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The effects of a polyphenol present in olive oil, oleuropein aglycone, in an experimental model of spinal cord injury in mice

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ABSTRACT

Several olive oil phenolic compounds, such us oleuropein have attracted considerable attention because of their antioxidant activity, anti-atherosclerotic and anti-inflammatory properties. The aim of this experimental study was to determine the effect of oleuropein aglycone, a hydrolysis product of oleuropein, in the inflammatory response, in particular in the secondary injury associated with the mouse model of spinal cord trauma. The injury was induced by application of vascular clips to the dura via a four-level T5–T8 laminectomy in mice. Oleuropein aglycone was administered in mice (100 μg/kg, 40 μg/kg, 20 μg/kg, 10% ethanol, i.p.) 1 h and 6 h after the trauma. The treatment with oleuropein aglycone significantly decreased: (1) histological damage, (2) motor recovery, (3) nuclear factor (NF)-κB expression and IKB-α degradation, (4) protein kinase A (PKA) activity and expression, (5) pro-inflammatory cytokines production such as tumor necrosis factor (TNF-α) and interleukin-1β (IL-1β), 6) inducible nitric oxide synthase (iNOS) expression, (7) neutrophil infiltration, (8) lipid peroxidation, (9) nitrotyrosine and poly-ADP-ribose (PAR) formation, (10) glial cell-derived neurotrophic factor (GDNF) levels, (11) apoptosis (TUNEL staining, FAS ligand expression, Caspase 3, Bax and Bcl-2 expression). Thus, we propose that olive oil phenolic constituents such as oleuropein aglycone may be useful in the treatment of various inflammatory diseases.

1. Introduction

Spinal cord injury (SCI) is a highly debilitating pathology [1]. Although innovative medical care has improved patient outcome, advances in pharmacotherapy for the purpose of limiting neuronal injury and promoting regeneration have been limited. High-dose corticosteroids, given within the first 8 h after injury and continued for 24–48 h, were regarded as part of the standard treatment regimen [2–5], although recently the results of studies, which supported the efficacy of corticosteroids in SCI, have come under scrutiny. One of the reasons why most of the treatments utilized in preclinical studies [6] have had limited success in clinical trials is the complexity of the secondary degenerative response. In fact, many treatments affect only one aspect of this response, and a successful treatment should probably target several of these mechanisms [6].

The primary traumatic mechanical injury to the spinal cord causes the death of a number of neurons that cannot be recovered and regenerated. Studies indicate that neurons continue to die for hours following traumatic SCI [7]. The events that characterize this successive phase to mechanical injury are called "secondary damage." The secondary damage is determined by a large number of cellular, molecular, and biochemical cascades. A large body of recent data suggests the presence of a local inflammatory response, which amplifies the secondary damage.

Moreover, various evidences have suggested that resident microglia and macrophages originating from blood are two key cell types related to the occurrence of neuronal degeneration in CNS after traumatic injury. In particular, when SCI occurs, microglia in parenchyma is activated and macrophages in circulation get across

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blood–brain barrier (BBB) to act as intrinsic spinal phagocytes. Therefore, these cells can release various neurotrophic peptides such as brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) [8] and laminin which are excellent substrates for growing neuritis. Concomitantly, different pro-inflammatory mediators such as proinflammatory cytokines [9] and reactive oxygen species (ROS) [10] are also produced. ROS can initiate a wide range of toxic oxidative reactions. These include initiation of lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, inhibition of membrane sodium/potassium ATPase activity, inactivation of membrane sodium channels, and other oxidative modifications of proteins [11]. Increased ROS production is also implicated in the activation of the transcription factors such as nuclear factor-κB (NF-κB) that plays a central and crucial role in inducing the expression of inflammatory cytokines [12]. In addition, many inflammatory conditions are associated with production of comparatively large amounts of oxide nitric (NO), produced by inducible nitric oxide synthase (iNOS), with consequent cytotoxic effects [11]. Various studies have clearly demonstrated that NO is also closely involved in the development of pathological processes in vivo such as post-traumatic spinal cord cavitations [13]. It is confirmed in vitro that NO-induced cell injury is mediated via either the necrotic or apoptotic pathway, depending upon the severity of the cellular damage. A study clearly demonstrated that NO produced by iNOS modulate the secondary inflammatory response following traumatic SCI [14]. Olive oil is an integral ingredient of the traditional Mediterranean diet and several studies attribute many of the healthy advantages of this diet to olive oil's unique characteristics [15]. It is well known for its minor components exerting either anti-inflammatory or antioxidant effects. Among the several minor constituents of virgin olive oil, there are vitamins such as α- and γ-tocopherols and β-carotene, phytosterols, pigments, terpenic acids, flavonoids such as luteolin and quercetin, squalene, and phenolic compounds, usually and incorrectly termed polyphenols. Oleuropein (Ole) is an ester of 2-(3,4-dihydroxyphenyl)ethanol (hydroxytyrosol) and has the oleosidic skeleton that is common to the secoiridoid glucosides of *Oleaceae* [16], mainly in its aglycone form, which makes the sugar moiety insoluble in oil. Many of the biological activities attributed to natural phenolic derivatives have anti-inflammatory components [17] so various health benefits seem to overlap with those attributed to nonsteroidal anti-inflammatory drugs [18]. Recently, Beauchamp et al. [19] reported that oleocanthal, the dialdehydic form of deacetoxy-ligstroside aglycone, a well-known phenolic compound contained in virgin olive oil, can inhibit cyclooxygenase enzymes COX-1 and COX-2. Conclusions made in that paper triggered a great interest in the scientific community.

Ole has several pharmacological properties including antioxidant, anti-inflammatory, anti-atherogenic, anti-cancer, antimicrobial, and antiviral, and for these reasons, it is commercially available as food supplement in Mediterranean countries [16]. Thus, ole scavenges superoxide anions, hydroxyl radicals and inhibits the respiratory burst of neutrophils and hypochlorous acid-derived radicals [20]. In addition to their antioxidant properties, polyphenolic compounds have been shown to exhibit a range of indirect actions that may be beneficial to health, including the inhibition of enzymes involved in the inflammatory process, the inhibition of platelet aggregation and inhibition of the metabolic activation of procarcinogens [21]. Therefore, the phenolic fraction is responsible for the stability and flavor of olive oil and is endowed with "pharmacological" properties as an additional, valuable marker of olive oil quality. In light of the above considerations and of increasing interest in the Mediterranean diet, in the present studies we decided to evaluate the effects of oleuropein aglycone (ole aglycone), a hydrolysis product obtained from oleuropein by the action of β-glucosidase on the parent glucoside, in a mice model of spinal cord injury.

2. Materials and methods

2.1. Animals

Male adult CD1 mice (25–30 g, Harlan Nossan, Milan, Italy) were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

2.2. SCI

Mice were anesthetized using chloral hydrate (400 mg/kg body weight). We used the clip compression model described by Rivlin and Tator [22]. A longitudinal incision was made on the midline of the back, exposing the paravertebral muscles. These muscles were dissected away exposing T5–T8 vertebrae. The spinal cord was exposed via a four-level T5–T8 laminectomy and SCI was produced by extradural compression of the spinal cord using an aneurysm clip with a closing force of 24 g. In the injured groups, the cord was compressed for 1 min. Following surgery, 1.0 cc of saline was administered subcutaneously in order to replace the blood volume lost during the surgery. During recovery from anesthesia, the mice were placed on a warm heating pad and covered with a warm towel. The mice were singly housed in a temperature-controlled room at 27 °C for a survival period of 10 days. Food and water were provided to the mice ad libitum. During this time period, the animals' bladders were manually voided twice a day until the mice were able to regain normal bladder function. Sham injured animals were only subjected to laminectomy.

2.3. Experimental design

Mice were randomized into 6 groups of 10 mice/group ($N = 60$ total animals).

SCI + vehicle group ($N = 10$): mice were subjected to SCI plus administration of 250 μl of vehicle (10% ethanol);
SCI + ole aglycone ($N = 10$): as the SCI + vehicle group but in which ole aglycone (250 μl) at the dose of 100 μg/kg 10% ethanol, was administered intraperitoneally 1 h and 6 h after SCI;
SCI + ole aglycone ($N = 10$): as the SCI + vehicle group but in which ole aglycone (250 μl) at the dose of 40 μg/kg 10% ethanol, was administered intraperitoneally 1 h and 6 h after SCI;
SCI + ole aglycone ($N = 10$): as the SCI + vehicle group but in which ole aglycone (250 μl) at the dose of 20 μg/kg 10% ethanol, was administered intraperitoneally 1 h and 6 h after SCI;
Sham + vehicle group ($N = 10$): mice were subjected to the surgical procedures as the above groups except that the aneurysm clip was not applied (only laminectomy) and they were treated i.p. with 250 μl of vehicle (10% ethanol);
Sham + ole aglycone ($N = 10$): identical to sham + vehicle group except that ole aglycone was administered intraperitoneally (250 μl) 1 h and 6 h after SCI.

As described below mice ($N = 10$ from each group for each parameters) were sacrificed at 24 h after SCI in order to evaluate

the various parameter. In a separate set of experiments other 10 animals for each group were observed until 10 days after SCI in order to evaluate the motor score. The dose of ole aglycone used here was based on previous our in vivo studies [23,24].

2.4. Light microscopy and histological score

Spinal cord tissues were taken at 24 h following trauma. Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin embedded and cut into 5 μm-thick sections. Tissue sections (thickness 5 μm) were deparaffinized with xylene, stained with Hematoxylin/Eosin (H&E), or with silver impregnation for reticulum and studied using light microscopy (Dialux 22 Leitz). The segments of each spinal cord were evaluated by an experienced histopathologist. Damaged neurons were counted and the histopathology changes of the gray matter were scored on a 6-point scale [25]: 0, no lesion observed, 1, gray matter contained 1–5 eosinophilic neurons; 2, gray matter contained 5–10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one third of the gray matter area); 5, moderate infarction; (one third to one half of the gray matter area); 6, large infarction (more than half of the gray matter area). The scores from all the sections from each spinal cord were averaged to give a final score for individual mice. All the histological studies were performed in a blinded fashion.

2.5. Measurement of spinal cord TNF-α and IL-1β levels

Portions of spinal cord tissues, collected at 24 h after SCI, were homogenized as previously described in phosphate buffered saline (PBS) containing 2 mmol/l of phenyl-methyl sulfonyl fluoride (PMSF, Sigma Chemical Co.) (D.B.A. s.r.l., Milan, Italy) and tissue TNF-α and IL-1β levels were evaluated. The assay was carried out by using a colorimetric, commercial kit (Calbiochem-Novabiochem Corporation, USA) according to the manufacturer instructions. All TNF-α and IL-1β determinations were performed in duplicate serial dilutions.

2.6. Measurement of spinal cord GDNF levels

Portions of spinal cord tissues, collected at 24 h after SCI, were homogenized as previously described [26] in 150 μl of ice-cold buffer (137 mM NaCl, 20 mM Tris, pH 8.0, 1% NP40, 10% glycerol, 1 mMphenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.5 mM sodium vanadate) and tissue GDNF levels were evaluated. The assay was carried out by using an enzyme-linked immunosorbent assay (ELISA) Kit (E90043Mu 96 Tests, Life Science Inc. D.B.A. s.r.l. Milan, Italy) according to the manufacturer instructions.

2.7. Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined in the spinal cord tissues as previously described [27] at 24 h after SCI. Following SCI, spinal cord tissues were obtained and weighed and each piece homogenized in a solution containing 0.5% (w/v) hexadecyltrimethyl-ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged for 30 min at 20,000 × *g* at 4 °C. An aliquot of the supernatant was then allowed to react with a solution of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2. The rate of change in absorbance was measured spectrophotometrically at 650 nm. MPO activity was defined as the quantity of enzyme degrading 1 μmol of peroxide per min at 37 °C and was expressed as units of MPO/mg of proteins.

2.8. Thiobarbituric acid-reactant substances measurement

Thiobarbituric acid-reactant substances measurement, which is considered a good indicator of lipid peroxidation, was determined, as previously described [28] in the spinal cord tissue at 24 h after SCI. Thiobarbituric acid-reactant substances were calculated by comparison with OD_{650} of standard solutions of 1,1,3,3-tetramethoxypropan 99% malondialdehyde bis (dymethyl acetal) 99% (MDA) (Sigma, Milan). The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

2.9. Lipid peroxidation

Lipid peroxides (LPO) were measured in portions of spinal cord tissues, collected at 24 h after SCI, by using LPO determination kit (OXIS International Inc., USA) according to the manufacturer instructions.

2.10. Grading of motor disturbance

The motor function of mice subjected to compression trauma was assessed once a day for 10 days after injury. Recovery from motor disturbance was graded using the Basso Mouse Scale (BMS) [29].

2.11. Protein kinase A (PKA) activity

PKA activity was determined in portions of spinal cord tissues, collected at 24 h after SCI, by using determination kit (R&D system Italy) according to the manufacturer instructions as previously described [30].

2.12. Immunohistochemical localization of nitrotyrosine, PAR, FAS ligand, Bax, Bcl-2

At 24 h after SCI, the tissues were fixed in 10% (w/v) PBS-buffered formaldehyde and 8 mm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3% (v/v) hydrogen peroxide in 60% (v/v) methanol for 30 min. The sections were permeabilized with 0.1% (w/v) Triton X-100 in PBS for 20 min. Non-specific adsorption was minimized by incubating the section in 2% (v/v) normal goat serum in PBS for 20 min. Endogenous biotin or avidin binding sites were blocked by sequential incubation for 15 min with avidin–biotin peroxidase complex (DBA). Sections were incubated overnight with (1) rabbit polyclonal anti-Bax (1:100 in PBS, w/v) (Santa Cruz Biotechnology, Inc.) (D.B.A. s.r.l., Milan, Italy), (2) rabbit polyclonal anti-Bcl-2 (1:100 in PBS, w/v) (Santa Cruz Biotechnology, Inc.) (D.B.A. s.r.l., Milan, Italy), (3) goat polyclonal anti-PAR antibody (1:100 in PBS, w/v) (Santa Cruz Biotechnology, Inc.) (D.B.A. s.r.l., Milan, Italy), (4) mouse monoclonal anti Fas Ligand (1:100 in PBS, w/v) (Monosan) (D.B.A. s.r.l., Milan, Italy), (5) rabbit polyclonal anti-nitrotyrosine (1:250 in PBS, w/v) (Millipore) (D.B.A. s.r.l., Milan, Italy). Sections were washed with PBS, and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and DBA. The counter stain was developed with DAB (brown color) and nuclear fast red (red background). A positive staining (brown color) was found in the sections, indicating that the immunoreactions were positive. To verify the binding specificity for nitrotyrosine, PAR, FAS-L, Bax, and Bcl-2, some sections were also incubated with only the primary antibody (no secondary) or with only the secondary antibody (no primary). In these situations no positive staining was found in the sections indicating that the immunoreactions were positive in all the experiments carried out. Immunocytochemistry photographs ($N = 5$) were assessed by densitometry using Imaging

Densitometer (AxioVision, Zeiss, Milan, Italy) and a computer program.

2.13. Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay

TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturer's instruction (Apotag, HRP kit DBA, Milan, Italy). Sections were incubated with 15 μg/ml proteinase K for 15 min at room temperature and then washed with PBS. Endogenous peroxidase was inactivated by 3% H_2O_2 for 5 min at room temperature and then washed with PBS. Sections were immersed in terminal deoxynucleotidyltransferase (TdT) buffer containing deoxynucleotidyl transferase and biotinylated dUTP in TdT buffer, incubated in a humid atmosphere at 37 °C for 90 min, and then washed with PBS. The sections were incubated at room temperature for 30 min with anti-horseradish peroxidase-conjugated antibody, and the signals were visualized with diaminobenzidine. The number of TUNEL positive cells/high-power field was counted in 5–10 fields for each coded slide.

2.14. Western blot analysis for IκB-α, NF-κB p65, IKB-kinase (IKK)-α, PKA, iNOS, GDNF, Caspase 3, Bax and Bcl-2

Cytosolic and nuclear extracts were prepared as previously described [31] with slight modifications. Spinal cord tissues from each mouse were suspended in extraction Buffer A containing 0.2 mM PMSF, 0.15 μM pepstatin A, 20 μM leupeptin, 1 mM sodium orthovanadate, homogenized at the highest setting for 2 min, and centrifuged at 1000 × *g* for 10 min at 4 °C. Supernatants represented the cytosolic fraction. The pellets, containing enriched nuclei, were re-suspended in Buffer B containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris–HCl pH 7.4, 1 mM EGTA, 1 mM EDTA, 0.2 mM PMSF, 20 μm leupeptin, 0.2 mM sodium orthovanadate. After centrifugation 30 min at 15,000 × *g* at 4 °C, the supernatants containing the nuclear protein were stored at −80 °C for further analysis. The levels of IκB-α, IKK-α, PKAα, iNOS, GDNF, Caspase 3, Bax and Bcl-2 were quantified in cytosolic fraction from spinal cord tissue collected after 24 h after SCI, while NF-κB p65 levels were quantified in nuclear fraction. The filters were blocked with 1x PBS, 5% (w/v) non fat dried milk (PM) for 40 min at room temperature and subsequently probed with specific Abs IκB-α (1:1000; Santa Cruz Biotechnology) (Biogenerica s.r.l. Catania Italy), or anti-Bax (1:500; Santa Cruz Biotechnology) (D.B.A s.r.l. Milan Italy), or anti-Bcl-2 (1:500; Santa Cruz Biotechnology) (D.B.A s.r.l. Milan Italy), or anti-NF-κB p65 (1:1000; Santa Cruz Biotechnology) (D.B.A s.r.l. Milan Italy), anti-iNOS (1:200; BD transduction) (Biogenerica s.r.l Catania Italy), anti-PKAα (1:500; Santa Cruz Biotechnology) (D.B.A s.r.l. Milan Italy), or anti-GDNF (1:500; Santa Cruz Biotechnology) (D.B.A s.r.l. Milan Italy), in 1 × PBS, 5% (w/v) non fat dried milk, 0.1% Tween-20 (PMT) at 4 °C overnight, and anti-IKK-α (1:1000; Cell Signaling), (D.B.A s.r.l. Milan Italy), or anti-Caspase 3 (1:1000; Cell Signaling) (D.B.A s.r.l. Milan Italy), in BSA al 5%, at 4 °C overnight. Membranes were incubated with peroxidase-conjugated bovine anti-mouse IgG secondary antibody or peroxidase-conjugated goat anti-rabbit IgG (1:2000, Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of proteic lysates, they were also incubated in the presence of the antibody against β-actin (1:10,000; Santa Cruz Biotechnology) (D.B.A s.r.l. Milan Italy).

The relative expression of the protein bands of IκB-α (∼37 kDa), NF-κB p65 (∼65 kDa), IKKα (∼85 kDa), PKAα (∼40 kDa), iNOS (∼130 kDa), GDNF (∼16 kDa), Caspase 3 (∼35 kDa), Bax (∼23 kDa), Bcl-2 (∼29 kDa), was quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (GS-700, Bio-Rad Laboratories, Milan, Italy) and a computer program (Molecular Analyst, IBM).

2.15. Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, U.K.). Ole aglycone was obtained from the controlled hydrolysis of oleuropein extracted from olive leaves by means the patented method reported by Procopio et al. [18]. All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

2.16. Statistical evaluation

All values in the figures and text are expressed as mean ± standard error of the mean [32] of *N* observations. For the in vivo studies, *N* represents the number of animals studied. In the experiments involving histology or immunohistochemistry, the figures shown are representative at least three experiments (histological or immunohistochemistry coloration) performed on different experimental days on the tissues section collected from all the animals in each group. The results were analyzed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A *p*-value of less than 0.05 was considered significant. BMS scale data were analyzed by the Mann–Whitney test and considered significant when $p < 0.05$.

3. Results

3.1. Ole aglycone reduces the severity of spinal cord trauma

The severity of the trauma at the level of the perilesional area, assessed by the presence of edema as well as alteration of the white matter and infiltration of leukocytes, was evaluated 24 h after injury by hematoxylin/eosin (H&E) staining. Significant damage was observed in the spinal cord tissue collected from SCI (Fig. 1B) when compared with sham-operated mice (Fig. 1A). Significant protection against the SCI was observed in ole aglycone-treated mice (Fig. 1C). The histological score (Fig. 1D) was evaluated in a dose dependent manner by an independent observer. In order to evaluate if histological damage to the spinal cord was associated with a loss of motor function, the modified BMS hind limb locomotor rating scale score was also evaluated in a dose dependent manner. While motor function was only slightly impaired in sham mice, mice subjected to SCI had significant deficits in movement (Fig. 1E). Ole aglycone treatment significantly ameliorated the functional deficits induced by SCI (Fig. 1E).

3.2. Effect of ole aglycone on the modulation on neurotrophic factors production

Glial cell-derived neurotrophic factor, GDNF has been shown to have a potent survival-promoting effect on various neuronal populations. In this study, we have also evaluated GDNF expression by western blot. High levels of GDNF was found in sham animals (Fig. 2A). Low expression was found in SCI operated mice compared with sham animals (Fig. 2A). The treatment with ole aglycone increased the expression of this neurotrophic factor in the spinal cord from SCI-subjected mice (Fig. 2A). In addition, spinal cord tissues levels of GDNF was evaluated by an ELISA kit. A substantial increase in GDNF levels was found in spinal cord tissues samples from SCI mice treated with ole aglycone collected 24 h after SCI (Fig. 2B). Spinal cord levels of GDNF were significantly attenuated in SCI-subjected mice (Fig. 2B). In sham animals was found a baseline spinal concentration of GDNF in spinal cord tissues.

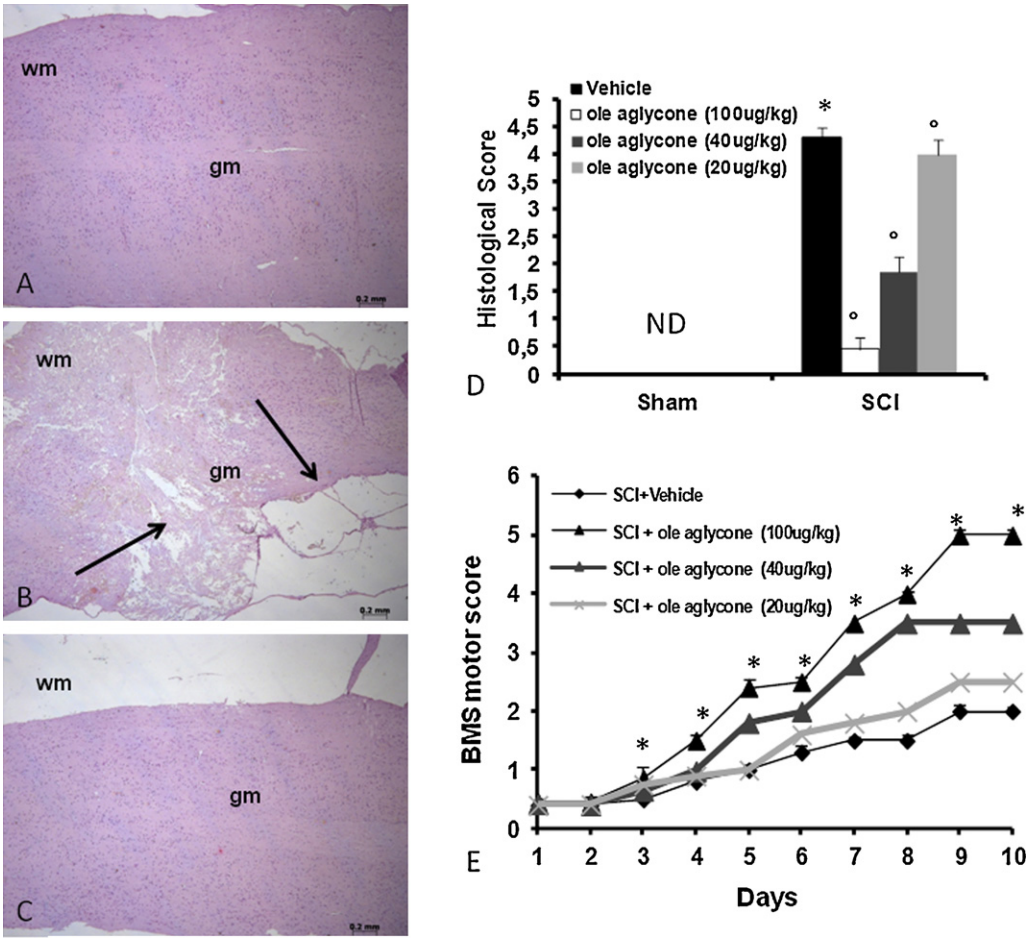

Fig. 1. Effect of ole aglycone treatment on histological alterations of the spinal cord tissue 24 h after injury. A significant damage to the spinal cord, from SCI operated mice at the perilesional area, was assessed by the presence of edema (see arrow) as well as alteration of the white matter 24 h after injury (B). Notably, a significant protection from the SCI was observed in the tissue collected from ole aglycone treated mice (C) when compared with sham-operated mice (A). The histological score was made by an independent observer in a dose dependent manner. Damaged neurons were counted and the histopathology changes of the gray matter were scored on a 6-point scale [25]. This figure is representative of at least 3 experiments performed on different experimental days on the tissues section collected from all the animals in each group. Values shown are mean ± s.e. mean of 10 mice for each group. $^{*}P < 0.01$ vs. SCI (D). The motor function of mice subjected to compression trauma was assessed once a day for 10 days after injury. Recovery from motor disturbance was graded using the Basso Mouse Scale [29]. Treatment with ole aglycone reduces the motor disturbance after SCI in a dose dependent manner. Values shown are mean ± s.e. mean of 10 mice for each group. $^{\circ}P < 0.01$ vs. SCI (E). wm: white matter; gm: gray matter; ND: not detectable.

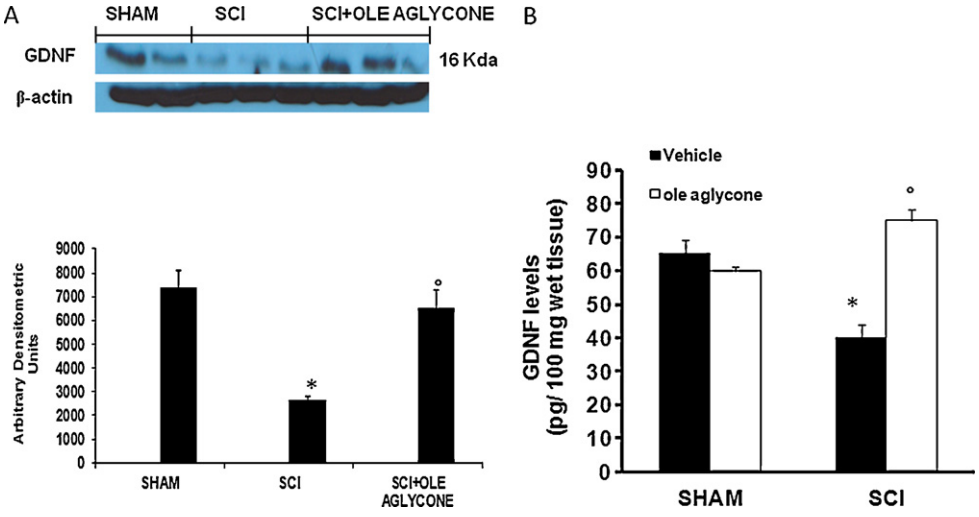

Fig. 2. Effect of ole aglycone treatment on GDNF expression 24 h after injury. In this study, we have evaluated GDNF expression by western blot and ELISA. SCI caused a significant decrease of GDNF expression in SCI mice (A and B). The treatment with ole aglycone increased this expression in the spinal cord from SCI-subjected mice (A and B). High levels of GDNF was found in sham animals (A and B). Moreover, The relative expression of the protein bands was standardized for densitometric analysis to β-actin levels. $^{*}P < 0.01$ vs. sham; $^{\circ}P < 0.01$ vs. SCI + vehicle. ND: not detectable.

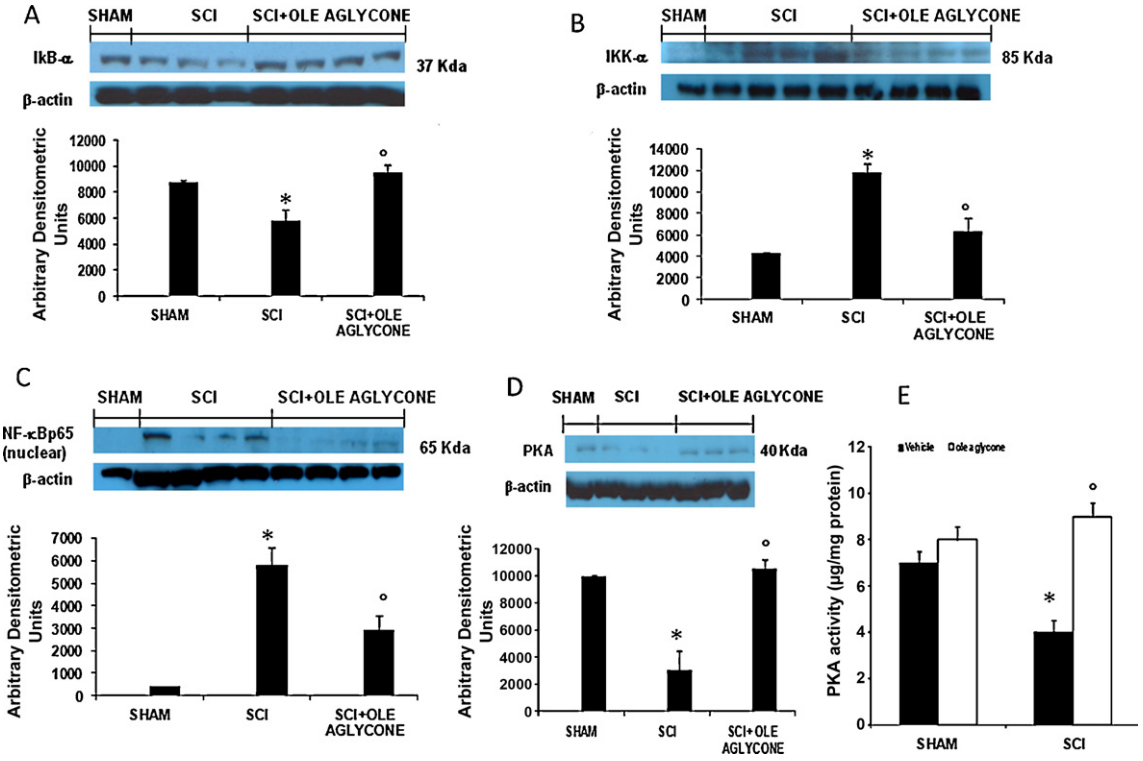

Fig. 3. Western blot analysis for IκB-α IKK-α, NF-κB p65, PKA and PKA activity. A basal level of IκB-α was also detected in the spinal cord from sham-operated animals (A) whereas IκB-α levels were substantially reduced in SCI mice (A). Ole aglycone treatment prevented the SCI-induced IκB-α degradation (A). In addition, SCI caused a significant increase in IKK-α (B) and nuclear NF-κB p65 (C) compared to the sham-operated mice. Ole aglycone treatment significantly reduced IKK-α and nuclear NF-κB p65 levels as shown in figures (B and C). Moreover, we have also evaluated the induction of PKA activity and its expression (D and E). Basal levels of PKA were found in sham animals (D and E). SCI caused a significant decrease of PKA expression and PKA activity in SCI mice (D and E). The treatment with ole aglycone increased the activity of PKA and its expression in the spinal cord from SCI-subjected mice (D and E). β-Actin was used as internal control. The relative expression of the protein bands was standardized for densitometric analysis to β-actin levels, and reported in figs. A–D are expressed as mean ± s.e.m. from $n = 5/6$ spinal cord for each group. $^{*}P < 0.01$ vs. sham; $^{\circ}P < 0.01$ vs. SCI + vehicle. ND: not detectable.

3.3. Effect of ole aglycone on IκB-α degradation and NF-κB p65 activation

We evaluated IκB-α degradation, nuclear NF-κB p65 activation by western Blot analysis to investigate the cellular mechanisms by which treatment with ole aglycone may attenuate the development of SCI.

A basal level of IκB-α was detected in the spinal cord from sham-operated animals (Fig. 3A) whereas IκB-α levels were substantially reduced in SCI mice (Fig. 3A). Consequently, IKK-α kinase expression, responsible of IKB-α phosphorylation, was significantly increased in spinal cord of SCI mice (Fig. 3B). Ole aglycone administration prevented the SCI-induced IκB-α degradation (Fig. 3A and B). NF-κB p65 levels in the nuclear fractions from spinal cord tissue were also significantly increased at 24 h after SCI compared to the sham-operated mice (Fig. 3C). Ole aglycone treatment reduced the levels of IKK-α and NF-κB p65 as shown in Fig. 3B and C.

3.4. Effect of ole aglycone on PKA expression and activity

In this study, we have also evaluated the changes in PKA activity and expression in spinal cord tissues collected at 24 h after SCI to investigate the cellular mechanisms by which treatment with ole aglycone may attenuate the development of SCI. A basal level of PKA was detected in the spinal cord from sham-operated animals (Fig. 3D and E), whereas PKA expression and activity were significantly reduced in SCI mice (Fig. 3D and E). Treatment with ole aglycone significantly restored PKA levels in SCI-operated mice (Fig. 3D and E).

3.5. Ole aglycone modulates TNF-α and IL-1β levels and MPO activity

To test whether ole aglycone modulates the inflammatory process through the regulation of secretion of pro-inflammatory cytokines, we analyzed spinal cord levels of TNF-α and IL-1β (Fig. 4A and B). A substantial increase in TNF-α and IL-1β production was found in spinal cord tissues samples collected from SCI mice 24 h after SCI (Fig. 4A and B). Spinal cord levels of TNF-α and IL-1β were significantly attenuated by the intraperitoneal injection of ole aglycone (Fig. 4A and B). In this study, we also investigated the effect of the treatment of ole aglycone on the infiltration of neutrophils by measuring tissue MPO activity. MPO activity was significantly elevated in the spinal cord at 24 h after injury in mice subjected to SCI when compared with sham-operated mice (Fig. 4C). In ole aglycone-treated mice, the MPO activity was significantly attenuated in comparison to that observed in SCI (Fig. 4C).

3.6. Effects of ole aglycone on lipid peroxidation, nitrotyrosine and PAR formation

Twenty-four hours after SCI, thiobarbituric acid-reactant substances levels and lipid peroxides were also measured in the spinal cord tissue as an indicator of lipid peroxidation. A significant increase of thiobarbituric acid-reactant substances (Fig. 5A) and LPO levels (Fig. 5B) were observed in the spinal cord collected at 24 h from mice subjected to SCI when compared with sham-operated mice. Thiobarbituric acid-reactant substances (Fig. 5A) and LPO levels (Fig. 5B) were significantly attenuated by the intraperitoneal injection of ole aglycone. In addition, spinal cord sections from sham-operated mice did not stain for nitrotyrosine

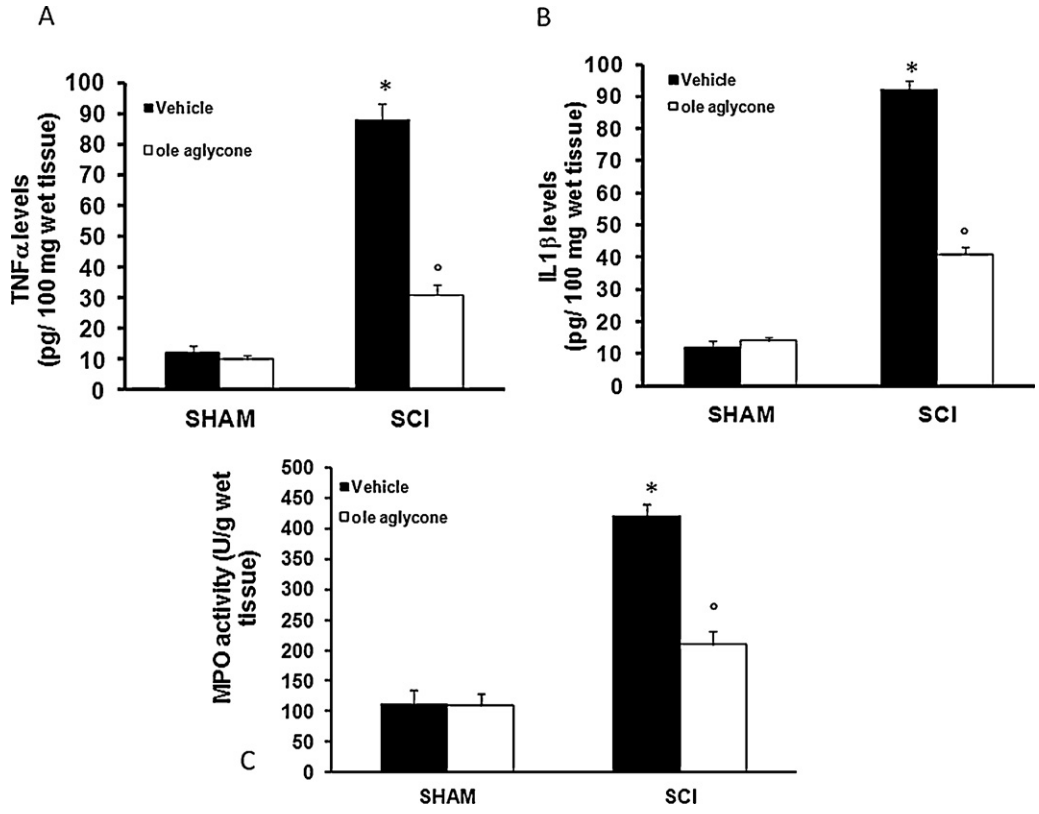

Fig. 4. Effects of ole aglycone on TNF-α and IL-1β levels and MPO activity. A substantial increase in TNF-α (A) and IL-1β (B) production was found in spinal cord tissue collected from SCI mice at 24 h. Spinal cord levels of TNF-α and IL-1β were significantly attenuated by ole aglycone treatment (A and B). Following the injury, MPO activity in spinal cord from SCI mice was significantly increased at 24 h after the damage in comparison to sham groups (C). Treatment i.p. with ole aglycone significantly attenuated neutrophil infiltration. Data are means ± s.e. means of 10 mice for each group. $^{*}P < 0.01$ vs. sham; $^{\circ}P < 0.01$ vs. SCI + vehicle. ND: not detectable.

and PAR (Fig. 6A, D and H), whereas spinal cord sections obtained from SCI mice exhibited positive staining for nitrotyrosine and PAR (Fig. 6B, E and H). The positive staining was mainly localized in inflammatory cells as well as in nuclei of Schwann cells in the white and gray matter of the spinal cord tissues. Ole aglycone treatment reduced the degree of positive staining for nitrotyrosine and PAR (Fig. 6C, F and H) in the spinal cord.

3.7. Effects of ole aglycone on iNOS expression

At 24 h after SCI, the appearance of iNOS in spinal cord homogenates was also investigated by Western blot. iNOS levels were appreciably increased in the spinal cord from mice subjected to SCI compared to sham animals (Fig. 6G). On the contrary, treatment with ole aglycone prevented the SCI-induced iNOS expression (Fig. 6G).

3.8. Effects of ole aglycone on FAS ligand expression

Immunohistological staining for FAS ligand in the spinal cord was also determined 24 h after injury. Spinal cord sections from sham-operated mice did not stain for FAS ligand (Fig. 7A and G) whereas spinal cord sections obtained from SCI mice exhibited positive staining for FAS ligand mainly localized in inflammatory

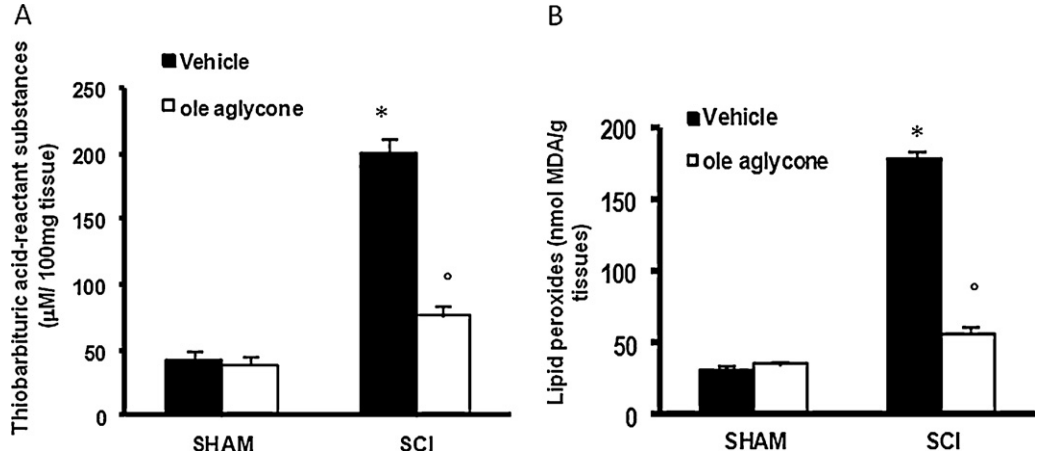

Fig. 5. Effect of ole aglycone on lipid peroxidation. A significant increase of thiobarbituric acid-reactant substances (A) and LPO levels (B) were observed in the spinal cord collected at 24 h from mice subjected to SCI when compared with sham-operated mice. Treatment i.p. with ole aglycone significantly attenuated hiobarbituric acid-reactant substances (A) and LPO levels (B) data are means ± s.e. means of 10 mice for each group. $^{*}P < 0.01$ vs. sham; $^{\circ}P < 0.01$ vs. SCI + vehicle. ND: not detectable.

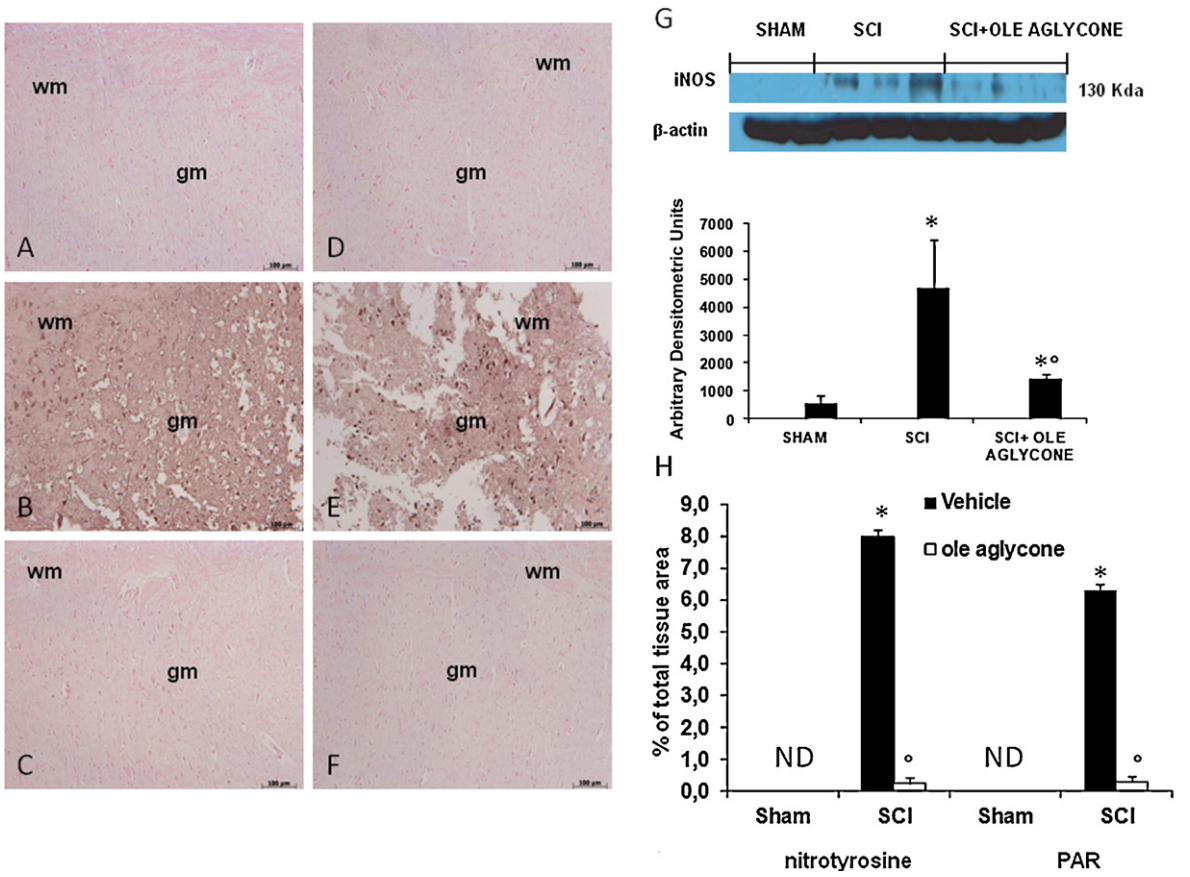

Fig. 6. Effects of ole aglycone on nitrotyrosine, PAR formation and iNOS expression. Spinal cord sections from sham-operated mice did not stain for nitrotyrosine (A). Sections obtained from vehicle-treated animals after SCI demonstrate positive staining for nitrotyrosine mainly localized in inflammatory cells, in nuclei of Schwann cells in the white and gray matter (B). Ole aglycone treatment reduced the degree of positive staining for nitrotyrosine (C) in the spinal cord. In addition, immunohistochemistry for PAR, an indicator of in vivo PARP activation, revealed the occurrence of positive staining for PAR localized in nuclei of Schwann cells in wm and gm of the spinal cord tissues from SCI mice (E). Spinal cord sections from sham-operated mice did not also stain for PAR (D). Ole aglycone treatment reduced the degree of positive staining for PAR (F) in the spinal cord. In addition, representative western blot showing no significant iNOS expression in spinal cord tissues obtained from sham-treated animals (G). iNOS levels were appreciably increased in the spinal cord from SCI mice (G). On the contrary, ole aglycone prevented the SCI-induced iNOS expression (G). The relative expression of the protein bands was standardized for densitometric analysis to β-actin levels. Densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for nitrotyrosine and PAR (H) from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days on the tissues section collected from all the animals in each group. $^{*}P < 0.01$ vs. sham; $^{\circ}P < 0.01$ vs. SCI + vehicle. wm: white matter; gm: gray matter; ND: not detectable.

cells as well as in nuclei of Schwann cells (Fig. 7B and G). Ole aglycone treatment reduced the degree of positive staining for FAS ligand in the spinal cord (Fig. 7C and G).

3.9. Effects of ole aglycone in the apoptosis in spinal cord after injury

To test whether spinal cord damage was associated to cell death by apoptosis, we also measured TUNEL-like staining in the perilesional spinal cord tissue. Almost no apoptotic cells were detected in the spinal cord from sham-operated mice (Fig. 7D and H). At 24 h after the trauma, tissues from SCI mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Fig. 7E and H). In contrast, tissues obtained from mice treated with ole aglycone treatment demonstrated no apoptotic cells or fragments (Fig. 7F and H).

3.10. The effect of ole aglycone on Bax, Bcl-2 and Caspase 3 expression

Samples of spinal cord tissue were taken at 24 h after SCI also to determine the immunohistological staining for Bax and Bcl-2. Spinal cord sections from sham-operated mice did not stain for Bax (Fig. 8A and G) whereas spinal cord sections obtained from SCI mice exhibited a positive staining for Bax (Fig. 8B and G). Ole aglycone treatment reduced the degree of positive staining for Bax in the spinal cord of mice subjected to SCI (Fig. 8C and G). In addition, spinal cord sections from sham-operated mice demonstrated Bcl-2 positive staining (Fig. 8D and G) while in SCI mice the staining was significantly reduced (Fig. 8E and G). Ole aglycone treatment attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice (Fig. 8F and G). At 24 h after SCI, the appearance of proapoptic protein, Bax, in spinal cord homogenates was also investigated by Western blot. Bax levels were appreciably increased in the spinal cord from mice subjected to SCI (Fig. 9A). On the contrary, treatment with ole aglycone prevented the SCI-induced Bax expression (Fig. 9A). By Western blot analysis were also analyzed Bcl-2 expression in homogenates from spinal cord of each mice. A basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (Fig. 9B). Twenty-four hours after SCI, the Bcl-2 expression was significantly reduced in spinal cord from SCI mice (Fig. 9B). Treatment of mice with ole aglycone significantly blunted the SCI-induced inhibition of anti-apoptotic protein expression (Fig. 9B). In addition, sequential activation of caspases plays a central role in the execution-phase of cell apoptosis. By western blot, we have evaluated the activation of Caspase 3. Caspase 3 levels were appreciably increased in the spinal cord from mice subjected to SCI

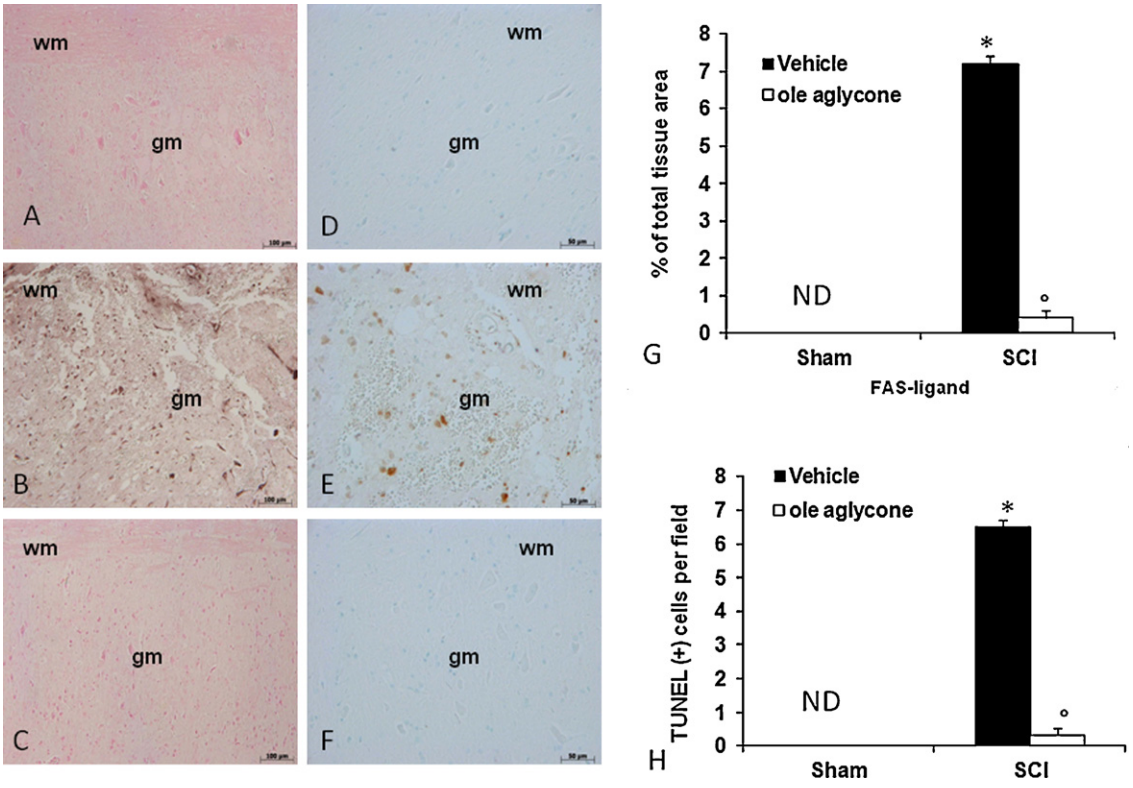

Fig. 7. Effect of ole aglycone on FAS-ligand expression and on TUNEL-like staining in the perilesional spinal cord tissue. Spinal cord sections were processed at 24 h after SCI to determine the immunohistological staining for Fas-ligand and TUNEL staining. Spinal cord sections from sham-operated mice did not stain for FAS ligand (A) whereas a substantial increase in Fas-ligand expression was found in inflammatory cells, in nuclei of Schwann cells in wm and gm of the spinal cord tissues from SCI mice at 24 h after SCI (B). Spinal cord levels of Fas-ligand were significantly attenuated in ole aglycone treated mice in comparison to SCI animals (C). Densitometry analysis of immunocytochemistry photographs (n = 5 photos from each sample collected from all mice in each experimental group) for Fas-ligand (G) from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days on the tissues section collected from all the animals in each group. $^{*}P < 0.01$ vs. sham; $^{\circ}P < 0.01$ vs. SCI + vehicle. Morever, almost no apoptotic cells were detected in the spinal cord from sham-operated mice (D). At 24 h after the trauma, SCI mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (E). In contrast, tissues obtained from mice treated with ole aglycone demonstrated no apoptotic cells or fragments (F). The number of TUNEL positive cells/high-power field was counted in 5–10 fields for each coded slide (H). Figure is representative of at least 3 experiments performed on different experimental days on the tissues section collected from all the animals in each group. $^{*}P < 0.01$ vs. sham; $^{\circ}P < 0.01$ vs. SCI + vehicle. wm: white matter; gm: gray matter; ND: not detectable. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Fig. 9C). On the contrary, treatment with ole aglycone prevented the SCI-induced Caspase 3 expression (Fig. 9C).

4. Discussion

Spinal cord injury is a highly debilitating pathology [1]. The primary injury refers to the mechanical damage leading to direct cell death and bleeding. Further progressive destruction of the tissue surrounding the necrotic core is known as secondary injury [33] that is determined by a large number of vascular, biochemical and cellular cascades including the breakdown of blood-spinal cord barrier with edema formation, ischemia and hypoxia, the release of vasoactive substances leading to alteration of spinal cord perfusion, the excitotoxicity leading to Ca^{2+} dependent, glutamate-associated neuronal cell death, the formation of free radicals and NO, a damage of mitochondrias with energy depletation, the invasion and activation of inflammatory cells such as (neutrophils, resident microglia, peripheral macrophages and astrocytes) which secrete lytic enzymes and cytokines contributing to further tissue damage, the apoptosis of oligodendrocytes and neurodegeneration [34].

The pharmacological properties of olive oil, the olive fruit and its leaves have been recognized as important components of medicine and a healthy diet because of their phenolic content [35]. Ole is the major constituent of the leaves and unprocessed olive drupes of *Olea europaea* and the majority of polyphenols found in olive oil or table olives are derived from its hydrolysis. It has been suggested that, of all the phenols present in olive oil, only the catechols (hydroxytyrosol and oleuropein, "ole") are very important [35]. These two phenols show dose-dependent activity and are considered potent antioxidants, demonstrating activity in the micro-molar range. Both are more potent at scavenging free radicals than the endogenous antioxidant vitamin E and the exogenous antioxidants dimethyl sulfoxide (DMSO) and butylated hydroxytoluene (BHT) [36]. Therefore, ole aglycone also has a powerful antioxidant effect as that shown by melatonin in the model of spinal cord injury [37].

Both hydroxytyrosol and ole have been shown to be scavengers of superoxide anions, and inhibitors of the respiratory burst of neutrophils and hypochlorous acid-derived radicals, but hydroxytyrosol was more effective than ole [20,38]. Both compounds also scavenged hydroxyl radicals, but in this case ole showed greater activity [38]. Thus, ole potently and dose-dependently inhibits copper sulfate-induced oxidation of low-density lipoproteins (LDL) [16]. Moreover, one study [39] has also reported that ole decreases or even prevents Aβ aggregation, which is inherent to Alzheimer's disease (AD) demonstrating neuroprotective effects.

Further pharmacological activity of ole includes diverse healing properties due to its vasodilatory, anti-platelet aggregation,

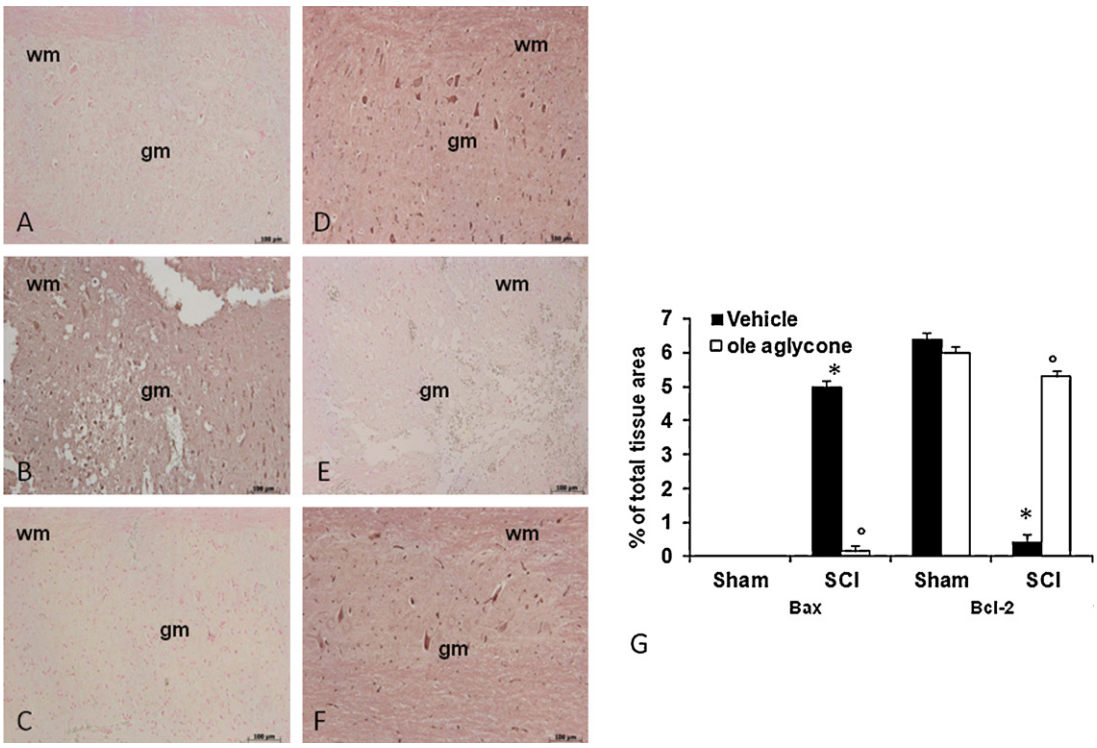

Fig. 8. Effect of ole aglycone on immunocytochemistry of Bax, Bcl-2. Spinal cord sections from sham-operated mice did not stain for Bax (A) whereas SCI caused, at 24 h, an increase in Bax expression (B). Ole aglycone treatment reduced the degree of positive staining for Bax in the spinal cord (C). On the contrary, positive staining for Bcl-2 was observed in the spinal cord tissues from sham-operated mice (D) while the staining was significantly reduced in SCI mice (E). Ole aglycone treatment attenuated the loss of positive staining for Bcl-2 in the spinal cord from SCI-subjected mice (F). Densitometry analysis of immunocytochemistry photographs ($n = 5$ photos from each sample collected from all mice in each experimental group) for Bax and for Bcl-2 (G) from spinal cord tissues was assessed. The assay was carried out by using Optilab Graftek software on a Macintosh personal computer (CPU G3-266). Data are expressed as % of total tissue area. This figure is representative of at least 3 experiments performed on different experimental days on the tissues section collected from all the animals in each group. $^{*}P < 0.01$ vs. sham; $^{\circ}P < 0.01$ vs. SCI + vehicle. wm: white matter; gm: gray matter; ND: not detectable.

hypotensive, anti-rheumatic, diuretic and antipyretic effects. Moreover, ole aglycone, may exert protective effects toward several malignancies, especially human breast cancer [16]. A more efficient anti-inflammatory role of the aglyconic, compared with the glycosidic form of ole possibly derives from the greater lipophilicity of the former, a property that should allow better cell membrane incorporation and/or interaction with other lipids [40]. Thus, oleuropein is a novel, naturally occurring antioxidant compound, which may possibly be used to resolve problems of clinical relevance. In agreement with other colleagues, the findings of this study strengthen the hypothesis that the health benefits of olive oil are related to the oleuropein derivatives that are present in olive oil [16]. For this reason, we investigate here the effects of ole aglycone, a hydrolysis product of oleuropein, in the modulation of secondary injury associated with SCI. We showed here that SCI resulted in edema and loss of myelin in lateral and dorsal funiculi. This histological damage was associated to the loss of motor function. In addition, GDNF has been shown to have a potent survival-promoting effect on various neuronal populations, including dopaminergic [41], noradrenergic [42], cortical [43], retinal ganglion [44], sensory [45], and motor neurons [46]. Moreover, GDNF induced the growth of motor and sensory axons and remyelination in laboratory rats with partial and complete spinal cord transections [47]. In a previous study, immunohistochemical analysis has also showed GDNF immunoreactivity to be present mainly in microglia and macrophages 1 day after injury, but not in neurons or astrocytes. This immediate upregulation of GDNF gene expression may be a component of an inflammatory process and probably exerts a protective effect on neurons following SCI [48]. We also demonstrated here by western blot and ELISA kit, that the treatment with ole aglycone increased the levels of neurotrophic factors such as GDNF.

The molecular mechanisms involved in the anti-inflammatory activities of polyphenols present in olive oil include several points within inflammatory cascade: (i) the inhibition of pro-inflammatory enzymes, such as cyclooxygenase (COX-2), lipoxygenase (LOX) and inducible nitric oxide synthase (iNOS), (ii) the inhibition of phosphoinositide 3-kinase (PI 3-kinase), tyrosine kinases, NF-κB, c-JUN and (iii) the activation of phase II antioxidant detoxifying enzymes, mitogen-activated protein kinase (MAPK), protein kinase C (PKC), serin/threonin protein kinase Akt/PKB as well as (iv) the modulation of several cell survival/cell-cycle genes [49]. Therefore, in this study, we provide evidence to indicate that administration of ole aglycone inhibits the development of SCI through inhibition of ROS and subsequently ROS-induced inflammatory pathways. One consequence of increased oxidative stress is the activation and inactivation of redox-sensitive proteins. Therefore, NF-κB is an attractive candidate to mediate the effects of ROS because the interaction between the inhibitory protein IκB and NF-κB proteins is regulated by protein kinases that contain several redox-sensitive cysteine residues in critical kinase domains [50]. Recent studies have observed that the acute consumption of olive oil decreased the activation of NF-κB system on mononuclear cells from healthy men [51] and that ole aglycone, *trans*-resveratrol, and hydroxytyrosol incubated with human umbilical vein endothelial cells inhibit LPS-triggered NF-κB and AP-1 activation [52]. NF-κB is normally sequestered in the cytoplasm, bound to regulatory proteins IκBs. In response to a wide range of stimuli including oxidative stress, infection, hypoxia, extracellular signals, and inflammation, IκB is phosphorylated by the enzyme IκB kinase

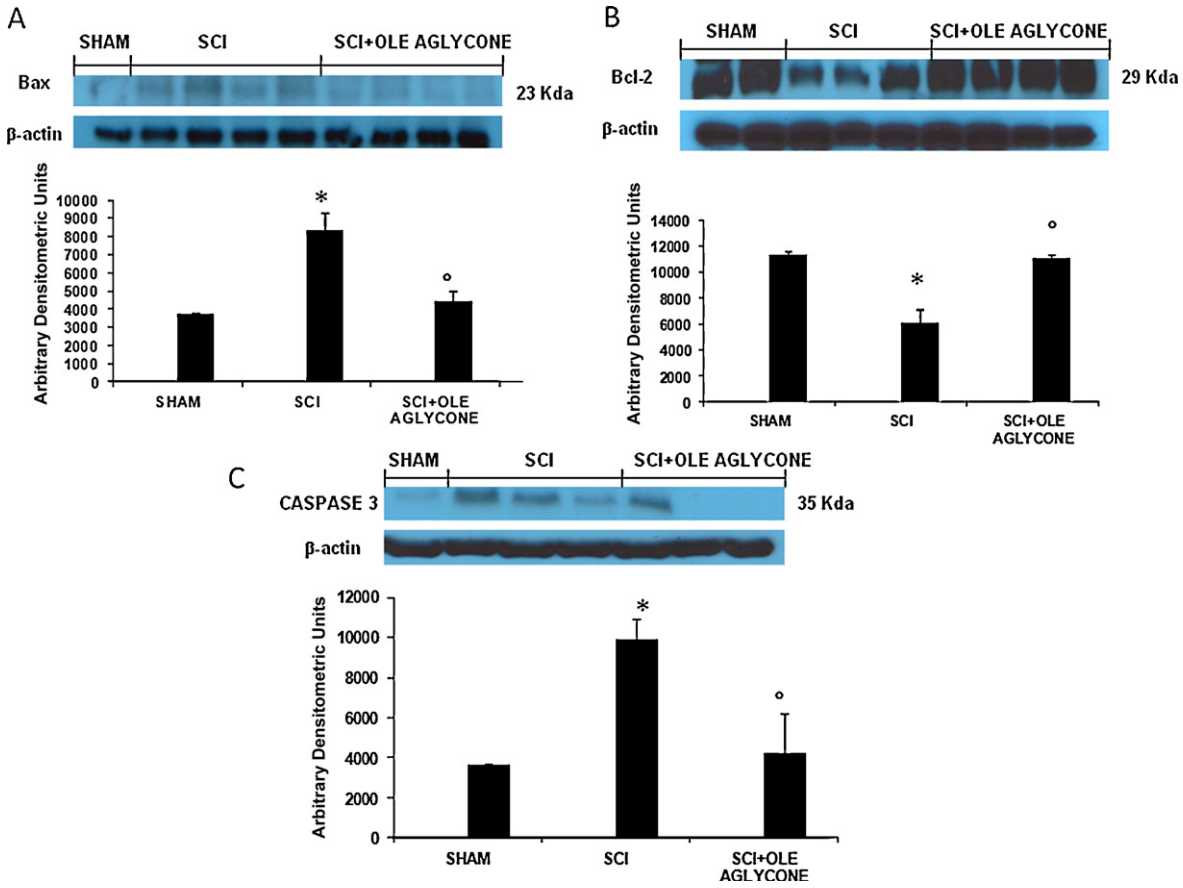

Fig. 9. Western blots for Bax, Bcl-2 and Caspase 3. Representative Western blots showing no significant Bax expression in spinal cord tissues obtained from sham-treated animals (A). Bax levels were appreciably increased in the spinal cord from SCI mice (A). On the contrary, ole aglycone prevented the SCI-induced Bax expression (A). Moreover, a basal level of Bcl-2 expression was detected in spinal cord from sham-operated mice (B). Twenty-four hours after SCI, Bcl-2 expression was significantly reduced in spinal cord from SCI mice (B). Ole aglycone treatment significantly reduced the SCI-induced inhibition of Bcl-2 expression (B). Moreover, we have also demonstrated Caspase 3 activation by western blot. SCI caused a significant increase in Caspase 3 expression (C) compared to the sham-operated mice (C). Ole aglycone treatment significantly reduced Caspase 3 levels as shown in figure (C). The relative expression of the protein bands was standardized for densitometric analysis to β-actin levels. $^{*}P < 0.01$ vs. sham; $^{\circ}P < 0.01$ vs. SCI + vehicle. ND: not detectable.

(IKK). The net result is the release of the NF-κB dimer, which is then free to translocate into the nucleus. In the study we report that SCI was associated with significant IκB-α degradation as well as increased nuclear expression of p65 in spinal cord tissue at 24 h after injury. Treatment with ole aglycone significantly reduced IκB-α degradation induced by the action of IKK enzyme as well as the consequent NF-κB translocation. Recent study also reported that the phenolic compound oleuropein enhances thermogenesis by increasing uncoupling protein (UCP1) content in brown adipose tissue and noradrenaline and adrenaline secretion in rats [53]. These hormones bind cell-surface receptors that are coupled to the activation of adenylate cyclase upon ligand binding. The resultant increase in cAMP leads to activation of protein kinase A (PKA) that is responsible of inhibition of the NF-κB transcriptional activity [54]. In that regard, we have also demonstrated that the treatment with ole aglycone determined the induction of PKA increasing both expression and activity in SCI mice.

NF-κB has an important function in the regulation of many genes responsible for the generation of mediators or proteins in secondary inflammation associated with SCI [55] such as (IL-1β, TNF-α, iNOS) [56]. A direct consequence of the inhibitory effect of ole aglycone on NF-κB activation is the reduction of proinflammatory mediators production under its control [57]. In that regard, it has been well demonstrated that in SCI the expression of proinflammatory cytokines (TNF-α and IL-1β) at the site of injury regulates the precise cellular events after SCI [9]. We have clearly confirmed a significant increase in TNF-α and IL-1β during SCI. On the contrary, no significant expression of TNF-α and IL-1β was observed in the spinal cord sections obtained from SCI-operated mice which received ole aglycone as previously demonstrated [23]. In this regard, Miles et al. [58] also demonstrated that ole glycoside significantly decreased the concentration of IL-1β in LPS-stimulated human whole blood cultures. One consequence of reduced cytokines production is a decrease of leukocyte activation and infiltration into inflamed tissue. We report here that SCI was associated with significant increase of neutrophil infiltration measured by MPO activity, while in ole aglycone-treated mice, the MPO activity was significantly attenuated in comparison to that observed in SCI. Several studies also showed that in the auricular edema induced by either arachidonic acid (AA) or 12-O-tetradecanoylphorbol acetate (TPA), the topical application of the olive oil compounds such as ole also produced an inhibition of the MPO in the inflamed tissue. [59] In addition, recent studies also showed that the potential cardioprotective activity of oleuropein in acute cardiotoxicity induced by doxorubicin treatment was determined in vivo in rats [60] by inhibiting lipid peroxidation products, decreasing oxidative stress and reducing iNOS in cardiomyocytes. In that regard, we have demonstrated that the treatment with ole aglycone significantly decreased thiobarbituric acid-reactant substances, MDA levels and iNOS expression in spinal cord of SCI-operated mice. Among the reactive oxygen species, peroxynitrite ($ONOO^{-}$) is known to play an important role in local and

systemic inflammatory response as well as neurodegenerative disease [61,62]. It is one of a number of toxic factors produced in the spinal cord tissues after SCI [62] likely contributes to secondary neuronal damage through pathways resulting from the chemical modification of cellular proteins and lipids. To probe the pathological contributions of $ONOO^-$ to secondary damage after SCI, we have used the appearance of nitrotyrosine staining in the inflamed tissue. We have observed in this study that the immunoassaying of nitrotyrosine is reduced in SCI operated mice treated with ole aglycone when compared with SCI operated mice. Increased nitrotyrosine staining is considered, therefore, as an indication of "increased nitrosative stress" rather than a specific marker of the peroxynitrite generation. Our results are also in according with a previous study in which it was demonstrated that the effect of oleuropein on DSS-induced colitis is associated with a decrease in the production of interleukins and expression of proteins, principally through reduction of NF-κB activation [63].

ROS produce strand breaks in DNA, which trigger energy-consuming DNA repair mechanisms and activate the nuclear enzyme PARP resulting in the depletion of its substrate NAD in vitro and a reduction in the rate of glycolysis. As NAD functions as a cofactor in glycolysis and the tricarboxylic acid cycle, NAD depletion leads to a rapid fall in intracellular ATP. There are several evidences that the activation of PARP may also play an important role in inflammation [64]. We demonstrate here that oleuropein aglycone treatment reduced the activation of PARP in spinal cord during the trauma as previously demonstrated [23,65]. In this regard, several studies demonstrated that hydroxytyrosol, a hydrolysis product of ole aglycone, also exerts an inhibitory effect on peroxynitrite-dependent DNA base modifications and tyrosine nitration [66]. Similarly, Salvini et al. [67] showed a 30% reduction of oxidative DNA damage in peripheral blood lymphocytes during intervention on postmenopausal women with virgin olive oil containing high amounts of phenols. Apoptosis is an important mediator of secondary damage after SCI [68]. It incurs its affects through at least two phases: an initial phase, in which apoptosis accompanies necrosis in the degeneration of multiple cell types and a later phase, which is predominantly confined to white matter and involves oligodendrocytes and microglia [69]. Chronologically, apoptosis initially occurs 6 h post-injury at the lesion center and last for several days associated with the steadily increased number of apoptotic cells in this region. In an effort to prevent or diminish levels of apoptosis, we demonstrate that the treatment with ole aglycone attenuates the degree of apoptosis, measured by TUNEL detection kit, in the spinal cord after the damage. Several studies have also demonstrated that hydroxytyrosol, a hydrolysis product of ole aglycone, protects cells from cell death induced by reactive oxygen intermediates, as occurs during ischemia and reperfusion injury [17]. Moreover various studies have upostulated that preserving Bax, a pro-apoptotic gene, plays an important role in developmental cell death [70] and in CNS injury [71]. Similarly, it has been shown that the administration of Bcl-xL fusion protein (Bcl-xL FP), (Bcl-2 is the most expressed anti-apoptotic molecule in adult central nervous system) into injured spinal cords significantly increased neuronal survival, suggesting that SCI-induced changes in Bcl-xL contribute considerably to neuronal death [72]. Based on these evidences, we have identified in SCI proapoptotic transcriptional changes, including upregulation of proapoptotic Bax and down regulation of antiapoptotic Bcl-2, by immunohystochemical staining and western blot. In particular, we demonstrated that the treatment with ole aglycone reduced Bax expression, while on the contrary, Bcl-2 is expressed much more in mice treated with ole aglycone. A lot of number of studies has linked apoptosis to thoracic SCI. To such purpose, furthermore, some authors have also shown that FAS and p75 receptors are expressed on oligodendrocytes, astrocytes and microglia in the spinal cord following SCI. Therefore, FasL plays a central role in apoptosis induced by a variety of chemical and physical insults [73]. Recently, it has been pointed out that FasL signaling plays a central role in SCI [74]. We confirm here that SCI leads to a substantial activation of FasL in the spinal cord tissues which likely contributes in different capacities to the evolution of tissues injury. In the present study, we found that ole aglycone treatment leads to a substantial reduction of FasL activation.

Finally, in this study we demonstrate that ole aglycone treatment significantly reduced the SCI-induced spinal cord tissues alteration as well as improve the motor function. The results of the present study enhance our understanding of the role of ROS in the pathophysiology of spinal cord cell and tissue injury following trauma, implying that the antioxidant and antinflammatory effect of the polyphenols present in olive oil such as oleuropein aglycone may be useful in the therapy of spinal cord injury, trauma and inflammation.

Conflict of interest

None.

Acknowledgments

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