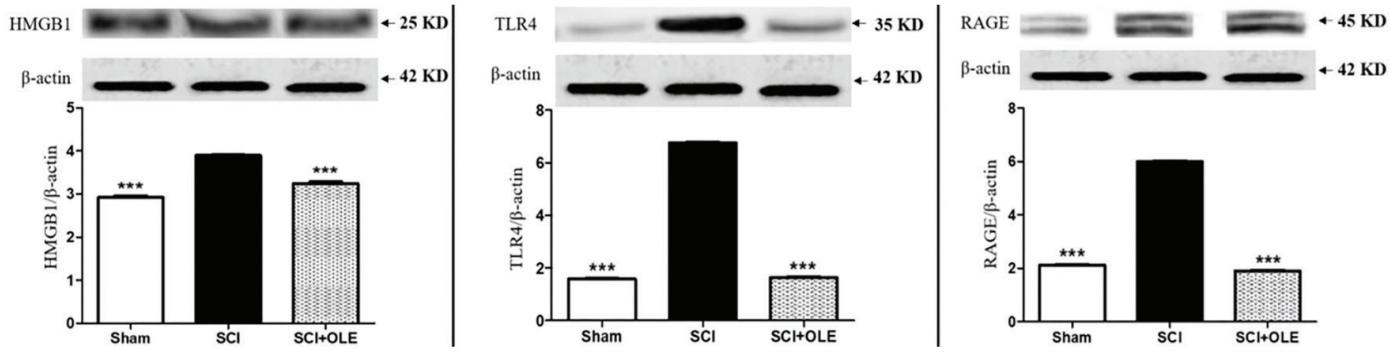

Figure 7. Western blot analysis of HMGB1, TLR4, and RAGE protein expression in spinal cord tissue from the sham, SCI, and SCI+OLE groups at 24 hours post-procedure

***: p<0.001 vs. SCI group, SCI: Spinal cord injury, OLE: Oleuropein

Tissue TOS levels were significantly elevated in the SCI group ($p<0.01$), whereas the increase in serum levels failed to achieve statistical significance ($p>0.05$). In the SCI+OLE group, TOS values decreased in both tissue and serum ($p<0.001$ and $p<0.05$, respectively) (Figure 10).

OSI values were higher in the SCI group relative to the sham group in both tissue ($p<0.01$) and serum ($p<0.05$), and were significantly reduced following OLE treatment in both tissue and serum compared with the SCI group ($p<0.001$) (Figure 10).

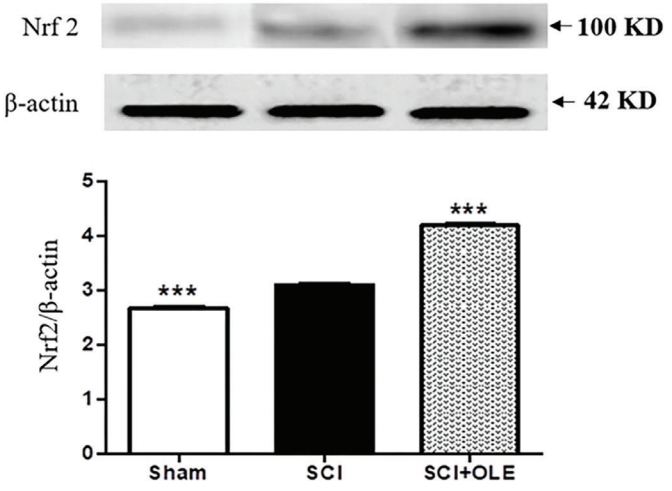

Figure 8. Western blot analysis of Nrf2 protein expression in spinal cord tissue from the sham, SCI, and SCI+OLE groups at 24 hours post-procedure

***: $p<0.001$ vs. SCI group, SCI: Spinal cord injury, OLE: Oleuropein

Table 2. Mean ± standard error values of HMGB1, TLR4, RAGE, Nrf2, Bax, Bcl-2, active caspase-3, and P53 western blot measurements

Parameters	Sham	SCI	SCI-OLE
HMGB1/β-actin	2.92±0.04	3.89±0.02	3.24±0.04
TLR4/β-actin	1.58±0.02	6.76±0.02	1.63±0.02
RAGE/β-actin	2.11±0.04	6.00±0.03	1.91±0.02
Nrf2/β-actin	2.68±0.03	3.11±0.02	4.19±0.03
Bax/β-actin	2.22±0.04	4.86±0.02	2.97±0.02
Bcl-2/β-actin	3.68±0.03	2.83±0.02	3.57±0.03
Active caspase-3/β-actin	1.75±0.03	4.95±0.03	3.34±0.04
P53/β-actin	2.65±0.02	4.31±0.02	2.99±0.02

SCI: Spinal cord injury, OLE: Oleuropein

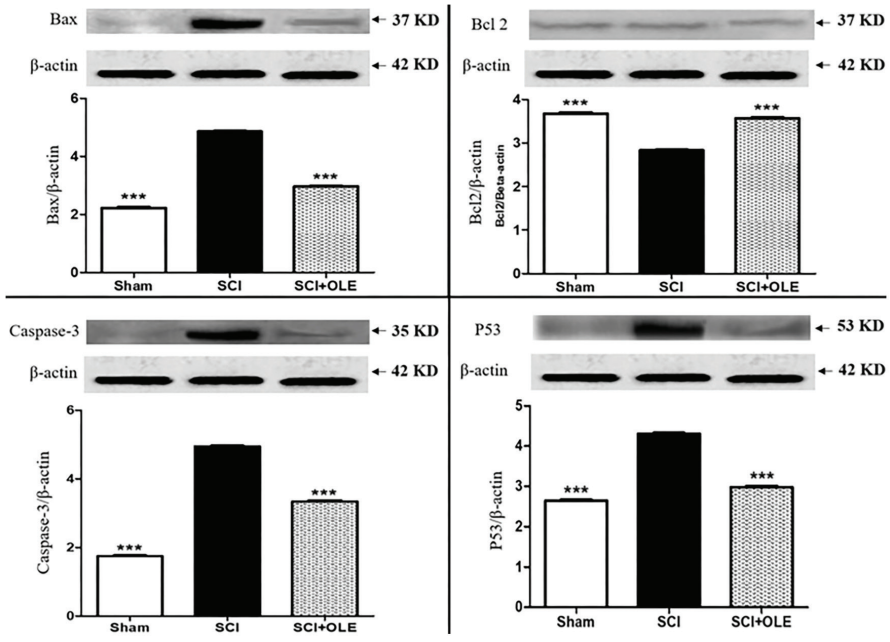

Figure 9. Western blot analysis of Bax, Bcl-2, caspase-3, and p53 protein expression in spinal cord tissue from the sham, SCI, and SCI+OLE groups at 24 hours post-procedure

***: $p<0.001$ vs. SCI group, SCI: Spinal cord injury, OLE: Oleuropein

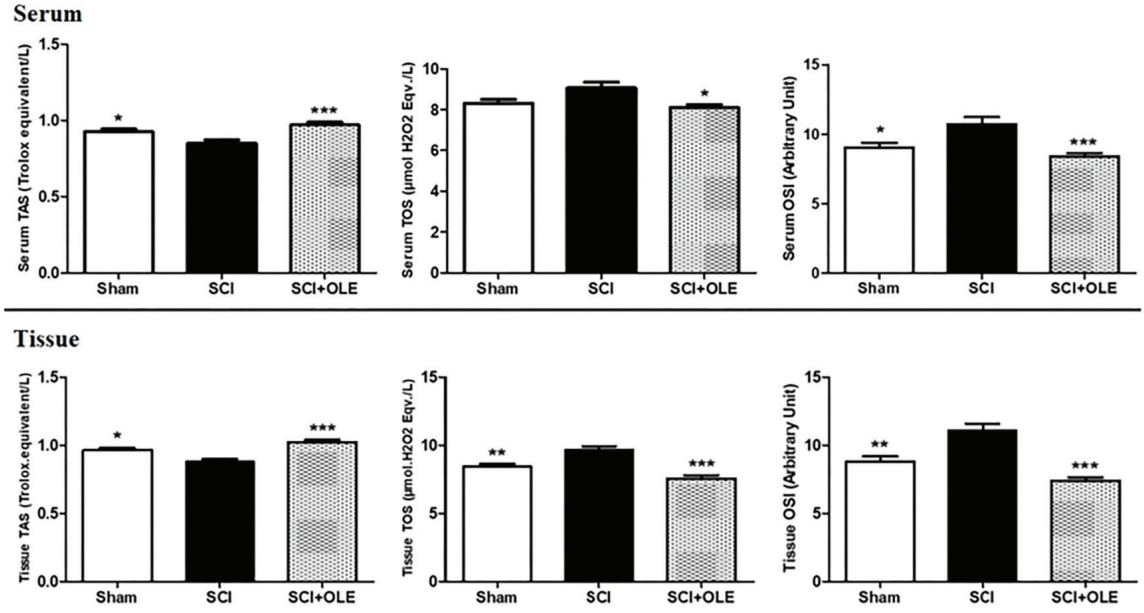

Figure 10. Serum and tissue total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) in the study groups at 24 hours post-procedure

*: p<0.05, **: p<0.01, ***: p<0.001 vs. SCI group, SCI: Spinal cord injury, OLE: Oleuropein

DISCUSSION

In this experimental SCI model, early administration of OLE produced a consistent protective profile at 24 hours. Compared with untreated SCI, OLE significantly reduced the levels of key mediators of damage-associated inflammatory signaling (HMGB1, RAGE, and TLR4), while NF-κB immunoreactivity was lower, but the reduction did not reach statistical significance. OLE also enhanced antioxidant defenses (increased Nrf2), improved systemic and tissue redox balance (higher TAS and lower TOS and OSI), and attenuated apoptosis, as evidenced by fewer TUNEL-positive cells and a shift in apoptosis-related proteins (reduced Bax, caspase-3, and p53, and increased Bcl-2). Given that secondary injury evolves within hours after SCI and is driven by the interplay of inflammatory signaling, oxidative stress, and programmed cell death, these convergent effects suggest that OLE may mitigate early mechanisms that promote secondary injury and potential lesion expansion[2,7,24,25].

Neuroinflammation is a major contributor to secondary injury after SCI, and DAMPs link tissue damage to innate immune activation. HMGB1 is a well-established DAMP that is passively released from damaged cells and actively secreted by immune cells, thereby amplifying sterile inflammation through receptors such as RAGE and TLRs (particularly TLR4), with downstream activation of pro-inflammatory transcriptional programs including NF-κB[7,8,24]. In this context, our observation that OLE reduced HMGB1, RAGE, and TLR4 levels supports the concept that OLE interferes with upstream danger-signaling pathways that propagate post-injury inflammation and subsequent neural tissue damage.

This interpretation aligns with prior SCI literature demonstrating that HMGB1 contributes to secondary injury and that interruption of the HMGB1-RAGE axis can limit pro-inflammatory macrophage/microglial polarization and provide neuroprotection[8,26]. Although NF-κB immunoreactivity decreased without reaching statistical significance in our immunohistochemical analysis, the direction of the effect remained consistent with a dampened pro-inflammatory state and may reflect methodological sensitivity, timing, or heterogeneous cellular contributions during the acute phase. Importantly, our results extend earlier work that examined OLE in SCI models. Khalatbary and Ahmadvand[27], Khalatbary and Zarrinjoei[28] demonstrated the anti-inflammatory effects of OLE in an experimental model of SCI, supporting OLE as a candidate modulator of post-injury inflammatory responses. Unlike prior OLE studies in SCI, which primarily focused on downstream cytokines and general oxidative or apoptotic markers, our study specifically investigates the upstream HMGB1-RAGE/TLR4 signaling axis and the Nrf2 antioxidant response, providing a more pathway-oriented interpretation of OLE's effects.

In addition, evaluating spinal cord tissue and serum in parallel strengthens the translational relevance of our results. The concordant direction of change across compartments suggests that circulating biomarkers may reflect, at least in part, intrathecal inflammatory and oxidative responses after SCI. This raises the possibility that serum HMGB1-related and redox markers could serve as surrogate indicators of neuroinflammation in clinical settings where direct tissue sampling is not feasible; however, prospective validation in humans and time-course analyses are required.

Oxidative stress is a key component of secondary injury after SCI. Nrf2 is a critical transcriptional regulator of antioxidant and cytoprotective responses and has emerged as a potential therapeutic target in SCI[25,29]. In our study, Nrf2 increased after SCI and was further upregulated by OLE treatment, as demonstrated by both immunohistochemistry and Western blot; this was accompanied by improved redox balance (higher TAS with lower TOS and OSI). These results suggest that OLE strengthens endogenous antioxidant defenses during the acute post-injury period, consistent with prior experimental evidence that OLE can activate Nrf2-related pathways and attenuate oxidative stress[30].

Besides HMGB1-related inflammatory signaling and oxidative stress, apoptosis contributes to lesion expansion and neural tissue damage after SCI, and inflammatory and oxidative signals can converge on intrinsic and extrinsic cell-death pathways. In our study, SCI increased apoptosis, whereas OLE significantly reduced TUNEL-positive cells and shifted apoptosis-associated protein expression toward survival (Bcl-2 increased; Bax, caspase-3, and p53 decreased). These results are biologically consistent with a combined reduction in upstream inflammatory danger signaling (HMGB1/RAGE/TLR4) and in oxidative burden (TOS/OSI); together with increased Nrf2 activity; all of these changes may reduce cellular susceptibility to apoptosis.

Study Limitations

Several limitations should be acknowledged. First, outcomes were assessed at a single early time point (24 hours), which captures acute inflammatory, oxidative, and apoptotic events, but does not determine whether OLE provides sustained neuroprotection or translates into long-term functional recovery. Second, this study did not include functional outcome assessments, radiological quantification of lesion extent, or detailed cell-type-specific analyses (e.g., neuronal versus glial localization), all of which would have provided additional evidence regarding the biological effects of OLE. Third, only one dose and dosing schedule were evaluated; therefore, dose-response relationships and the optimal therapeutic window remain undefined. The exclusive use of female Sprague-Dawley rats may limit the generalizability of the findings to other sexes and strains. Although the observed changes in HMGB1, TLR4, RAGE, NF-κB, and Nrf2 are consistent with modulation of these pathways, the present study does not establish pathway-specific causality. Therefore, the findings should be interpreted as evidence of pathway-associated changes rather than definitive proof that these pathways mediate the neuroprotective effects of OLE.

Future studies should evaluate longer-term outcomes, incorporate functional assessments and histological lesion characterization, and examine whether OLE modulates microglial/macrophage polarization and blood-spinal cord barrier integrity-processes closely linked to HMGB1-RAGE/TLR4 signaling[7,8]. Exploring downstream Nrf2 target genes (e.g., *HO-1, GCLC, NQO1*) would also clarify whether the observed Nrf2 upregulation reflects transcriptional activation of antioxidant defense programs.

CONCLUSION

Overall, our results demonstrate that OLE exerts early antioxidant, anti-inflammatory, and anti-apoptotic effects following experimental SCI, potentially through coordinated modulation of HMGB1-associated receptor signaling and activation of Nrf2-related antioxidant defenses.

Ethics

Ethics Committee Approval: This study's methods and protocols were authorized by the Bezmialem Vakıf University Animal Experiments Local Ethics Committee (approval no: 2015/63, date of approval: 27 February 2015).

Informed Consent: Animal study.

Footnotes

Author Contributions

Surgical and Medical Practices: F.C., E.A., Ş.E., H.H., S.C., Concept: F.C., A.H., Design: F.C., A.H., Data Collection and/or Processing: F.C., E.A., Ş.E., H.H., E.G.G., H.D., S.C., A.K., E.İ.A., O.B.B.E., A.H., Analysis or Interpretation: E.G.G., H.D., S.C., A.K., E.İ.A., O.B.B.E., A.H., Literature Search: F.C., A.H., Writing: F.C., E.G.G., H.D.

Conflict of Interest: The authors declare that they have no conflict of interest.

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