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Inhibitors of poly (ADP-ribose) polymerase modulate signal transduction pathways and secondary damage in experimental spinal cord trauma.

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Running Title: PARP inhibitor reduces post-traumatic spinal cord injury

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List of abbreviations: Poly(ADP-ribose) polymerase (PARP); nuclear factor-κB (NF-κB); spinal cord injury (SCI); reactive oxygen species (ROS); adenosine triphosphate (ATP); 3-aminobenzamide (3-AB); 1,5-dihydroxyisoquinoline (5-hydroxyisoquinolin-1(2H)-one) (1,5-DHIQ); 3,4-dihydro-5-(4-(1-piperidinyl)butoxyl)-1(2H)-isoquinolinone), (DPQ); 5-aminoisoquinolinone [5-aminoisoquinolin-1(2H)-one] (5-AIQ); Electrophoretic mobility-shift assay (EMSA); poly(ADP-ribose) (PAR); Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL): deoxynucleotidyltransferase (TdT) Myeloperoxidase (MPO);

ABSTRACT

Poly(ADP-ribose) polymerase, (PARP), a nuclear enzyme activated by strand breaks in DNA, plays an important role in the tissue injury associated with stroke and neurotrauma. The aim of our study was to evaluate the therapeutic efficacy of *in vivo* inhibition of PARP in an experimental model of spinal cord trauma, which was induced by the application of vascular clips (force of 24 g) to the dura via a four-level T5-T8 laminectomy. Spinal cord injury in mice resulted in severe trauma characterized by edema, neutrophil infiltration (measured as an increase in myeloperoxidase activity) and apoptosis (measured by TUNEL coloration). Infiltration of spinal cord tissue with neutrophils was associated with a marked increase in immunoreactivity for PAR, index of PARP activation, in the spinal cord tissue. These inflammatory events were associated with the activation of nuclear factor-κB (NF-κB) at 4h after spinal cord damage. Treatment of the mice with the PARP inhibitors, 3-aminobenzamide (3-AB) or 5-aminoisoquinolinone (5-AIQ) significantly reduced the degree of (1) spinal cord inflammation and tissue injury (histological score), (2) PAR formation, (3) neutrophil infiltration and (4) apoptosis. Treatment with these PARP inhibitors also reduced DNA binding of NF-κB and of IκB-α degradation. In a separate set of experiments we have also demonstrated that PARP inhibitors significantly ameliorated the recovery of limb function (evaluated by motor recovery score). Taken together, our results clearly demonstrate that treatment with PARP inhibitors reduces the development of inflammation and tissue injury events associated with spinal cord trauma.

Introduction

Post-traumatic inflammatory reactions may play an important role in the secondary injury processes after spinal cord injury (SCI) (Bartholdi and Schwab,1995). The primary traumatic mechanical injury to the spinal cord causes the death of a number of neurons that cannot be recovered or regenerated to date. However, neurons continue to die for hours after SCI, and this represents a potentially avoidable event. This secondary neuronal death is determined by a large number of cellular, molecular, and biochemical cascades. One such cascade that has been touted to contribute importantly to the evolution of this secondary damage is the local inflammatory response in the injured spinal cord. Although the neuraxis is considered somehow privileged under an immunological point of view and poorly influenced by inflammatory processes, a large body of recent data suggests the presence of a local inflammatory response, and that aspects of this response to injury amplify the secondary damage (Popovich et al., 1994). The cardinal features of inflammation, namely infiltration of inflammatory cells (polymorphonuclear neutrophils, macrophages, and lymphocytes), release of inflammatory mediators, and activation of endothelial cells leading to increased vascular permeability, edema formation, and tissue destruction have been well and extensively characterized in animal SCI models.

The production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide and hydroxyl radicals, as well as peroxynitrite, also contribute to the tissue injury observed during inflammation, neurodegenerative disease and post-traumatic inflammatory reactions after SCI (Xu et al., 2001). ROS and peroxynitrite also cause DNA damage, which results in the activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP), depletion of NAD^+ and ATP and ultimately cell death (Szabó and Dawson 1998). Therefore, recently it been demonstrated that spinal cord injury induced PARP activation (Scott et al., 1999) PARP is a ubiquitous, chromatin-bound enzyme, which is abundantly present in the nuclei of numerous cell types (Szabó and Dawson 1998). Continuous or excessive activation of PARP produces extended chains of ADP-ribose on nuclear proteins and results in a substantial depletion of intracellular

NAD^+ and subsequently, adenosine triphosphate (ATP), leading to cellular dysfunction and ultimately, cell death (Chiarugi, 2002). Chemically distinct inhibitors of PARP activity such as benzamides [e.g., 3-aminobenzamide (3-AB), nicotinamide] and isoquinolinones [e.g., 1,5-dihydroxyisoquinoline (5-hydroxyisoquinolin-1(2*H*)-one) (1,5-DHIQ), 3,4-dihydro-5-(4-(1-piperidinyl)butoxyl)-1(2*H*)-isoquinolinone), (DPQ)] can reduce the degree of injury associated with inflammation and these investigations have provided the basis for potential clinical applications of PARP inhibitors (Virag and Szabo 2002; Southan and Szabò 2003).

Therefore, several PARP inhibitors have been previously examined as potential novel therapeutic interventions against tissue injury associated with experimental inflammatory models. Specifically, these studies have demonstrated that the chemically distinct PARP inhibitors GPI6150, PJ34 and 3-AB can attenuate PARP activation and provide beneficial actions against tissue injury and dysfunction *in vivo* during inflammation (Mazzon et al., 2002, Cuzzocrea et al., 2002a; Li et al., 2004). However, in contrast isoquinolinone derivatives such as 1,5-DHIQ, DPQ, 3-AB, and nicotinamide are weak inhibitors of PARP activity that do not readily cross cell membranes (Szabó and Dawson, 1998). Furthermore, although the potency of recently developed PARP inhibitors has improved greatly, most lack good solubility in water, making it difficult to find a biocompatible vehicle for utilization *in vivo*. Thus, there is still a great need for the development of potent, water-soluble inhibitors of PARP activity. Much effort has been made to develop new PARP inhibitors with better potency, selectivity and water-solubility and there are now 13 chemical classes of PARP inhibitors (Virag and Szabo 2002; Southan and Szabò 2003). Twelve years ago, Suto et al (Suto et al., 1991) used a cell-free preparation of PARP (purified 900-fold from calf thymus) to demonstrate that 5-aminoisoquinolinone [5-aminoisoquinolin-1(2*H*)-one] (5-AIQ) is a water-soluble inhibitor of PARP activity. As previously published reports of the synthesis of 5-AIQ reported problems of low yield and unreliability (Suto et al., 1991), McDonald and colleagues have recently developed a novel and more efficient method for the synthesis of 5-AIQ (McDonald et al., 2000). We have previously demonstrated that 5-AIQ can reduce ischemia/reperfusion injury of the heart, intestine

and liver (Wayman et al., 2001; Mota Filipe et al., 2002), and 5-AIQ has been shown to provide beneficial effects in rodent models of heart transplantation (Szabo et al., 2002) and lung injury (Cuzzocrea et al., 2002a).

Consistent with these findings, the objective of the present study was to investigate the biological effects of pharmacological inhibition of PARP in a mouse model of SCI with two structurally unrelated inhibitors of PARP, 3-AB, as reference inhibitor and 5-AIQ as new synthetic inhibitor were tested. In order to gain a better insight into the mechanism(s) of action of the observed anti-inflammatory effects of 3-AB or 5-AIQ, we have evaluated the following endpoints of the inflammatory process: (1) clinical score, (2) NF-κB activation, (3) activation of the nuclear enzyme PARP, (4) neutrophil infiltration, (5) apoptosis (TUNEL coloration) and (6) spinal cord tissue histopathology. We observed that pharmacological inhibition of PARP activity resulted in a significant reduction of secondary damage and that this therapeutic efficacy was associated with the prevention of NF-κB activation.

Material and Methods

Animals

Adult male 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).

Spinal cord injury (SCI)

Mice were anaesthetized using chloral hydrate (40 μg/kg body weight). 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 T6-T7 laminectomy and SCI was produced by extradural compression of the spinal cord using an aneurysm clip with a closing force of 24 g. Following surgery, 1 ml of saline was administered subcutaneously in order to replace the blood volume lost during surgery. During the surgery and the 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. In all injured groups, the spinal cord was compressed for 1 min. Sham injured animals were only subjected to laminectomy.

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Experimental groups

Mice were randomly allocated into the following groups: (i) *SCI + saline group*. Mice were subjected to SCI plus administration of saline (*N*=40); (ii) *3-AB group*. Same as the *SCI + saline group* but 3-AB at a dose of 10 mg/kg was administered (i.p.) 30 min, 1 and 6 h after SCI (*N*=40); (iii) *5-AIQ group*. Same as the *SCI + saline group* but 5-AIQ at a dose of 3 mg/kg was administered 30 min, 1 and 6 h after SCI (*N*=40); (iv) *Sham + saline group*. Mice were subjected to the surgical procedures as above group except that the aneurysm clip was not applied (*N*=40); (v) *Sham + 3-AB group*. Identical to *Sham + saline group* except for the administration of 3-AB (i.p.) at 30 min, 1, and 6 h after sham SCI (*N*=40). (vi) *Sham + 5AIQ group*. Identical to *Sham + saline group* except for the administration of 5AIQ (i.p.) at 30 min, 1, and 6 h after sham SCI (*N*=40). In the experiments regarding the motor score the animals were treated with 3-AB or 5-AIQ (i.p.) 30 min, 1 and 6 h after SCI and daily until day 9. At different time points (see figure 1) the mice (*N*=10 from each group for each of the 3 time points) were sacrificed in order to evaluate the various parameters as described below.

Preparation of whole extracts

All the extraction procedures were performed on ice using ice-cold reagents. Tissues from each mouse were suspended in 6 ml of a high-salt extraction buffer (20 mM pH 7.9 HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM phenylmethylsulphonylfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and homogenized at the highest setting for 2 min in a Polytron PT 3000 tissue homogenizer. The homogenates were chilled on ice for 15 min and then vigorously shaken for a few minutes in the presence of 20 μl of 10 % Nonidet P-40. After centrifugation at 13,000 x g at 4°C for 5 min, the protein concentration in the supernatant was determined by the Bio-Rad (Bio-Rad) protein assay kit and then it was aliquoted and stored at –80°C.

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Electrophoretic mobility-shift assay (EMSA)

Double-stranded oligonucleotides containing the NF-κB recognition sequence (5'-GAT CGA GGG GAC TTT CCC TAG-3') were end labeled with γ-[^{32}P]ATP (ICN Biomedicals). Aliquots of whole extracts collected 4 hours after SCI (20 μg of protein for each sample) were incubated for 30 min with radiolabeled oligonucleotides (2.5 - 5.0 x 10^4 cpm) in 20 μl reaction buffer containing 2 μg poly dI-dC, 10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1mM ethylenediaminotetraacetic acid, 1mM DL-dithiothreitol, 1 mg/ml bovine serum albumin, 10 % glycerol. The specificity of the DNA/protein binding was determined for NF-κB by competition reaction in which a 50 fold molar excess of unlabeled wild-type, mutant or Sp-1 oligonucleotide was added to the binding reaction 10 min before addition of radiolabeled probe. Protein-nucleic acid complexes were resolved by electrophoresis on 4 % nondenaturing polyacrylamide gel in 0.5 % Tris borate ethylenediaminotetraacetic acid buffer at 150 V for 2 h at 4°C. The gel was dried and autoradiographed with intensifying screen at –80°C for 20 h. Subsequently, the relative bands were quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM). The time of 4 h after SCI was chosen in agreement with other studies (Kim et al., 2001)

Western blot analysis for IκB-α

The levels of IκB-α were quantified in whole extracts 4 hours after SCI, by immunoprecipitation followed by Western blot analysis according to the manufacturers instructions (Celbio, Milan, Italy).

Briefly, proteins were then transferred onto nitrocellulose membranes, according to the manufacturers instructions. Briefly, the membranes were saturated by incubation at 4^{o}C overnight with 10 % (w/v) non-fat dry milk in PBS and then incubated with anti-IκB-α (1:1000) for 1 h at room temperature. Membranes were washed three times with 1% (w/v) Triton X-100 in PBS and

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then incubated with anti-rabbit immunoglobulins coupled to peroxidase (1:1000). The immune complexes were visualized using the ECL chemiluminescence method (Amersham, Buckinghamshire, U.K.). Subsequently, the relative expression of the proteins were quantified by densitometric scanning of the X-ray films with GS-700 Imaging Densitometer (Bio-Rad) and a computer program (Molecular Analyst, IBM).

Immunohistochemical localisation of poly(ADP-ribose)

Evidence of poly(ADP-ribose) (PAR) formation (an indicator of PARP activation) was determinedby immunohistochemistry as previously described (Di Paola et al., 2004). Twenty four hours after SCI, tissues were fixed in para-formaldehyde solution (4 % in PBS 0.1 M) at room temperature and 10 μm sections were prepared from paraffin embedded tissues. After deparaffinization, endogenous peroxidase was quenched with 0.3 % (v/v) H_2O_2 in 60 % (v/v) methanol for 30 min. The sections were permeablized 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 and biotin (DBA, Milan, Italy). The sections were then incubated overnight with primary anti-PAR or with control solutions including buffer alone or non specific purified rabbit IgG. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). The counterstain was developed with DAB (brown colour) and nuclear fast red (red background). The time of 24 h after SCI was chosen in agreement with other studies (Bao and Liu, 2002)

To verify the binding specificity of the relevant antibodies for PAR, some sections were also incubated with the primary antibody only (no secondary antibody) or with secondary antibody only (no primary antibody). 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 photos from each sample collected from all mice in each experimental group)

were assessed by densitometry as previously described (Di Paola et al., 2004) using Optilab Graftek software on a Macintosh personal computer.

Terminal Deoxynucleotidyltransferase-Mediated UTP End Labeling (TUNEL) Assay.

TUNEL assay was conducted by using a TUNEL detection kit according to the manufacturers instructions (Apotag, HRP kit DBA, Milan, Italy). Briefly, 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.

Myeloperoxidase activity

Myeloperoxidase (MPO) activity, an indicator of polymorphonuclear leukocyte (PMN) accumulation, was determined as previously described (Di Paola et al., 2004) 4 h after SCI. The time of 4 h after SCI was chosen in agreement with other studies (Hamada et al., 1996). At the specified time 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 x *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/min at 37°C and was expressed in milliunits/g of wet tissue.

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Light microscopy

Spinal cord biopsies were taken at 24 h following trauma. The biopsies were fixed for 24 h in paraformaldehyde solution (4 % in PBS 0.1M) at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections (thickness 5 μm) were deparaffinized with xylene, stained with haematoxylin/eosin and studied using light microscopy (Dialux 22 Leitz). All the histological studies were performed in a blinded fashion.

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 modified murine Basso, Beattie, and Bresnahan (BBB) hind limb locomotor rating scale (Joshi and Fehlings, 2002). The following criteria were considered: **0=**No hind limb movement; **1=**Slight (<50% range of motion) movement of 1-2 joints; **2=**Extensive (>50% range of motion) movement of 1 joint and slight movement of one other joint; **3=**Extensive movement of 2 joints; **4=**Slight movement in all 3 joints; **5=**Slight movement of 2 joints and extensive movement of 1 joint; **6=**Extensive movement of 2 joints and slight movement of 1 joint; **7=**Extensive movement of all 3 joints; **8=**Sweeping without weight support or plantar placement and no weight support; **9=**Plantar placement with weight support in stance only or dorsal stepping with weight support; **10=**Occasional (0-50% of the time) weight-supported plantar steps and no coordination (Front/hind limb coordination); **11=**Frequent (50-94% of the time) to consistent (95-100% of the time) weight-supported plantar steps and no coordination; **12=**Frequent to consistent weight-supported plantar steps and occasional coordination; **13=**Frequent to consistent weight-supported plantar steps and frequent coordination; **14=**Consistent weight-supported plantar steps, consistent coordination and predominant paw position is rotated during locomotion (lift off and contact) or frequent plantar stepping, consistent coordination and occasional dorsal stepping; **15=**Consistent plantar stepping and coordination, no/occasional toe clearance, paw position is parallel at initial contact; **16=**Consistent plantar

stepping and coordination (Front/hind limb coordination) and frequent toe clearance and predominant paw position is parallel at initial contact and rotated at lift off; **17=**Consistent plantar stepping and coordination and frequent toe clearance and predominant paw position is parallel at initial contact and lift off; **18=**Consistent plantar stepping and coordination and consistent toe clearance and predominant paw position is parallel at initial contact and rotated at lift off; **19=**Consistent plantar stepping and coordination and consistent toe clearance and predominant paw position is parallel at initial contact and lift off; **20=**Consistent plantar stepping, coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off and trunk instability; **21=**Consistent plantar stepping, coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off and trunk stability.

Materials

Unless otherwise stated, all compounds were obtained from Sigma-Aldrich Company Ltd. (Poole, Dorset, U.K.). Primary anti-PAR antibody was obtained from Alexis (Milan, Italy). Biotin blocking kit, biotin-conjugated goat anti-rabbit IgG, anti PAR antibodies and avidin-biotin peroxidase complex were obtained from DBA (Milan, Italy). Antibodies against IκB-α and IκB-β were purchased from Santa Cruz Biotechnology (Milan, Italy). All other chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline [0.9% (w/v) NaCl; Baxter Healthcare Ltd., Thetford, Norfolk, U.K.].

Statistical evaluation

All values in the figures and text are expressed as mean ± standard error of the mean (SEM) for *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 of at least three experiments performed on 3 different experimental days. Data sets were examined by one- or two-way analysis of variance, and individual group means were then compared with Student's unpaired

t-test. BBB scale data were analyzed by the Mann-Whitney U-test and considered significant if $P<0.05$.

Results

Effect of PARP inhibition on the severity of spinal cord trauma

Twenty four hours after the trauma a significant damage to the spinal cord at the perilesional zone was observed as assessed by the presence of edema as well as an alteration of the white matter (Figure 2b) when compared with spinal cord tissue collected from sham-operated mice (Figure 2a). Notably, a significant protection of the SCI was observed in the tissues collected from the 3-AB treated mice (Figure 2c) and from 5-AIQ treated mice (Figure 2d). In order to evaluate if histological damage to the spinal cord was associated a loss of motor function the modified BBB hind limb locomotor rating scale score was evaluated. While motor function was only slightly impaired in sham mice, animals undergoing SCI had significant deficits in hind limb movement (Figure 3). In contrast, a significant recovery of hind limb motor disturbances was observed in the SCI-operated mice which were treated with the two PARP inhibitors (Figure 3).

Effect of PARP inhibition on apoptosis in spinal cord tissue after trauma

To test whether the tissue damage was associated with cell death by apoptosis, we measured TUNEL-like staining in the perilesional spinal cord tissue. Almost no apoptotic cells were detectable in the spinal cord tissue of sham-operated mice (Figure 4a). At 24 h after the trauma, tissues obtained from SCI-operated mice demonstrated a marked appearance of dark brown apoptotic cells and intercellular apoptotic fragments (Figures 4b, b1) associated with a specific apoptotic morphology characterized by the compaction of chromatin into uniformly dense masses in perinuclear membrane, the formation of apoptotic bodies as well as the membrane blebbing (see particles b2) . In contrast, tissues obtained from SCI-operated mice treated with 3-AB (Figure 4c) or with 5-AIQ (Figure 4c) demonstrated a small number of apoptotic cells or fragments.

Effect of PARP inhibitors on neutrophil infiltration and PARP activation in the spinal cord

SCI in vehicle-treated mice was also characterized by an increase in MPO activity, indicative of neutrophil infiltration into the inflamed tissue (Figure 5). Infiltration of leukocytes into the white matter has been suggested to contribute significantly to the SCI releasing free oxygen and nitrogen radicals, and favoring PARP activation (Cuzzocrea et al., 2002b). In our study, immunohistochemistry for PAR, as an indicator of *in vivo* PARP activation, revealed the occurrence of positive staining for PAR localized in various cells in the grey matter mice subjected to SCI (Figures 6b,7). However, pharmacological inhibition of PARP prevented neutrophil infiltration, as assessed by MPO activity (Figure 5) and reduced PARP activation (Figures 6,7). In comparative experiments, no significant increase of infiltrated neutrophils (Figure 5) as well as a positive staining for PAR (Figures 6 b,7) was observed in sham-operated mice.

Effect of PARP inhibitors on IκB-α degradation and NF-κB translocation

To investigate the cellular mechanisms by which treatment with 3-AB or 5-AIQ may attenuate SCI, we evaluated IκB-α degradation and NF-κB translocation, one of the major transcription factors involved in the signal transduction of inflammation (La Rosa et al., 2004).

The appearance of IκB-α in homogenates of spinal cord tissues was investigated by immunoblot analysis at 4 h after SCI. A basal level of IκB-α was detectable in the homogenated spinal cord tissues from sham-operated mice (Figure 8). IκB-α levels were substantially reduced in the spinal cord of injured mice (Figure 8). PARP inhibition prevented such SCI-mediated IκB-α degradation and the IκB-α band remained unchanged 4 h after SCI in both the 3-AB and 5-AIQ treated mice (Figure 8). To detect NF-κB/DNA binding activity, whole extracts from spinal cord tissue of each mouse was analyzed by EMSA. A low basal level of NF-κB/DNA binding activity was detected in nuclear proteins from tissues of sham-operated mice (Figure 8). The DNA binding activity significantly increased in whole extracts obtained from spinal cord tissues of vehicle-treated

mice 4 h after SCI (Figure 9). Treatment of mice with 3-AB or with 5-AIQ caused a significant inhibition of SCI-induced NF-κB/DNA binding activity as revealed by specific EMSA (Figure 9).

The specificity of NF-κB/DNA binding complex was demonstrated by the complete displacement of NF-κB/DNA binding in the presence of a 50 fold molar excess of unlabeled NF-κB probe (W.T. 50x) in the competition reaction. In contrast a 50 fold molar excess of unlabeled mutated NF-κB probe (Mut. 50x) or Sp-1 oligonucleotide (Sp-1 50x) had no effect on this DNA-binding activity (data not shown).

Discussion

In this report we demonstrate that pharmacological inhibition of PARP exerts beneficial effects in a mouse model of SCI. The main findings of the current study are that treatment with 3-AB or 5-AIQ, two structurally unrelated PARP inhibitors attenuates: (i) the degree of PARP activation in the injured spinal cord, (ii) the infiltration of the injured spinal cord with neutrophils, (iii) cell apoptosis and (iv) spinal cord damage. These protective effects were associated with inhibition of DNA binding of the transcription factor NF-κB in the inflamed spinal cord. Thus, we propose that the anti-inflammatory activity of PARP inhibitors may be mediated, at least in part, by inhibition of the transcription of certain pro-inflammatory mediators, which are regulated by NF-κB. All of these findings support the view that PARP plays a detrimental role in the development and persistence of post-traumatic injury associated with SCI in the mouse.

Spinal cord trauma initiates a sequence of events that lead to secondary neuronal cell damage. While the precise mechanisms responsible remain undefined, several studies have implicated ROS in the secondary neuronal damage of SCI (Xu et al.,2001). Traditionally, oxidants have been considered to exert their effects via a direct toxic action on target cells. However, recent studies have also suggested a contributory role for oxidants in gene induction. NF-κB is a pleiotropic transcription factor activated by low levels of ROS and inhibited by antioxidants (Schwartz et al., 1996). Consensus binding sequences for NF-κB have been identified in the promoter regions of several genes implicated in the pathogenesis of acute and chronic inflammation (Bowie and O'neill, 2000).

In our experimental model of SCI, in agreement with previous reports (Kim et al., 2001; La Rosa et al., 2004), we found that DNA binding activity of NF-κB is increased after spinal cord damage. Therefore, NF-κB DNA binding activity is associated with a significant IκB-α degradation after SCI.

Thus, our data support the well-established hypothesis that NF-κB may represent an important therapeutic target in the treatment of SCI (Bethea et al 1998). In this regard, we have

recently demonstrated that pyrrolidine dithiocarbamate (PDTC), an antioxidant reported to be a potent inhibitor of NF-κB *in vitro,* significantly reduced secondary injury after spinal cord trauma by acting as an NF-κB inhibitor (La Rosa et al., 2004). Our present data show that the amelioration of SCI by pharmacological inhibition of PARP was associated with inhibition of NF-κB activation. Although it is difficult to establish the definitive mechanism by which the PARP inhibitors reduce the DNA binding of these nuclear factors in *in vivo* experiments, our data support the possibility that PARP may be an important modulator of transcription during inflammation.

Moreover, in this study, the transient loss of IkB-α, which occurs in injured spinal cord from SCI-operated mice was prevented by PARP inhibitor treatment which correlated well with the inhibition of NF-κB activation suggesting that PARP inhibitors may also inhibit NF-κB activation via stabilization of IκB-α.

Our results are in agreement with other reports which have clearly demonstrated a role of poly (ADP-ribosyl)ation in signal transduction (Scott et al., 1999; Zingarelli et al., 2003). Recently, it has been demonstrated that PARP-deficient cells are defective in NF-κB-dependent transcriptional activation (Hassa and Hottiger, 1999; Oliver et al., 1999). Similarly, pharmacological inhibitors of PARP abolish mRNA expression of iNOS, interleukin-6 and TNF-α *in vitro* cultured cells (Hauschildt et al., 1997). Furthermore, recently Zingarelli and colleagues have clearly demonstrated that NF-κB DNA binding is completely abolished in heart from PARP-deficient mice subjected to ischemia and reperfusion (Zingarelli et al., 2003), as well as, in the colon from rats subjected to experimental colitis treated with two different PARP inhibitors (Zingarelli et al., 2003). The specific mechanism of PARP activation in regulating transcription needs future studies. Changes in cellular energetics after PARP activation may interfere with calcium sequestration and biosynthetic processes. Poly (ADP-ribosyl)ation may lead to the relaxation of chromatin with the consequence that genes become more accessible to RNA-polymerase (De Murcia et al., 1988).

Numerous binding sequences of NF-κB on various genes with important immunologic functions characterize this transcription factor as a pluripotent factor in the inflammatory response (Xie et al., 1994). Furthermore, the activation of NF-κB is a common end-point of various signal transduction pathways, including the activation of phosphatidylcholine-specific phospholipase C, protein kinase C, protein tyrosine kinases, and mitogen activated protein kinases and other signaling factors (Novogrodsky et al., 1994). Binding of NF-κB to the respective binding sequence on genomic DNA encoding for different pro-inflammatory genes, results in a rapid and effective transcription of these genes (Xie et al., 1994). Another potential mechanism by which PARP inhibition improved secondary damage in our experimental model of SCI is the reduction of neutrophil recruitment into the site of inflammation. Accumulation and activation of inflammatory cells are some of the initial events of tissue injury and are regulated at the transcriptional level. For example, expression of adhesion molecules, such as P-selectin, E-selectin and intercellular adhesion molecule-1 (ICAM-1), is regulated by genes responsive to NF-κB.

Therefore, we may hypothesize, as previously demonstrated (Zingarelli et al., 1998; Cuzzocrea et al., 2002a) that pharmacological inhibition of PARP may also inhibit recruitment of inflammatory cells at the transcription level. The discovery of the concept that PARP regulates neutrophil trafficking may provide new insights in the interpretation of recent reports demonstrating the protective effect of PARP inhibition in experimental models of shock, ischemia-reperfusion injury and inflammation. For instance, there is good evidence that PARP activity (including 3-AB and 5-AIQ used in this study) reduces the upregulation of adhesion molecules (e.g. P-selectin and ICAM-1) in regional myocardial ischemia and reperfusion of the heart (Zingarelli et al., 1998), the gut (Di Paola et al., 2004), the kidney (Chatterjee et al., 1999), as well as in the inflamed lung (Cuzzocrea et al., 2002a). Therefore, recently it has been point out that the ability of PARP inhibitors in reducing infiltration of activated and PAR+ PMNs/monocytes into damaged tissues, may be independent of PARP activity. Consistent with this is the study by Scott and collegues (Scott et al., 2003) showing

that peroxynitrite-induced oligodendrocyte cell death is PARP independent. Recent studies have demonstrated the induction of apoptosis in different cell line in response to ROS, peroxynitrite and nitric oxide (Leist et al., 1997). Apoptosis may occur from several hours to several days after injury in some locations thus the suppression of cell death is clinically relevant. Following SCI, apoptosis appears in the vicinity of the area affected by mechanical contusion, and since the chronological course of cell death is controlled, delayed cell death in spinal white matter (Abe et al., 1999). We demonstrate here that PARP inhibitors inhibits apoptotic cell death in spinal cod tissue from mice subjected to trauma (as determined by Tunnel coloration). Thus, our *in vivo* findings support the view that inhibition of PARP directly protects cells by preventing the activation of the apoptosis pathway. However, the role of PARP in apoptosis remains to be determined since conflicting data have been reported. It has been proposed that preventing PARP activation increased the sensitivity of cells to apoptosis-inducing agents (Pieper et al., 1999). Therefore, PARP undergoes site-specific proteolysis during apoptosis. In this regards, recently it have been clearly demonstrated that PARP mediated DNA repair is initiated in the cortex following experimental brain injury in the rat in the acute posttraumatic period but that subsequent PARP activation does not occur, possibly owing to delayed apoptosis-associated proteolysis, which may impair the repair of damaged DNA (LaPlaca et al., 1999). In addition we clearly demonstrate that the two PARP inhibitors clearly improve the motor function associated with SCI. This evidene is in agreement with recent studies which have clearly point out that the genetic (using PARP deficent mice) or the pharmacological inhibition of PARP improved the motor and memory function after brain trauma as well as the neuropathic pain (Besson et al., 2003a; LaPlaca et a., 2001; Mao et al., 1997). In conclusion, our results indicate that PARP inhibitors have strong anti-inflammatory properties resulting in reduced: 1) PMN infiltration, 2) activation of NF-κB, and 3) the degree of tissue injury.

Finally the important demonstration of the PARP involvement in the neurological consequence of traumatic brain injury (Besson et al., 2003a,b; LaPlaca et a., 2001) as well as in spinal cord injury

(Scott et al., 1999) et to considered PARP as a promising therapeutic target in clinical treatment of brain trauma.

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Legends for Figures

Figure 1. Mice were sacrificed at different time points in order to evaluate the various parameters. n=10 mice from each group for each time point. See Material and Methods for further explanations.

Figure 2. **Effect of PARP inhibitors on histological alteration of the spinal cord tissue 24 h after injury.**

Twenty four hours after the trauma a significant damage to the spinal cord at the perilesional zone was observed as assessed by the presence of edema, as well as an alteration of the white matter (**b**) when compared with spinal cord tissue collected from sham-operated mice (**a**). Notably, a significant protection of the spinal cord injury was observed in the tissue collected from the 3-AB treated mice (**c**) and from 5-AIQ treated mice. This figure is representative of at least 3 experiments performed on different experimental days.

Figure 3. **Effect of PARP inhibitor treatment on the recovery of hind limb motor disturbance after spinal cord injury.** The degree of motor disturbance was assessed every day until 7 days after SCI by Basso, Beattie, and Bresnahan criteria. 3-AB or 5-AIQ treatment significantly reduced the motor disturbance after SCI. Values shown are mean ± s.e.mean of 10 mice for each group. **P*<0.01 *versus* SHAM, °*P*<0.01 *versus* SCI.

Figure 4. A few apoptotic cells were observed in the spinal cord tissue from sham-operated mice (**a**). The number of apoptotic cells (see arrows) increased at 24 h after SCI (**b** see particle **b1**) associated with a specific apoptotic morphology characterized by the compaction of chromatin into uniformly dense masses in perinuclear membrane, the formation of apoptotic bodies as well as the membrane blebbing (see particles **b**2). In contrast, only a few apoptotic cells were seen in spinal cord tissue from 3-AB (**C**) or 5-AIQ (**D**) treated mice. Section **E** demonstrates the positive staining

in the Kit positive control tissue (normal rodent mammary gland). Figure is representative of at least 3 experiments performed on different experimental days.

Figure 5. **Effect of PARP inhibitors on myeloperoxidase activity in the spinal cord 4 h after injury.** Myeloperoxidase (MPO) activity was significantly increased in the spinal cord 4 h after injury. 3-AB or 5-AIQ treatment significantly reduced the SCI-induced increase in MPO activity. Values shown are mean ± s.e.mean of 10 mice for each group. **P*<0.01 *versus* SHAM, °*P*<0.01 *versus* SCI.

Figure 6. **Immunohistochemical localization of PARP activation.** Administration of 3-AB **(c)** or 5-AIQ **(d)** to SCI-induced mice produced a marked reduction in the immunostaining for PAR (indicative of PARP activation) in spinal cord tissue, when compared to positive PAR **(b)** staining obtained from the spinal cord tissue of mice 24 h after the injury. No positive staining was observed in the tissue section from sham-operated mice (**a**). This figure is representative of at least 3 experiments performed on different experimental days.

Figure 7. **Typical Densitometry evaluation**

Densitometry analysis of immunocytochemistry photographs (n=5 photos from each sample collected from all mice in each experimental group) for PAR from spinal cord 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. ND: not detectable. **P*<0.01 *versus* SHAM, °*P*<0.01 *versus* SCI.

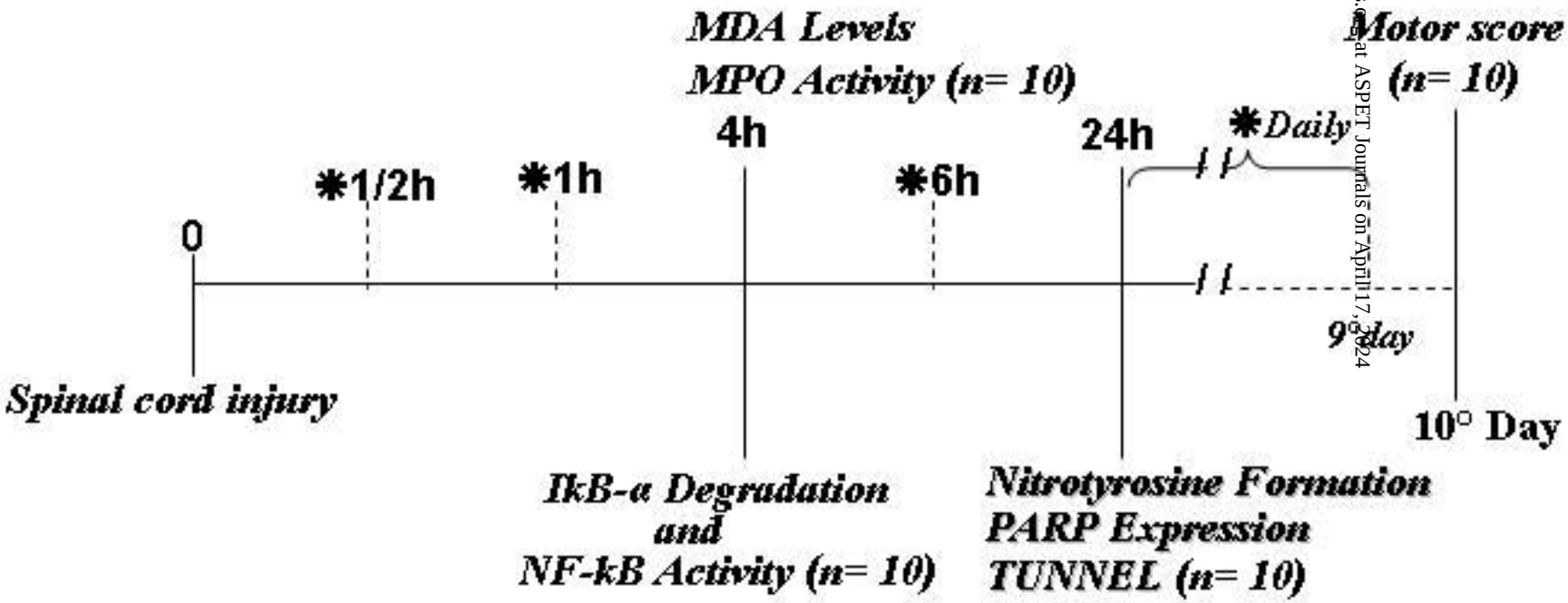
Figure 8. **Representative Western blots of IκB-α degradation (a), as well as the densitometric analysis (b).** Western blot analysis shows the effect of 3-AB or 5-AIQ on degradation of IκB-α in spinal cord tissue collected at 4 h after injury administration. **Sham:** basal level of IκB-α band was

present in the tissue from sham-operated mice. **SCI**: IκB-α band has disappeared in the tissue from spinal cord inured mice. **SCI + 3-AB**: IκB-α band remained unchanged in the tissue from spinal cord inured mice which received 3-AB. **SCI + 5-AIQ**: IκB-α band remained unchanged in the tissue from spinal cord inured mice which received 5-AIQ. Immunoblotting in panel A is representative of one spinal cord out of 10 analyzed. The results in panel B are expressed as mean ± s.e.mean from 10 blots **P*<0.01 *versus* SHAM, °*P*<0.01 *versus* SCI.

Figure 9. **Effect of PARP inhibitors on NF-κB/DNA binding activity in mice spinal cord**

Whole extracts from injured (**SCI**) or non-inflamed (**sham**) mice spinal cord were prepared as described in Materials and Methods and incubated with ^{32}P-labelled NF-κB probe. Representative EMSA of NF-κB shows the effect of 3-AB (**SCI + 3-AB**), as well as the effect of 5-AIQ (**SCI + 5-AIQ**) on NF-□B/DNA binding activity evaluated in spinal cord tissue 4 h after the injury. Data illustrated are from a single experiment and are representative of 3 separate experiments.

Figure 1



* Administration of 3-AB and 5-AIQ

Figure 2

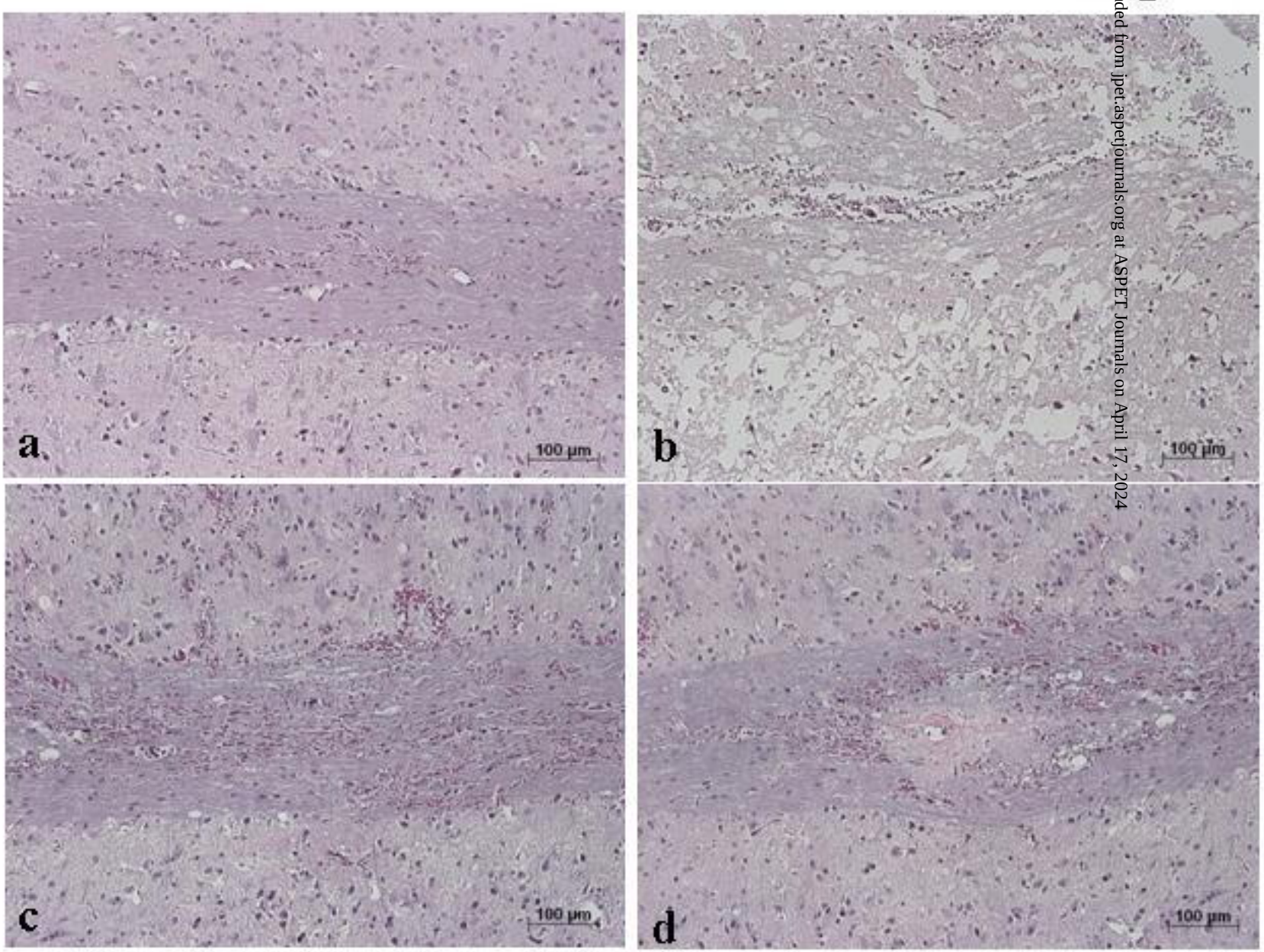
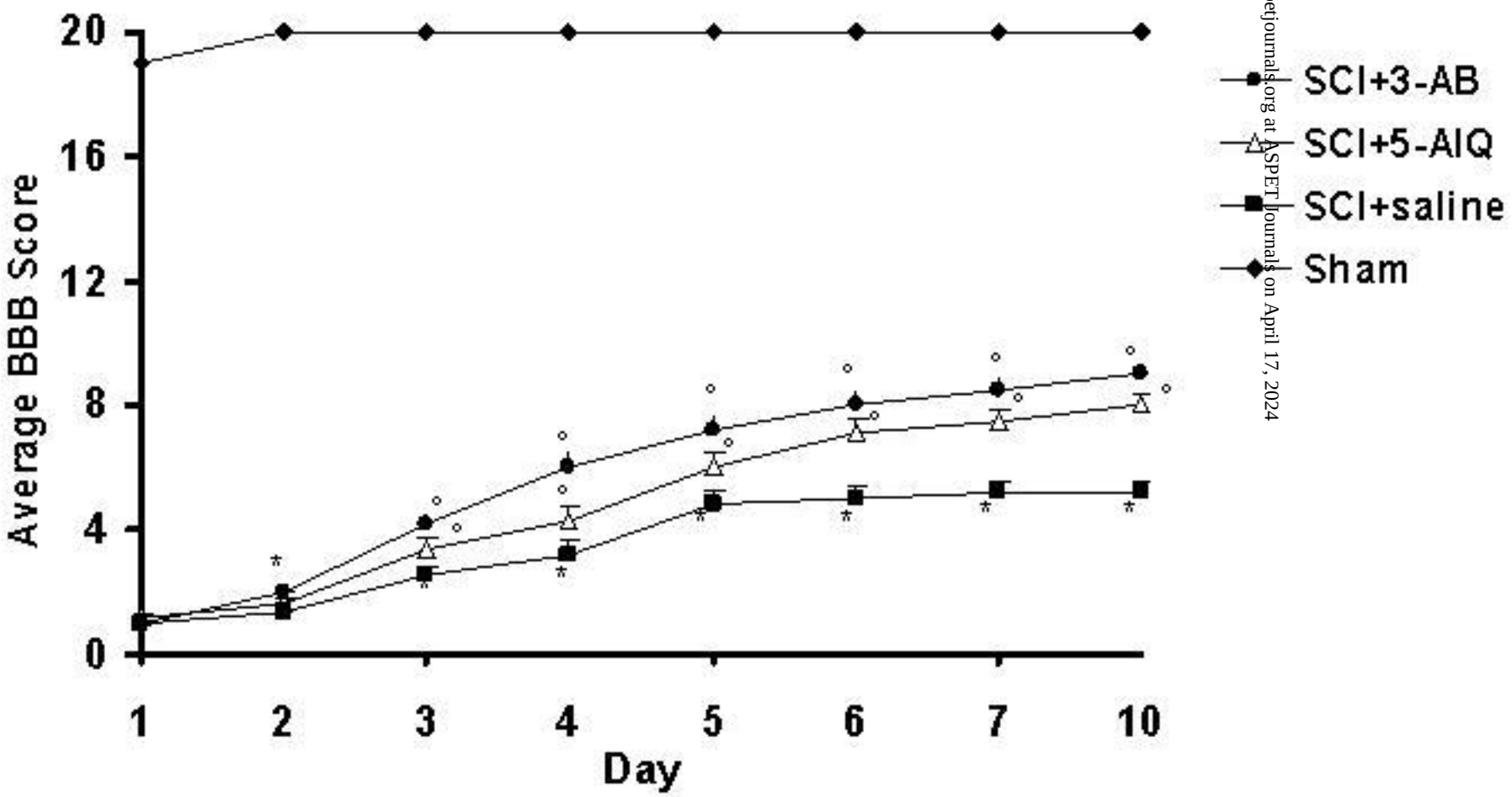


Figure 3



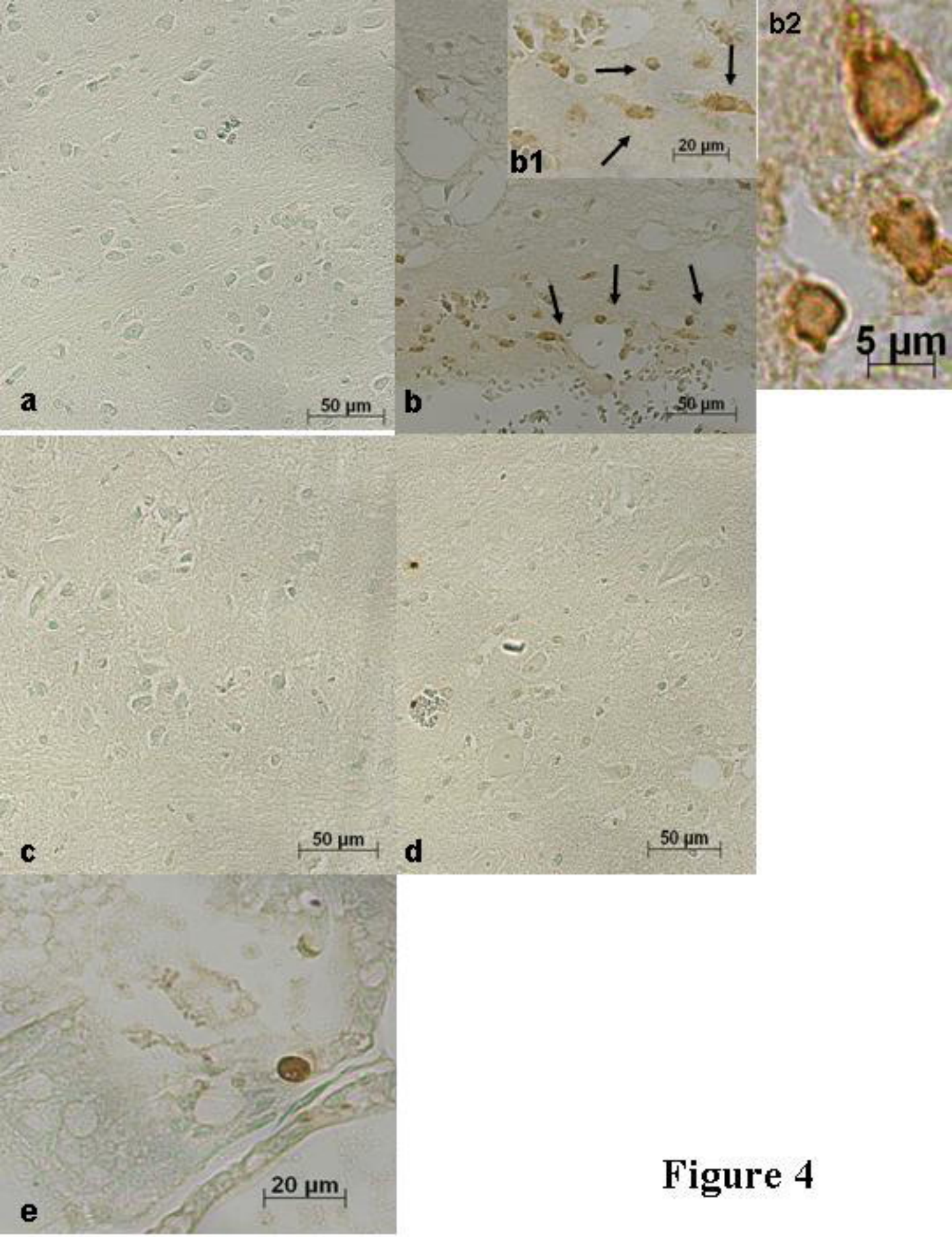


Figure 4

Figure 5

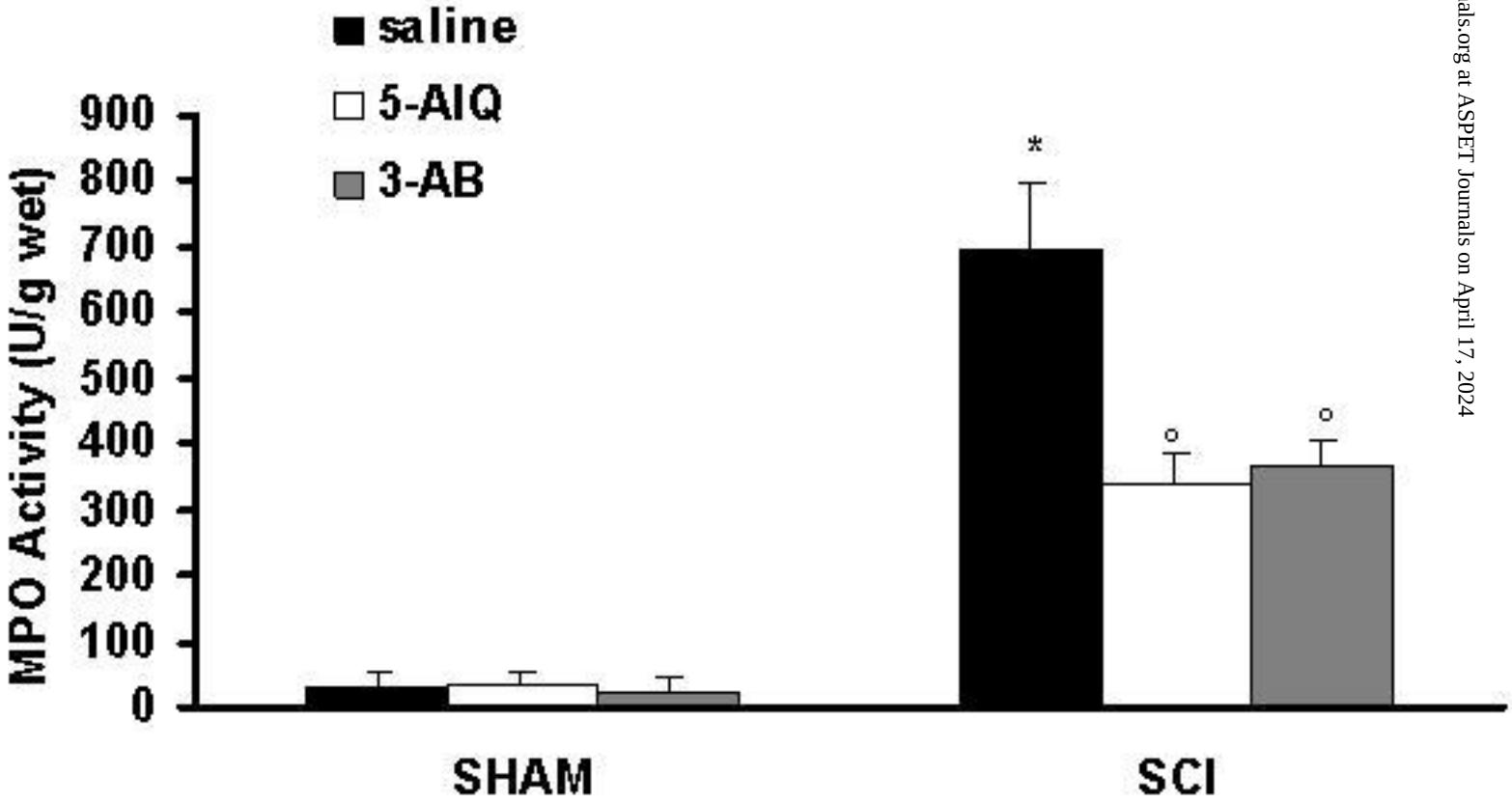


Figure 6

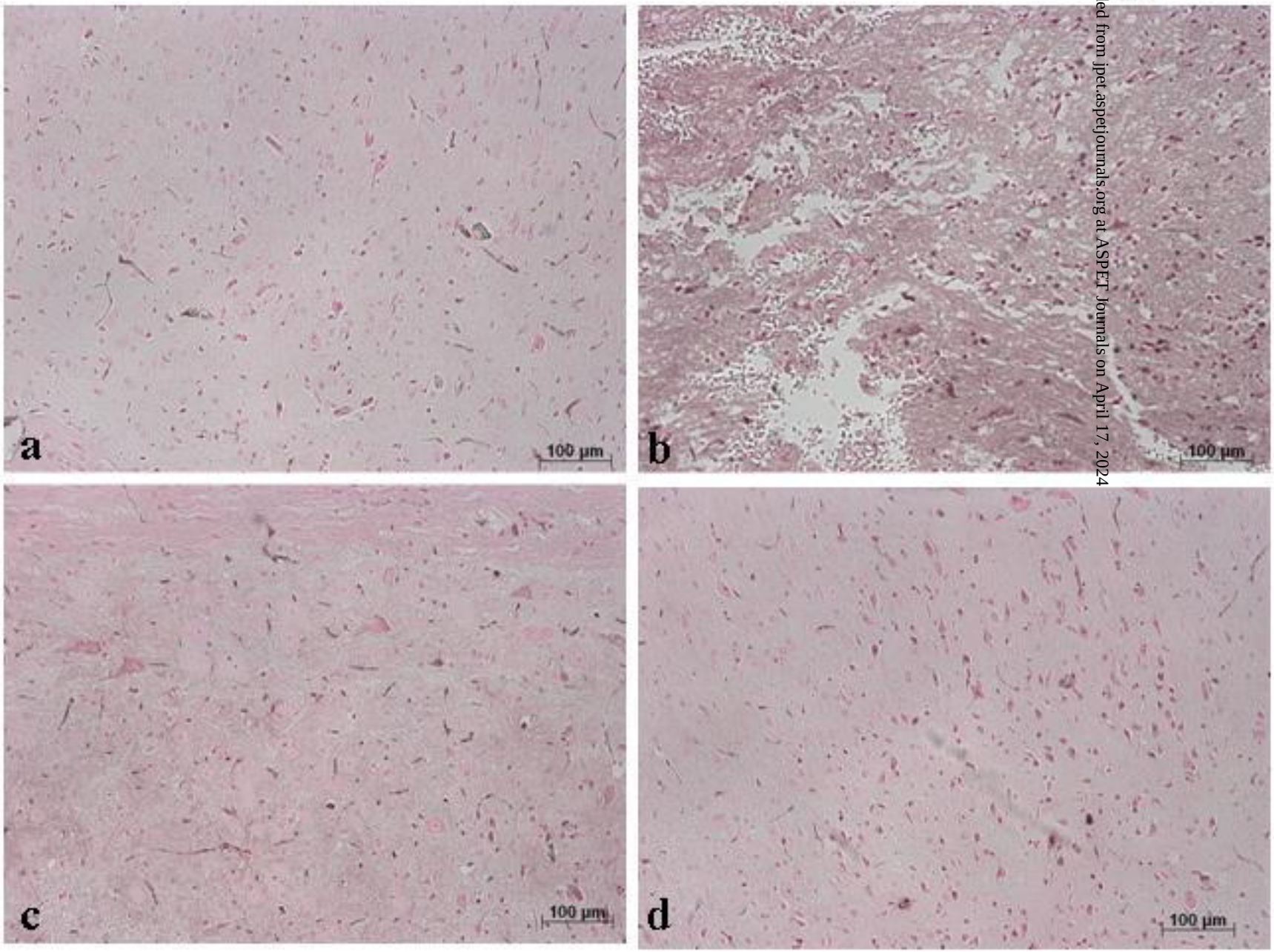


Figure 7

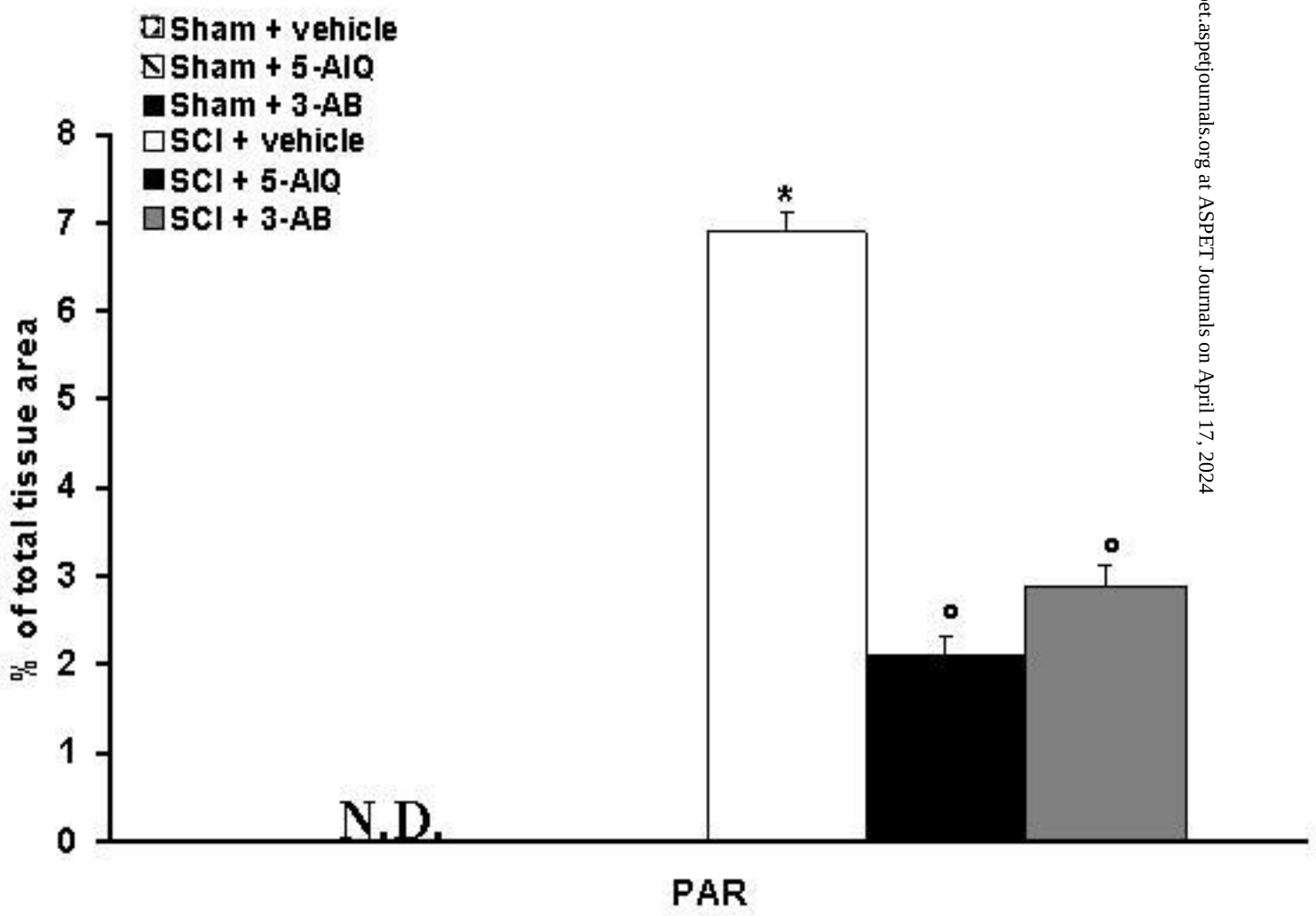


Figure 8

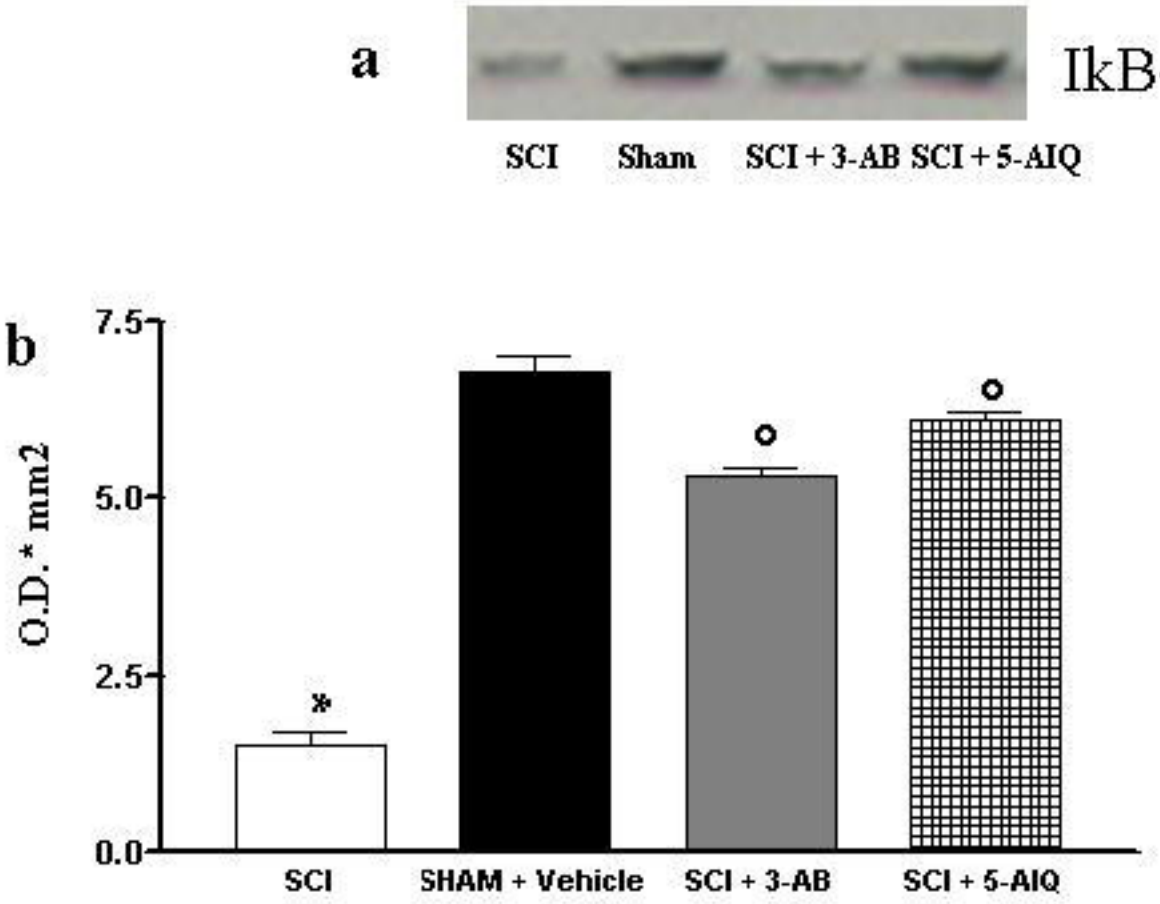


Figure 9