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THE PROTECTIVE ROLE OF MELATONIN IN AN ORGANOTYPIC MODEL OF SPINAL CORD INJURY SECONDARY DAMAGE

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Abstract

Spinal cord injury (SCI) is characterized to be a two-step process composed by the primary lesion consisting of the initial trauma and the secondary damage, characterized by multiple processes including inflammation, oxidative stress and cell death that lead to a significant expansion of the original damage and to an increase of the functional deficit. Among the aforementioned processes, the oxidative stress plays a significant role in pathophysiology of SCI. In this study, we evaluated the role of melatonin, potent antioxidant and immunomodulator indoleamin, on the oxidative stress, the tissue viability and the neuritic plasticity deriving from the gray matter in an experimental model of organotypic cultures. These cultures consisted of Sprague Dawley rat spinal cord slice treated with hydrogen peroxide (H2O2). In five experimental groups, A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm); B) Stressed Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM); C) Control Group treated with melatonin (10-5M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours; D) Treated Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10-5M) for 24 hours; E) Treated Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10-5M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours.

We investigated the slice cellular death by propidium iodide (PI) assay, the slice vitality by MTT assay, the superoxide dismutase (SOD) and total thiols (SH) levels for the contrast to the oxidative stress, the neuronal (NeuN) and the synaptophysin (Syp) immunopositivity. Melatonin significantly decreased the number of dead cells, increased slice vitality, mainly in slices treated before H2O2 exposition. Melatonin enhanced SOD immunopositivity, contrasted total thiols decrease, attenuated Syp reduction and increased NeuN immunopositivity. Overall, these findings suggest that melatonin may exert a potentially beneficial effect upon the progression of SCI secondary damage, protecting the tissue from a further degeneration.

1 INTRODUCTION

1.1 Human Spinal Cord Development

The central nervous system in humans and other vertebrates derives from a specialized epithelium, the *neural plate*, a paramedian zone thickened and elongated of the external germinal sheet, or *ectoderm*. The ectoderm, along the lateral edges of the neural plate, forms bilaterally a ribbon-shaped structure, the *primitive neural crest*, which divides the primitive neural ectoderm from the primitive general somatic ectoderm (Purves et al., 2001). With the growth of the neural plate, its lateral margin raise up to form the neural folds, while its median region goes to form the *neural groove*. In subsequent phases of the development, the neural groove deepen itself and neural folds approach to each other until the merging on the median line, giving rise to the *neural tube*. The transformation of the neural plate in the neural tube is the primary neurulation. The cephalic end of the neural tube loses the cylindrical shape and widens in encephalic vesicles, sketches of the various parts of the brain; the remaining part of the neural tube or rather the tapered caudal region, gives origin to the spinal cord. The neural crest develops into the sensory neurons of the peripheral nervous system and the postganglionic neurons of the autonomic nervous system. The wall of the neural tube consists of neuroepithelial cells, which, after closure of the neural tube, divide and therby increase rapidily in number, and differentiate into neuroblasts. The neuroblast cells accumulate themself around the neuroepithelial layer and form the so called Mantle Layer which later will form the gray matter of the spinal cord. In particular, while the paraxial mesoderm is developing into somites, the more inferior portion of the neural tube differentiates into the ependymal, mantle, and marginal layers of the future spinal cord. The future central spinal cord canal region is surrounded by ependymal layer. Cells of the nervous system will derive from the mantle layer (neurons and gJia), and axons of tract cells will arise from the outer marginal layer of the tube (Cramer and Darby, 2013). The motor areas of the spinal cord will derive from the ventral part of the mantle layer (basal plates). The dorsal part (alar plates) will form the sensory areas. The intermediate layer between the basal and the alar plates contains neurons of the autonomic nervous system. The marginal layer will form the white matter of the spinal cord (Eyre and Clowry, 2002; Jinkins 2000).

1.2 Spinal Cord Anatomy

1.2.1 Gross Anatomy

The spinal cord togheter with the brain is part of the central nervous system (CNS). It occupies the upper two thirds of the vertebral canal extendending itself from the medulla oblongata in the brainstem to the lumbar region of the vertebral column. It begins at the level of the occipital bone (or superior margin of the atlas) until the junction between the first and the second lumbar vertebra (Standring et al., 2012). It is covered by the three membranes of the CNS, i.e., the dura mater, arachnoid and the innermost pia mater and it is around 45 cm (18 in) in men and around 43 cm (17 in) long in women weighing about 30 g. Spinal cord has two enlarged portions: a cervical enlargement (C4 and T1) which receives sensory input from the upper limbs and sends motor output to the upper limbs and a lumbar enlargement (T9 and T12) which receives sensory input from the lower limbs and sends motor output to the lower limbs. A number of fissures and sulci marks the external surface of the spinal cord: an anterior median fissure, a posterior median sulcus, a posterolateral sulcus. The anterior median fissure extends the length of the anterior surface with an average depth of 3 mm. The posterior median sulcus extends along the posterior surface and the posterolateral sulcus is located from 1,5 to 2,5 mm laterally on each side of the posterior median sulcus on the posterior surface marking where the posterior rootles of spinal nerves enter the cord. A fine filament of connective tissue continues inferiorly from the apex of the conus medullaris, the distal end of the cord. Contrary to the brain, in the spinal cord, the grey matter is surrounded by the white matter at its circumference. The gray matter is rich in nerve cell bodies, wich form longitudinal columns along the cord. This one can be divided into the dorsal horn, intermediate grey, ventral horn and a centromedial region surrounding the central canal (central grey matter). It appears with a characteristic butterfly shaped structure that occupies the central portion of the cord. The white matter that sorrounds the gray matter is conventionally divided into the dorsal, dorsolateral, lateral, ventral and ventrolateral funiculi: these are constiuted by the nerve cell processes which form large bundles or tracts that ascend and descend in the cord (For detailed informations about gray and white matter see the next paragraphs). From the spinal cord emerge posterior and anterior roots. The posterior roots contain centripetal processes of neurons located in the dorsal root ganglia that carry sensory informations. The anterior roots are formed by axons of neurons present in anterior and lateral gray spinal columns emerging

with a series of smaller rootlets in a longitudinal area of 3 mm and carrying motor informations from the CNS to perifery. According to its rostrocaudal location the spinal cord can be divided into four parts: cervical, thoracic, lumbar and sacral. Moreover, it can be considered like a segmental structure from which originate 31 spinal cord nerve segments:

8 cervical segments forming 8 pairs of cervical nerves (C1 spinal nerves exit spinal column between occiput and C1 vertebra; C2 nerves exit between posterior arch of C1 vertebra and lamina of C2 vertebra; C3–C8 spinal nerves through IVF above corresponding cervica vertebra, with the exception of C8 pair which exit via intervertebral foramen between C7 and T1 vertebra)

12 thoracic segments forming 12 pairs of thoracic nerves (exit spinal column through intervertebral foramina below corresponding vertebra T1–T12)

5 lumbar segments forming 5 pairs of lumbar nerves (exit spinal column through intervertebral foramina, below corresponding vertebra L1–L5)

5 sacral segments forming 5 pairs of sacral nerves (exit spinal column through intervertebral foramina, below corresponding vertebra S1–S5)

1 coccygeal segments (Standring et al., 2012)

1.2.2 Internal organization

Transversally, the human spinal cord is divided in an incomplete manner into two symmetrical halves with a posterior median solcus and an anterior median fissure. It is mada up by an external portion of white matter and a central portion of gray matter. The amount of gray matter reflects the number of neurons present and is proportionately greater at the level of the cervical and lumbar bulges as there are neurons that innervate the limbs. The amount of white matteri is greater at the cervical part of the cord and decreases progressively to lower levels because the descendants beams lose fibers in the caudal direction and those ascending accumulate in the rostral sense (Standring et al., 2012).

1.2.2.1 Gray matter of the human spinal cord

Three-dimensionally, the gray matter of the spinal cord appears like a fluted column whose morphology in cross-section is often compared to the shape of a butterfly or a letter H. The gray matter consists of four cellular masses connected together, the two anterior horns and the two posterior horns, which project respectively towards the ventrolateral and dorsolateral surface of the spinal cord. The posterior horn is the point at which the primary afferent fibers arrive, entered in the spinal cord through the posterior roots of the spinal nerves. Behind the head of the posterior horn, between this one and the outer surface of the bone, there is the dossier or zone of Lissauer, a quadrangular area of the white matter of the spinal cord, where primary afferent fibers ascend and descend for a short distance before end in the adjacent gray matter. The anterior horn instead, contains efferent neurons whose axons lead in the anterior roots of the spinal nerves. The gray matter of the spinal cord is a complex set of cellular bodies of neurons, of neuronal processes and synapses, neuroglia and blood vessels. Gray matter neurons are multipolar and vary for dimensions, lenght and axons and dendritic disposition; some are intrasegmental (they are inside the same neuromere, others are intersegmental if their ramifications go along more neuromeri. In the gray matter of the human spinal cord, cell clusters associated with spinal nerves can be distinguished. Moreover, a pattern of lamination is exhibited within the gray matter and this is due to the various sizes and shapes of neurons. Bror Rexed proposed a classification based on 10 layers (laminae of Rexed). These laminae are numbered in a posterior-anterior order and are distinguished on the base of the morphology, size, cytologic features and density of neuronal cells. Laminae from I to IV, in general, are concerned with exteroceptive sensation and comprise the dorsal horn, whereas laminae V and VI are concerned primarily with proprioceptive sensations. Lamina VII is equivalent to the intermediate zone and acts as a relay between muscle spindle to midbrain and cerebellum, and laminae VIII-IX comprise the ventral horn and contain mainly motor neurons. The axons of these neurons innervate mainly skeletal muscle. Lamina X surrounds the central canal and contains neuroglia (Standring et al., 2012).

Laminae I-IV are located in the dorsal part of the posterior horn and represent the main point of termination of primary afferent coutaneous fibers and of their collateral ones. The Rexed Lamina I (lamina marginalis) consists of a thin layer of cells that cap the tip of the dorsal horn and is reticular aspect is due to complex array of big and small nervous fibers. The Rexed Lamina

II, bigger than the first lamina, has small neurons densely grouped. It is distinguishable for the total absence of myelinated fibers. This lamina matches with substantia gelatinaosa (of Rolando). Some authors believe that substantia gelatinosa contains in whole or in part the lamina III. The Rexed Lamina III is formed by bigger and more variable less densely grouped cells with respect to lamina II and contains also more myelinated fibers. The nucleus proprius of the posterior horn corresponds with some cellular groups inside laminae III and IV. The Rexed Lamina IV is the thickest of the first four laminae, loosely organized and heterogeneous and appears crossed by fibers.

Laminae V and VI are at the base of the posterior horn and receive the majority of the primary proprioceptive afferent fibers, widespread projections from the motor and sensory cortex as well as stimuli from subcortical levels. The Rexed Lamina V is thick and covers a broad zone extending across the neck of the dorsal horn and is divided into one lateral third and two medial thirds. Both portions have a mixed cellular population with more prominent cells in the lateral one. The Rexed Lamina VI is a broad layer which is best developed in the cervical and lumbar enlargements and is divided also into one medial third and two lateral thirds. In the two lateral thirds there are bigger cells but more loosely organized with respect to the medial part. Laminae VII-IX vary greatly at the level of cervical and lumbar enlargements. The Rexed Lamina VII includes most of the intermediate horn (lateral) and contains prominent nuclei of Clarke column (dorsal column or thoracic column) and intermediomedial and intermediolateral cellular groups. It occupies a large heterogeneous region. Its shape and boundaries vary along the length of the cord. The Rexed Lamina VIII includes an area at the base of the ventral horn, but its shape differs at various cord levels. In the cord enlargements, the lamina occupies only the medial part of the ventral horn. It's an accumulation of propriospinal neurons receiving fibers from the adjacent laminae and descending connections from interstitiospinal, vestibulospinal, reticulospinal and medial longitudinal fasciculus. The Rexed Lamina IX is a group of α and γ motoneurons and several interneurons. Its size and shape differ at various cord levels. In the cord enlargements the number of α motor neurons increase and they form numerous groups. The α motor neurons are large and multipolar cells and give rise to ventral root fibers to supply extrafusal skeletal muscle fibers, while the small γ motor neurons give rise to the intrafusal muscle fibers. The α motor neurons are somatotopically organized. The Rexed Lamina X sorround the central ependymal canal and is formed by gray anterior and posterior commisures (Standring et al., 2012).

1.2.2.2 White matter of the human spinal cord

Around the gray matter there is the white matter containing myelinated and unmyelinated nerve fibers for the conduction of information up (ascending) and down (descending) the cord. Generally we can distinguish three types of fibers in the white matter: long ascending nerve fibers from the column cells making synaptic connections to neurons in various brainstem nuclei, cerebellum and dorsal thalamus; long descending nerve fibers from the cerebral cortex and various brainstem nuclei to make synapses within the different Rexed laminae of the spinal cord gray matter; the shorter nerve fibers interconnecting various spinal cord levels such as for example the fibers responsible for the coordination of flexor reflexes. More precisely the white matter is divided into the dorsal (or posterior) column (or funiculus), lateral column and ventral (or anterior) column, each of which containing axonal tracts related to a specific function. The dorsal columns (also called posterior funiculi), which are the major ascending tracts in the spinal cord carrying mechanosensory information from the first-order sensory neurons in dorsal root ganglia to the dorsal column nuclei, are important for well-localized fine touch and conscious proprioception. This portion is made up of the gracile fasciculus (lower body sensory impulses) and the cuneate fasciculus (upper body sensory impulses). After reaching their respective nuclei in the medulla oblongata and after the crossing over as the internal arcuate fibers, they take the name of the medial lemniscus, so we can talk about the posterior column-medial lemniscus pathway (Purves et al., 2001). The lateral regions of spinal cord white matter convey motor information from the brain to the spinal cord. The lateral colums infact include axons that travel from the cerebral cortex to contact spinal motor neurons. These pathways are also referred to as the cortico-spinal tracts. Moreover, there is also the rubrospinal tract which is one of the several major motor control pathways. Going from lateral to ventral we have the neospinothalamic tract (or lateral spinothalamic tract) which is located more laterally, and carries pain, temperature and touch information from somatic and visceral structures. The dorsal and ventral spinocerebellar tracts carry unconscious proprioception information from muscles and joints of the lower extremity to the cerebellum. The ventral (and ventrolateral or anterolateral) columns carry both ascending information about pain and temperature, and descending motor information (reticulospinal, vestibulospinal, olivospinal and the anterior corticospinal tract). In the ventral column (funiculus) there are four principal tracts: the paleospinothalamic tract (or anterior spinothalamic tract) carries pain, temperature, and information associated with touch to the brain stem nuclei and to the diencephalon; the

spinolivary tract carries information from Golgi tendon organs to the cerebellum; the spinoreticular tract and the spino-tectal tract. There are also intersegmental nerve fibers that travel for several segments and are located as a thin layer around the gray matter; these are the fasciculus proprius, the spinospinal or archispinothalamic tract (Standring et al., 2012).

1.3 Spinal Cord Injury

Spinal Cord Injury (SCI) is a catastrophic event consisting in a damage to the spinal cord that results in a change, either temporary or permanent, in the cord's normal motor, sensory, or autonomic function with strong physical, psychological and social well-being impact for the patient. It can be caused by different reasons: the most common are trauma (car accident, gunshot, falls, sports injuries, etc) or diseases and degenerations (transverse myelitis, polio, spina bifida, Friedreich's ataxia, tumor). A trauma causes fracturation of the vertrebral column with possible dislocation of bone fragments and consecutive contusion/compression of the spinal cord, which leads to the irreversible loss of function since the spinal cord, as all Central Nervous System (CNS) has limited capacity to self-renew: injury of the adult CNS, infact, is devasting because of the inability of central neurons to regenerate correct axonal and dendritic connections. Much of the prognosis and recovery depend on the type, the location and severity of the injury. Motor control and sensory input are lost below the level of the lesion (Barnabe-Heider and Frisen 2008) with long lasting consequences. Depending on lesion features, subjects have different types of symptoms and effects: loss of voluntary movement, appearance of involuntary muscle spasm, loss of sensation, balance, loss of control of breathing, autonomic functions, bladder, sexual and bowel control (Raisman 2001). People with a SCI are two to five times more likely to die prematurely than people without a SCI, with worse survival rates in low- and middle-income countries. If in the past SCI was a mortal condition (during the First World War 90% of patients who suffered a spinal cord injury died within one year of wounding and only about 1% survived more than 20 years). In the present, the better understanding and management of SCIs have led to a reduction in mortality and a higher incidence of incomplete spinal cord damage in those who survive (Grundy and Swain, 2002). SCI is associated with lower rates of school enrollment and economic participation, and it carries substantial individual and societal costs.

1.4 Epidemiology of Spinal Cord Injury

In the media center regarding Spinal Cord Injury of World Health Organization website it's reported that every year, around the world, between 250.000 and 500.000 people suffer a SCI. Data of global prevalence are difficult to estimate, but estimated annual global incidence is between 40 to 80 cases per million population. Males are most at risk in young adulthood (20-29 years) and older age (70+). For females risk is major in adolescence (15-19) and older age (60+). Singh (Singh et al., 2014) refers that the incidence, the prevalence and causation of SCI differs between developing and developed countries. The increase of aging in westernized countries can lead to an increasing public health problem. The prevalence of SCI is highest in the United States of America with 906 per million and lowest in Rhone-Alpes region, France (250 per million) and Helsinki, Finland (280 per million). Annual incidences are above 50 per million in Hualien County in Taiwan, in the central Portugal region (58 per million) and in Minnesota (54.8 per million) and are lower than 20 per million in Taipei, Taiwan (14.6 per million), the Rhone-Alpes region in France (12.7 per million), Aragon, Spain (12.1 per million), Southeast Turkey (16.9 per million) and Stockolm, Sweden (19.5 per million). The highest national incidence was 49.1 per million in New Zealand and the lowest incidence were in Fiji (10.0 per million) and Spain (8.0 per million) (Singh et al., 2014). Following data of National Spinal Cord Injury Statistical Center (NSCISC – Annual Statistical Report 2014) it is estimated that in the United States of America, regarding gender, the 80.7 percent of all reported SCI occurred among males. The most common age of injury was 19 years. A quarter of all injuries occurred between the ages of 17 and 22 years (24.3%), and nearly half of all injuries occurred between the ages of 16 and 30 (48.9%) while 10.7 percent occurred at age 60 or older. Mean age was 34.7 years. Considering etiology for males and females, the incidence rate in developing countries is much lower compared to that in developed countries (Chiu et al. 2010) and the three leading causes of SCI were the same: auto accidents, falls and gunshot wounds. Among males, diving accidents ranked fourth followed by motorcycle accidents. However, for females, medical/surgical complications ranked fourth and motorcycle accidents ranked fifth. Significant gender differences are evident in five etiologies: auto accidents (29.3% for males, 48.3% for females); motorcycle accidents (6.9% males, 2.5% females); diving accidents (7.0% males, 2.1% females); hit by falling objects (3.3% males, 0.6% females) and medical/surgical complications (2.2% male, 5.1% females) (NSCISC – Annual Statistical Report 2014). Regarding the level of injury overall, 53.9 percent of patients had cervical lesions, 35.2 percent had

thoracic lesions, and 10.5 percent had lumbar lesions and 0.4 percent had sacral lesions. Close to half (45.6%) of the patients in the database were discharged with cervical lesions at C4 (14.8%), C5 (15.3%), C6 (10.4%) or C7 (5.1%). The next most common levels of lesion at discharge was T12 (6.3%) and L01 (4.9%) (NSCISC – Annual Statistical Report 2014). Considering causes of death among the deceased patients, it's reported the leading ones are the diseases of the respiratory system (67.4% of these were cases of pneumonia). The second one consists in infective and parasitic diseases: usally cases of septicemia (89.2%) associated with decubitus ulcers, urinary tract or respiratory infections. Cancer ranks third followed by hypertensive and ischemic heart disease. Afterwords there are unintentional injuries (disease of the digestive system, cerebrovascular and pulmonary diseases), than suicide and diseases of genitourinary system (NSCISC – Annual Statistical Report 2014).

1.5 Classification of Spinal Cord Injury

In general terms injury can be classified neurologically as ‘’complete’’ or ‘’incomplete’’ following the sacral sparing definition. If there is the presence of sensory or motor function in the most caudal sacral segments it is possible to refer to a ‘’sacral sparing’’ (i.e. preservation of light touch or pin prick sensation at the S4-5 dermatome, DAP or voluntary anal sphincter contraction). If there is an absence of sacral sparing (i.e. sensory and motor function in the lowest sacral segments, S4-5) a complete injury is present. If there is the presence of sacral sparing (i.e. some preservation of sensory and/or motor function at S4-5) there is an incomplete injury (Kirshblum et al., 2011). However, SCI can be classified according to the American Spinal Injury Association (ASIA) Classification, as a ASIA A, B, C, or D. In 1982 was published for the first time an international classification on spinal cord injury by the American Spinal Injury Association (ASIA) called International Standards for Neurological Classification of Spinal Cord Injury (ISNCSCI). Today ISNCSCI is still widely used to document sensory and motor impairment after SCI (Marino et al., 2010 ; 2003). The type of impairment is based on neurological responses, touch and pinprick sensations tested in each dermatome, and strength of the muscles that control ten key motions on both sides of the body, including hip flexion (L2), shoulder shrug (C4), elbow flexion (C5), wrist extension (C6), and elbow extension (C7). The five categories of the ASIA Impairment Scale are below described :

A = Complete. No sensory or motor function is preserved in the sacral segments S4-5.

B = Sensory Incomplete. Sensory but not motor function is preserved below the neurological level and includes the sacral segments S4-5 (light touch or pin prick at S4-5 or deep anal pressure) AND no motor function is preserved more than three levels below the motor level on either side of the body.

C = Motor Incomplete. Motor function is preserved below the neurological level, and more than half of key muscle functions below the neurological level of injury (NLI) have a muscle grade less than 3 (Grades 0-2).

D = Motor Incomplete. Motor function is preserved below the neurological level**, and at least half (half or more) of key muscle functions below the NLI have a muscle grade > 3.

E = Normal. If sensation and motor function as tested with the ISNCSCI are graded as normal in all segments, and the patient had prior deficits, then the AIS grade is E. Someone without an initial SCI does not receive an AIS grade.

** For an individual to receive a grade of C or D, i.e. motor incomplete status, they must have either (1) voluntary anal sphincter contraction or (2) sacral sensory sparing with sparing of motor function more than three levels below the motor level for that side of the body. The International Standards at this time allows even non-key muscle function more than 3 levels below the motor level to be used in determining motor incomplete status (AIS B versus C).

1.6 Spinal Cord Injury Pathophysiology

The pathophysiology of acute spinal cord injury (SCI) involves primary and secondary mechanisms of injury. Primary injury consists in the initial mechanical damage while secondary injury it is characterized by secondary changes due to vascular, biochemical and cellular events (Oymbo, 2011).

1.6.1 Primary injury mechanisms

The initial trauma causes the primary injury. It happens unexpectly and can be attributed to four morphological types: impact with persistent compression, impact alone with transient compression, distraction and laceration or transection (Oyinbo 2011). But what's the meaning of a primary injury? The initial impact is not a static damaging moment, because in addition to the resident cells, the injury causes delayed damage to the surviving cells. The initial impact leads to immediate haemorrhage and rapid cell death at the site of injury followed by a cascade of secondary events causing further tissue loss and dysfunction. This phase is very short because the secondary damage phase starts within seconds of the injury with local and systemic events. The spinal cord injury can be classified also on the base of the tissue biological response to the damage: so the temporal sequence is distinguished in acute (seconds to minutes after the injury), secondary (minutes to weeks after the injury), and chronic (months to years after the injury) (Oyinbo 2011). The primary injury mechanisms belong to the acute phase and are characterized by systemic hypotension, spinal shock, vasospasm, ischemia, plasma membrane compromise, derangements in ionic homeostasis, and accumulation of neurotransmitters (Tator et al. 1998, Hulsebosch 2002).

1.6.2 Secondary injury mechanisms

The initial trauma, or primary injury to the spinal cord, starts a sequence of pathological events collectively referred to as secondary injury. The secondary damage is mediated by multiple injury processes including inflammation, autoimmune response, vascular events, apoptosis, free radical-induced cell death, and glutamate excitotoxicity (Akakin et al., 2013). This process begins after few seconds from the primary injury and continues for several weeks leading to an expansion of the tissue destruction (Wilson et al., 2013). The secondary injury process involves

swelling and hemorrhage, which leads to increased free radicals and decreased blood flow, causing even greater cell membrane dysfunction and cell death. More precisely Oyinbo (Oyinbo et al., 2011) well describes how there are approximately 25 well-established secondary injury mechanisms in SCI (Ramer et al. 2005; Tator 1998): apoptosis, astroglial scar launch, calcium influx in cells, central cavitation, central chromatolysis, compression and vertebral column instability, conduction block and spinal shock due to leakage of fast K+ into the ECF, deficient expression of myelin associated genes after SCI, demyelination of residual axons and demyelination of subpial rim of surviving axons, derangements in ionic homeostasis, energy failure and decreased ATP production, excessive noradrenaline secretion, fluid accumulation / oedema at the lesion site, glutamatergic excitotoxicity, haemorrhage, immune cells invasion and release of cytokines, inflammation, ischemia / reperfusion-induced endothelial damage, lipid peroxidation / oxidative stress, neurite growth-inhibitory factors e.g., Nogo-A, Rho-A, oligodendrocyte myelin glycoprotein (OMgp) myelin- associated glycoprotein (MAG), and chondroitin sulfate proteoglycans, neurogenic shock, nitrous oxide excess, oligodendrocytes to secondary apoptotic death, plasma membrane compromise / increases in plasma membrane permeability, systemic hypotension due to sympathetic loss, TNF-α production at the site of SCI, vasospasm and microcirculatory inconsistencies. All these events contribute to enlarge the area of trauma even exacerbating the consequent functional deficits. After this phase occours the chronic injury phase which refers to a time when the spinal cord anatomy has been irreversibly changed. At the center of lesion continues apoptosis with a massive tissue degeneration that leads to the development of a cystic lesion cavity. The activation of microglia and the continuous invasion of macrophages and fibroblasts causes the formation of the glial scar tissue (Kao et al., 1977).

1.7 Oxidative stress

The oxidative stress could be defined as an imbalance between the presence of high levels of reactive oxygen species (ROS), reactive nitrogen species (RNS), and the biological system's ability to readily detoxify the reactive intermediates through its antioxidative defense mechanisms (Fatima et al., 2015). These alterations in the normal redox state can damage all cellular components including lipids, proteins and DNA. The primary toxic element to consider for the oxidative stress is the diffusion of free radicals, in particular peroxides and reactive oxygen species [O2− (superoxide radical), OH (hydroxyl radical) and H2O2 (hydrogen peroxide)]. The levels of oxidative stress strongly increase during the onset of SCI and during the first week (Margaritis et al., 2009): ROS are considered a key contributor to the posttraumatic oxidative damage. The intracellular calcium ions (Ca^{++}) accumulation is very important because impairs mitochondrial function creating dysfunctions in the electron transport chain and this means an increase of ROS production (Sullivan et al., 1999). Even reactive nitrogen species are important for the disruption of normal cellular mechanisms. Reactive nitrogen species (RNS) have a significant role in the pathogenesis of many diseases: nitric oxide (NO), infact, generated by the enzyme inducible NO synthase, is one of the most important and widely studied reactive nitrogen species (Fatima et al., 2015). For example, for Hall (Hall 2011), in the injured spinal cord the reactive nitrogen species peroxynitrite (PN) and its highly reactive free radicals are key initiators of lipid peroxidation and protein nitration. Moreover, regarding NO, Estevez (Estevez et al., 1998) describes as an excessive NO production has cytotoxic effects and induces neuronal apoptosis secondary to neural degeneration and neurodysfunction. To counteract these mechanisms there are many protective systems consisting of antioxidant molecules: superoxide dismutase (SOD), glutathione (GSH), peroxidase, glutathione reductase, thioredoxin, thiols and disulfide bonding are the 'buffering systems' in every cell (Fatima et al., 2015). These substances are able to neutralize the ROS species, thank to their scavenging activity and therefore have a fundamental role in the protection of damaged nervous cells. Recent studies, infact, have shown that oxidative stress may have an important role in the pathophysiology of SCI and in particular it may play an important role in the secondary injury: the diffusion of free radicals has been proposed to be a key contributor to the central nervous system (CNS) posttraumatic oxidative damage because it changes negatively protein structure, DNA, and cell membrane (Naseem and Parvez, 2014) leding to a progression of the initial injury.

1.8 Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is the major secretory product of the pineal gland which is principally associated with the regulation of the circadian rhythms (Reiter et al., 1995; Rezzani et al., 2014). In the pineal gland, melatonin is synthesized by pinealocytes and this is the main source of melatonin in the mammal blood and central nervous system with peaks during the night. Melatonin is produced also by other tissues and organs as the retina, Harderian gland, gut, bone marrow, platelets, astrocytes, glial cells, lymphocytes, pancreas, kidneys, and skin. It was isolated by Lerner and his coworkers in 1956 in the extract of bovine pineal tissue with skin lightening properties (Reiter et al., 2007; Zawilska et al., 2009; Naseem and Prvez, 2014). The biosynthesis of melatonin begins with the acetylation of serotonin by N-acetyltransferase producing N-acetylserotonin, which is then methylated by hydroxyindole-O-methyltransferase to form melatonin. Its activity is mediated by two high affinity specific receptors located in the cellular membrane, MT1 (or Mel1A or MTNR1A) and MT2 (or Mel1B or MTNR1B) and two nuclear receptors, RZR/ROR. An additional melatonin receptor subtype MT3 (or Mel1C or MTNR1C) has been identified in amphibia and birds (Sugden et al., 2004). The membrane receptors MT1 and MT2 belong to the seven-transmembrane G protein-coupled receptor (GPCR) superfamily and are primarily found in the mammalian hypotalamus suprachiasmatic nucleus which represents the endogenous circadian master clock. These receptors are also expressed in other organs partecipating in several physiological and neuroendocrine processes. Nuclear melatonin signaling is mediated via the transcription factor RZR/ROR, which is an orphan member of the nuclear receptor superfamily. Within this superfamily, the two subtypes of retinoid Z receptor (RZR α and β) and the three splicing variants of retinoid orphan receptor (ROR α1, α2, and α3) form a subfamily (Steinhilber et al., 1995). RZR/ROR binds as a monomer to DNA, but also forms homodimers on appropriate binding sites. In the regulatory regions of many genes, RZR/ROR binding sites have been identified. 5-lipoxygenase, p21WAF1/CIP1, apolipoprotein A-1, N-myc and Purkinje cell protein 2 may be functionally important target genes (Wiesenberg et al., 1998). Melatonin is also recognized as a potent antioxidant and immunomodulatory substance: it has been successfully used as a potent antioxidant molecule against many pathophysiological states (Rezzani et al., 2005; Favero et al., 2014;). The cytoprotective capacity of melatonin is, in part, derived from its high radical direct scavenging activity, which reduces oxidative damage, contributes to its anti-inflammatory properties and stimulates the activities of several antioxidative enzymes

(Reiter et al., 2010; Akakin et al., 2013). Moreover, it has been shown that these radical-scavenging properties related to melatonin have neuroprotective effects in the CNS (Domìnguez-Alonso et al., 2011). As well described by Hardeland (Hardeland et al., 2009) and Esposito (Esposito et al., 2010) melatonin under oxidation reactions results in the formation of cyclic 3-hydroxymelatonin (C3-OHM). This compound works as a radical scavenger and scavenges two ·OH resulting into the formation of N1-acetyl-N2-formyl- 5-methoxykynuramine (AFMK) (Naseem and Prvez, 2014). But AFMK is also formed directly from the melatonin when the melatonin itself reacts with H2O2. Moreover, AMK (N1-acetyl-5-methoxykynuramine) is considered a potent free radical scavenger of nitric oxide (NO) compared to its precursor AFMK which is formed from AFMK via pyrrole ring cleavage (Esposito et al., 2010). One important feature is that this molecule is both lipophilic and hydrophilic, therefore it can have effects not only in every cell but also within every subcellular compartment (Reiter et al., 1995). Moreover, among the different morphophysiological barriers melatonin is also able to pass readily through the blood-brain-barrier and to accumulate in the central nervous system at substantially higher levels than exist in the blood (Tan, 2010). Therefore, also in this compart it cold potentially exhibit the strong protective effects, especially under the conditions of elevated oxidative stress or intensive neural inflammation. In the nervous system melatonin for example shows antinflammatory properties decreasing proinflammatory cytokines (Ritz and Hausmann 2008), regulates mitogen-activated protein kinases (MAPKs) (Scheff et al., 2013; Ji et al., 2009), regulates nitric oxide synthase and reduces oxidative stress (Reiter et al., 2010). Different molecules have been used for the treatment of spinal cord oxidative stress: these include methylene blue, mexilitine, thiopental, β-glucan, cyclosporine A, erythropoietin and α-lipoic acid (Toklu et al., 2010). They have different properties because some are true chemical antioxidants (methylene blue, edaravone, α-lipoic acid), others induce indirectly endogenous antioxidant defenses (for example, N-acetylcysteine), others reduce the mitochondrial dysfunction and its associated free-radical leakage (cyclosporine A) or improve of cell membrane repair mechanisms (polyethylene glycol). Because the scientific research wants to find new molecules able to limit the oxidative damages, melatonin, due to its features, may be a good candidate for the treatment of the spinal cord secondary damage.

2 AIM

Therefore, the aim of this study was to evaluate in vitro the melatonin treatment of the secondary damage in spinal cord injuries.

In an experimental model of organotypic cultures of rat spinal cord slices it was evaluated the effect of melatonin administration on oxidative stress, on slice vitality and on neuritic plasticity deriving from the gray matter, before and after the treatment with hydrogen peroxide (H2O2).

The H2O2 was used to experimentally mimic homogeneously on the entire slice the oxidative stress typical of the secondary damage and subsequent to the primary lesion. Moreover, we wanted to consider the mechanisms by which melatonin acts on SCI because this could improve the role of this molecule in the scientific field.

3 MATERIAL AND METHODS

3.1 Animals

Three- to six-week-old rats (Charles River Italy) were used for preparation of organotypic spinal cord slices cultures.

3.2 Organotypic cultures

Rats were sacrificed by decapitation and the spinal cord was aseptically extracted and placed in ice-cold dissecting medium. In particular, after the sacrifice, during the dissection procedure the skin surface was sprayed with 95% ethanol and working under a laminar flow hood a dorsal midline incision was made using a scalpel. After the retraction of skin flaps and the back and neck muscles dorsal dissection, the spinal column was exposed. The spinal column was dissected in a Petri with ice-cold dissecting medium. The spinal cord was dissected opening ventrally the vertebral column and being very careful to not damage the nervous tissue during the extraction procedure. A midline incision was made along the length of the spine using fine microdissecting scissors. In this part of setting, it's very important working with cold temperatures using cold media to maintain a good preservation of the nervous tissue. A 1.5-2.0 cm length of spinal cord was rapidly dissected out from the thoracolumbar region and placed in ice-cold working solution (pH 7.2) (96% of HBSS/HEPES - Ca2+ and Mg2+ free - 2 mmol/L L-glutamine, 5 mg/ml glucose, 1%, 0.4% penicilin-streptomycin). The cord was transferred onto the chopping plate of a pre-set McIlwain tissue chopper and sliced in the trasversal plane (350 µm thickness): ten to twenty slices could be derived from each spinal cord. Slices were transposed to a Petri-dish containing new ice-cold working solution. Meanwhile, slice culture inserts (Millicell-CM membranes) in six-well plates were pre-equilibrated with 1.1 ml of culture medium prewarmed at 37 °C (DMEM 50%, HEPES, HBSS 25%, inactivated Horse Serum 25%, 2 mmol/L L-glutamine, 5 mg/ml glucose and 0.4% penicillin-streptomycin, pH 7.2). Five individual slices were transferred to each Millicell culture insert membrane using a plastic Pasteur pipette and were incubated in humidified 95% O2/5% CO2 at 37 °C changing the medium every two days. Five experimental group were used in this study: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm); B) Stressed Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours; D) Treatment Group (H2O2+MEL-POST) – Organotypic

spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours; E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours. Before fixation spinal cord slices were cultured totally for 9 days in vitro (DIV). The first group followed normal culture conditions for all nine DIV. The second group followed eight DIV of normal culture conditions and one DIV of H2O2 subministration. The third group followed eight DIV of normal culture conditions and one DIV of melatonin treatment. The fourth group followed seven DIV of normal culture conditions, one DIV of H2O2 subministration and one DIV of melatonin treatment. The fifth group followed seven DIV of normal culture conditions, one DIV of H2O2 subministration and one DIV of melatonin treatment.

3.3 Propidium Iodide (PI) assay

The fluorescent exclusion dye propidium iodide (PI) is a polar compound which is widely used as a vital dye in tissue culture systems because enters cells with damaged cell membranes and binds to nucleic acids of dying ones. His brightly red fluorescence can be visualized using a confocal microscope. After the treatments, the different culture media were changed and 1.1 ml of normal culture medium with 5 µg/mL propidium iodide (PI) was added to the slices for each well. To ensure sufficient diffusion of PI, the medium was added below the Millicel-filter as well as on the top of the culture, 1.1 ml each. After three hours spinal cord slices were washed with PBS and fixed in 4% paraformaldehyde (PFA) for 24 hours: 1.1 ml below the membrane and 2 ml on the top of the slices. Always working in low light conditions to not compromise the fluorescence of the dye, after a washing in PBS, the slices were shifted from the membrane with a tiny brush to 24-well culture plates filled with PBS. They were permeabilized with 1% Triton X-100 (Sigma) overnight, put on glass slides and counterstained with 4',6-diamidino-2-phenylindole (DAPI) for the visualization of cellular nuclei. As described by Yoon (Yoon et al., 2010) bridge mounting technique was used to avoid slice cultures from being squashed. The PI fluorescence images of the slice cultures were recorded using a laser scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany) at the final 100X magnification. Digitally fixed images of slices were analyzed using an image analyzer (Image Pro-Plus 9.1.4, Milan, Italy) and measurements were made as the percentage of area for ten random fields on every section.

3.4 MTT assay

The viability of organotypically cultured spinal cord tissue slices was systematically examined with a mitochondrial assay using the tetrazolium dye, 3-(4,5-dimethlythiazol-2-yl)-2,5-dihphenyltetrazolium bromide (MTT). The MTT assay is based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals by metabolic active cells. After the experimental treatments, the supernatant was removed and replaced by fresh culture medium (1.1 ml/chamber) to which 0.1 ml MTT stock solution (5 mg/ml in PBS, pH 7.4) was added (MTT final concentration: 0.5 mg/ml). The slices were incubated for four hours under standard culture conditions. Afterwards, they were washed with PBS, and transferred into a 96-well plate, where pure dimethyl sulfoxide (DMSO) (100 µl/slice/well) was added for dissolving formazane crystals according to Mozes (Mozes et al., 2012). After 1 hour, 70 µl of DMSO solution from each well was transfered to another 96-well plate. The absorbance of the samples (70 µL) was recorded at 540 nm and 620 nm and the metabolic activity was calculated as follows: (A540– A620) / slice volume (volume of elliptical cylinder = (slice length/2)*(slice width/2)*slice thickness) = 100% in control (untreated slices). Each analysis was performed in duplicate per five times.

3.5 Total Thiols Assay

For the determination of total thiols (-SH), after the period of culture, slices of different groups have been homogenized by agitation in Tris–HCl buffer (10mM, pH 7.4) in the following proportion: for each experimental group six spinal cord slices were removed from membranes and put with 300 µl of TRIS-HCl buffer for 15 minutes on ice bath. The homogenate was centrifuged at 3000g for 5 minutes and the supernatant fraction (S1 - 100 µl) was used for the assay with Ellman's reagent (Ellman, 1959) [5,5'-dithiobis- (2-nitrobenzoic acid) or DTNB] (100 µl), which is a chemical used to quantify the concentration of thiol groups in a sample, and 1M phosphate-buffered saline pH 7.4 (PBS) (400 µl). After 10 minutes samples were assayed spectrophotometrically at 412nm. Using glutathione a standard curve was constructed in order to calculate the thiols in the tissue samples (Oliveira VA et al., 2014).

3.6 Immunofluorescence Analysis

Organotypic spinal cord slice cultures were processed for multiple cell type markers in order to study the immunopositivity of different molecules involved in the oxidative stress and the neuronal and synaptic condition. At the end of the culture, the wholemount spinal cord slices were fixed in 4% paraformaldehyde (PFA) dissolved in PBS for 24 hours: after having removed the tissue culture and briefly whased the slices with PBS, 1.1 mL of 4% PFA were poured below the membrane and another 1.1 mL of 4% PFA was gently poured on top of the slices respectively (Sypecka et al., 2015). After a washing with PBS they have been shifted with a tiny and fine brush from the membrane to a 24-well culture plate for another washing with PBS. Therefore the slices were permeabilized with 1% Triton X-100 overnight (Yoon et al., 2010) and the day after blocked for 1 hour at room temperature with a 1% BSA diluted in PBS. The different primary antibody were: rabbit polyclonal anti-NeuN (1:200 - abcam), mouse monoclonal anti-Syp (D-4) (1:100 – Santa Cruz), rabbit polyclonal anti-SOD-1 (1:200 - Santacruz). After the incubation with the primary antibody, the sections were sequentially incubated with appropriate fluorescent secondary antibodies diluted 1:200 in PBS: anti-rabbit Alexa-Fluor 488 (green fluorescent dye), anti-mouse Alexa-Fluor 488 (green fluorescent dye), anti-rabbit Alexa-Fluor 555 (red fluorescent dye) (Invitrogen, Carlsbad, CA, USA). Cell nuclei were stained with DAPI (Sigma) for 10 minutes. After finally being fast washed, the slides were immersed in fluorescent mounting medium (Dako) and mounted through the bridge mounting technique (Yoon et al., 2010) to avoid slice cultures from being squashed. The localization of immunopositivity was evaluated on digitally images acquired with laser scanning confocal microscopy (LSM 510, Zeiss, Oberkochen, Germany) at a final magnification of 100X and 300X. Digitally fixed images of slices were analyzed using an image analyzer (Image Pro-Plus 9.1.4, Milan, Italy) and measurements were made as the percentage of area in ten random fields for five image on every section.

3.7 Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). Differences among groups were analyzed by a one-way analysis of variance (ANOVA test), using Bonferroni's multiple comparison test for post-hoc analysis. The level of significance was accepted at $*p < 0.05$. Origin v9.0 software was used for all statistical analyses performed.

4 RESULTS

4.1 Propidium Iodide analysis

The immunopositivity deriving from the PI intake, a marker for the membrane integrity, describes different percentages of death cells measured in the gray matter of spinal cord slices among the five experimental groups (Fig.1 - 2) and it was calculed as percentage of immunopositive area with respect to a standard area. Quantification of image analysis shows that the injured group (B – H2O2) has the highest percentage of dead cells with respect to the other groups (1,42±0,14). The control group (A - CTR) presents a significantly less cell death (*0,43±0,08) comparing to the injured group (B – H2O2) indicating that H2O2 causes cellular death. The number of cells in the third group, the control treated with melatonin (C – CTR+MEL) shows a number of dead cells (*0,76±0,12) slightly and not significantly smaller than the control (A – CTR) but significantly smaller with respect to the injured group (B – H2O2). The two groups exposed to H2O2 but treated with melatonin (D – H2O2+MEL-PRE; E – H2O2+MEL-POST) show a significant smaller number of dead cells with respect to the injured group (B – H2O2). The fourth group (D – H2O2+MEL-POST), post-treated with melatonin shows a number of dead cells (*0,76±0,12) not significantly higher with respect to the control group (A – CTR) and the control with melatonin (C – CTR+MEL), but significantly smaller with respect to the second group (B – H2O2). The fifth group, (E – H2O2+MEL-PRE) pre-treated with melatonin shows a number of dead cells (*0,65±0,06) not significantly higher with respect to the control group (A – CTR) and the control with melatonin (C – CTR+MEL), but significantly smaller with respect to the second group (B – H2O2). Between the two treated group, the number of dead cells in the pre-treated one (E – H2O2+MEL-PRE) is smaller but not significantly with respect to the post-treated one (D – H2O2+MEL-POST). Data are expressed as means ± SEM. *P<0.05 vs H2O2. (Fig.1 - 2)

4.2 MTT analysis

MTT assay was used for the spinal cord slices vitality analysis. The slices vitality is influenced by the mithocondrial activity and the number of dead cells. According to Mozes, the control was fixed as 100% (Mozes et al., 2012). The quantification of data shows that the control group (A – CTR) has an significant higher vitality (100±22% fixed as a standard) compared to the second group composed by sliced exposed to H2O2 (10±23%) (B – H2O2). The first group (A – CTR) (100±22%) has a decreased vitality compared to the third group (115±5%) (C - CTR+MEL), to the fourth group (140±35%) (D – H2O2+MEL – POST) and to the fifth group (113±7%) (E – H2O2+MEL – PRE). The second group (B – H2O2) (1±23%) has a very decreased vitality compared to the other groups: significantly decreased compared to the third (115±5%) (C - CTR+MEL), not significantly decreased compared to the fourth (140±35%) (D – H2O2+MEL – POST) and significantly decreased compared to the fifth (113±7%) (E – H2O2+MEL – PRE). The third group, formed by control slices treated with melatonin (115±5%) (C - CTR+MEL) has a vitality significantly higher with respect to the second group (B – H2O2) (1±23%); it has a vitality slightly higher compared to the fifth group, pre-treated with melatonin and exposed to H2O2 (113±7%) (E – H2O2+MEL – PRE); it has a decreased vitality compared to the melatonin post-treated group (140±35%) (D – H2O2+MEL – POST). The fourth group has the highest percentage (140±35%) (D – H2O2+MEL – POST) among the five different experimental groups, but this is not significant. The fifth group (113±7%) (E – H2O2+MEL – PRE), has a lower vitality compared to the third (115±5%) (C - CTR+MEL) and the fourth group (140±35%) (D – H2O2+MEL – POST), but higher compared with the first (A – CTR) (100±22%) and significantly higher compared to the second group (B – H2O2) (1±23%). (Fig. 3)

4.3 Immunofluorescence

4.3.1 Neuronal analysis (NeuN)

The immunopositivity for the neuronal antibody anti-NeuN, a marker expressed not only in the nucleus of neurons but also in their cytoplasm analyses the number of neurons in the gray matter of spinal cord slices after the different experimental conditions (Fig.4 - 5). The immunopositivity is calculed as the percentage (%) of immunopositive area with respect to a standard area. Quantification of image analysis performed after statystical analysis shows that the injured group (B – H2O2) (0,28±0,07) has the smallest percentage of neurons with respect to the other groups (0,28±0,07) but is only significant with respect to the third group (C – CTR+MEL) (0,89±0,03). The control group (A - CTR) (0,77±0,04) has an elevated NeuN immunopositivity (0,77±0,04) compared to the injured group (B – H2O2) (0,28±0,07) indicating that H2O2 causes neuronal death but this is not statistically significant. Immunopositivity of the control group is also slightly higher compared to the fourth one (H2O2+MEL-POST) (0,75±0,11) and the fifth one (H2O2+MEL-PRE) (0,69±0,13). The third group - control group treated with melatonin (C – CTR+MEL) - shows the significant and highest percentage of NeuN immunopositivity (0,89±0,03) compared to all the other groups: (A - CTR) (0,77±0,04), (B – H2O2) (0,28±0,07), (H2O2+MEL-POST) (0,75±0,11), (H2O2+MEL-PRE) (0,69±0,13). The NeuN immunopositivity of the fourth group (H2O2+MEL-POST) (0,75±0,11), exposed to H2O2 and post-treated with melatonin is not significantly higher than the second group (B – H2O2) (0,28±0,07) and the fifth group (H2O2+MEL-PRE) (0,69±0,13). It is lower than the first (A - CTR) (0,77±0,04) and significantly lower than the third one (C – CTR+MEL) (0,89±0,03). The NeuN immunopositivity of the fifth group (0,69±0,13) (E – H2O2+MEL-PRE) is higher compared to the second group (B – H2O2) (0,28±0,07) and is lower compared the first one (A - CTR) (0,77±0,04), the fourth (H2O2+MEL-POST) (0,75±0,11) and significantly than the third one (C – CTR+MEL) (0,89±0,03). Data are expressed as means ± SEM. *P<0.05 vs CTR+MEL. (Fig. 4 - 5)

4.3.2 Synaptophysin analysis (Syp)

The synaptophysin (Syp) immunopositivity, a marker expressed in pre-synaptic vescicles of neurons was studied around the gray matter going to examine the synaptic extensions from the gray matter into the white matter of the spinal cord slices. The quantification of Syp was expressed as percentage of immunopositive area with respect to a standard area. The injured group (B – H2O2) has the smallest percentage of Syp immunopositivity with respect to the other groups (0,22±0,03). The control group (A - CTR) presents a Syp immunopositivity statistically higher (*0,57±0,06) compared to the injured group (B – H2O2) indicating a greater presence of synaptic vescicles. The third group, represented by the control treated with melatonin (C – CTR+MEL) shows the highest Syp immunopositivity (*0,64±0,12) among the groups. This is significantly greater compared to the stressed group (B – H2O2) and also greater, but not significantly, than the normal control (A- CTR) and the two groups exposed to H2O2 but treated with melatonin (D – H2O2+MEL-PRE; E – H2O2+MEL-POST). These last two groups (D – H2O2+MEL-PRE; E – H2O2+MEL-POST) have a Syp immunopositivity (0,42±0,08) (0,34±0,05) smaller than the control (A – CTR) and the control treated with melatonin (C – CTR+MEL). Moreover they have a greater Syp immunopositivity compared to the injured group (B – H2O2), but not significant. Between these two last melatonin treated group, the percentage of Syp immunopositivity in the pre-treated one (E – H2O2+MEL-PRE) is higher but not significantly with respect to the post-treated one (D – H2O2+MEL-POST). Data are expressed as means ± SEM. *P<0.05 vs H2O2. (Fig.6 - 7)

4.3.3 Superoxide dismutase analysis (SOD-1)

Immunofluorescence detection showed that the SOD-1 immunostaining positivity was different among the five experimental groups. SOD immunopositivity of the first group (A – CTR) is very low (0,06±0,02) compared to the other groups: the second one (B - H2O2) (0,70±0,14), the third one (C - CTR+MEL) (0,23±0,05), the fourth one (D - H2O2+MEL-POST) (0,95±0,15), the fifth one (E - H2O2+MEL-PRE) (0,83±0,08). The group exposed to H2O2 (B - H2O2) (* ° 0,70±0,14) has a SOD immunopositivity significantly higher compared to control group (A – CTR) (0,06±0,02) and compared to the control group treated with melatonin (C - CTR+MEL) (0,23±0,05). It has an immunopositivity not significantly smaller compared to the fourth (D - H2O2+MEL-POST) (0,95±0,15) and the fifth groups (E - H2O2+MEL-PRE) (0,83±0,08). The third group (C - CTR+MEL) (0,23±0,05) shows an lower SOD immunopositivity compared to the second one (B - H2O2) (0,70±0,14), the fourth one (D - H2O2+MEL-POST) (0,95±0,15) and the fifth group (E - H2O2+MEL-PRE) (0,83±0,08); it shows a not significant increase of SOD immunopositivity with respect to the first group (A – CTR) (0,06±0,02). The fourth group (* ° D - H2O2+MEL-POST) (0,95±0,15) shows the highest immunopositivity compared to the other groups: a significant quantification compared to the first group (A – CTR) (0,06±0,02) and the third group (C - CTR+MEL) (0,23±0,05) but not significant compared the second one (B - H2O2) (0,70±0,14), to the fourth one (D - H2O2+MEL-POST) (0,95±0,15) and to the fifth one (E - H2O2+MEL-PRE) (0,83±0,08). The fifth group (* ° E - H2O2+MEL-PRE) (0,83±0,08) shows a SOD immunopositivity significantly higher compared to the first group (A – CTR) (0,06±0,02) and the third group (C - CTR+MEL) (0,23±0,05); it is not significantly higher compared to the second group (B - H2O2) (0,70±0,14); it is not significantly lower than the fourth group (D - H2O2+MEL-POST) (0,95±0,15). Data are expressed as means ± SEM. *P<0.05 vs CTR; °P<0.05 vs CTR+MEL. (Fig.8 -9)

4.4 Total thiols determination (total SH)

Total thiols (totalSH) levels have been calculed (µMol X gram) in spinal cord slices for the different experimental groups. It is shown that the control (A - CTR) (233,04±4,74) significantly has the highest total SH level compared to the other groups: the second one exposed to H2O2 (*B – H2O2) (233,04±4,74), the third one composed by control slices with melatonin (*C - CTR+MEL) (277,65±6,6), the fourth one exposed to H2O2 and post-treated with melatonin (*D - H2O2+MEL-POST) (272,74±8,01) and the fifth exposed to H2O2 but pre-treated with melatonin (*E - H2O2+MEL-PRE) (292,7±7,48). The second group (B – H2O2) has the lowest level of total SH levels (*°233,04±4,74). It is significantly lower compared to the the first group (A - CTR) (233,04±4,74) and the third one (C - CTR+MEL) (277,65±6,6). It is lower than the fourth (D - H2O2+MEL-POST) (272,74±8,01) (E - H2O2+MEL-PRE) (292,7±7,48) and the fifth group but not significantly. The total SH level of the third group, the control treated with melatonin (*C - CTR+MEL) (277,65±6,6), is significantly higher compared to the second group (°B – H2O2) (233,04±4,74), composed by slices exposed to H2O2. It is also significantly lower with respect to the control group (A - CTR) (233,04±4,74). It is slightly higer compared to the fourth group exposed to H2O2 and post-treated with melatonin (*D - H2O2+MEL-POST) (272,74±8,01) and is lower compared to the fifth group exposed to H2O2 and pre-treated with melatonin (E - H2O2+MEL-PRE) (292,7±7,48). The total SH level of the fourth group (*D - H2O2+MEL-POST) (272,74±8,01) is significantly lower compared to the control (A - CTR) (233,04±4,74). It is lower compared to the third group (C - CTR+MEL) (277,65±6,6) and the fifth group (E - H2O2+MEL-PRE) (292,7±7,48). It is higher compared to the second group (B – H2O2) (233,04±4,74). The total SH level of the fifth group (*E - H2O2+MEL-PRE) (292,7±7,48) is significantly lower compared to the control (A - CTR) (233,04±4,74). It is not significantly higher with respect to the second group (B – H2O2) (233,04±4,74), the third group (C - CTR+MEL) (277,65±6,6) and the fourth group (D - H2O2+MEL-POST) (272,74±8,01). Data are expressed as means ± SEM. *P<0.05 vs CTR; °P<0.05 vs CTR+MEL. (Fig. 10)

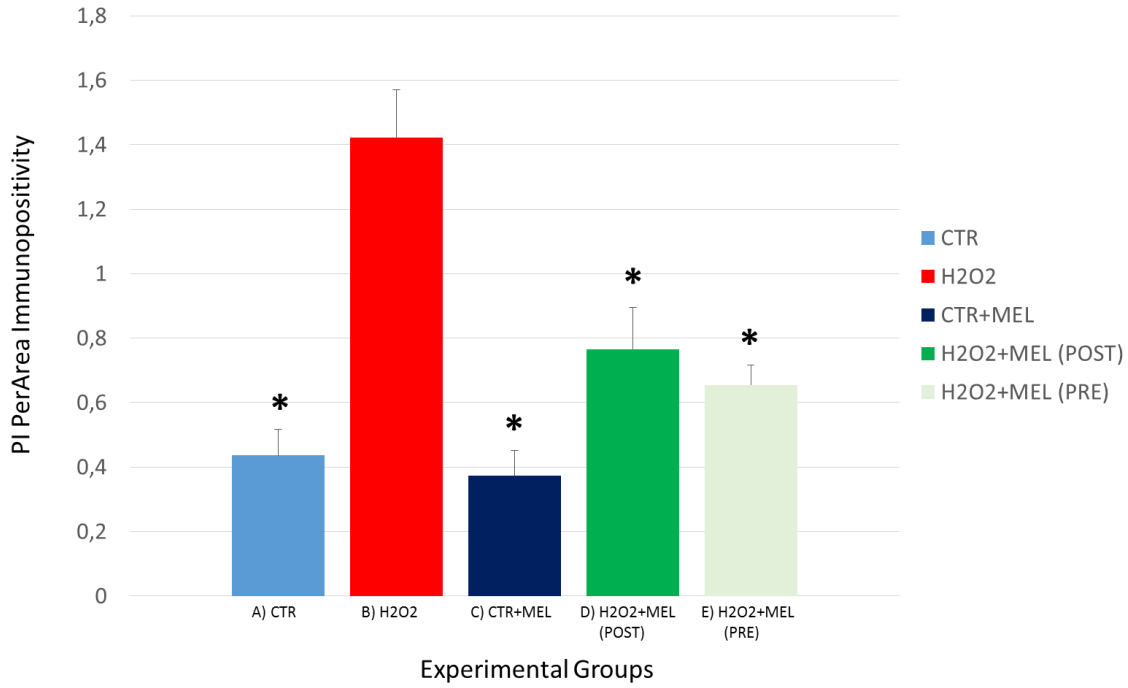

Fig.1

Cell death percentage analysis by positive immunostaining (PerArea - %) after Propidium Iodide (PI) intake. Graph comparing the five experimental groups: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm) (0,43±0,08); B) Injured Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) (1,42±0,14); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours (0,37±0,07); D) Treatment Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours (0,76±0,12); E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours (0,65±0,06). Data are expressed as means ± SEM. *P<0.05 vs H2O2.

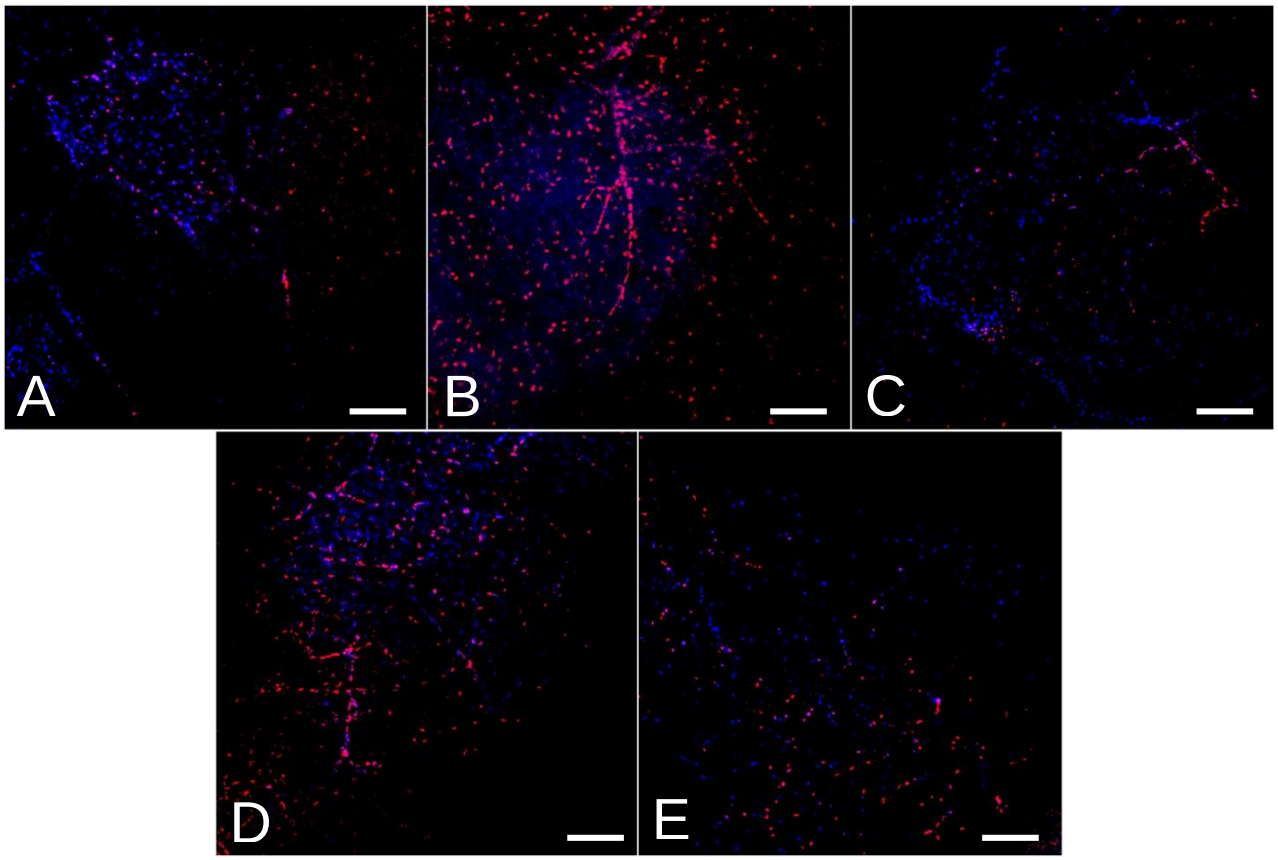

Fig. 2

(A-E) Immunofluorescence analyses (100X) of Propidium Iodide intake (PI) (red staining) comparing the five experimental groups: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm); B) Injured Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours; D) Treatment Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours; E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours. (Bar=100µm).

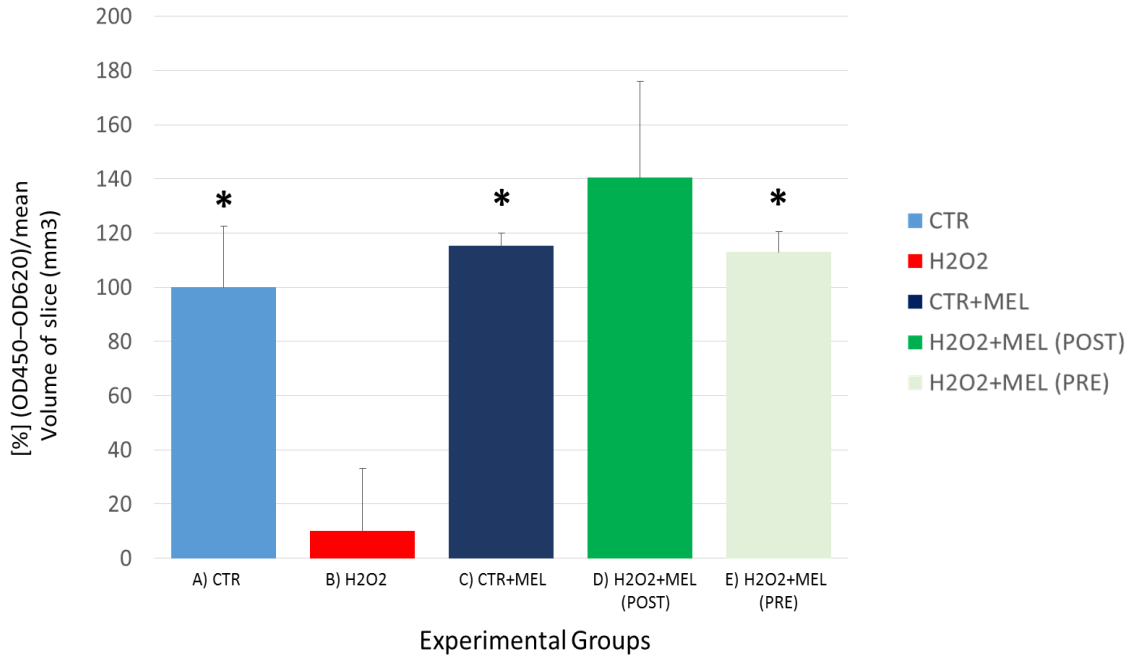

Fig. 3

Percentage analysis of slice vitality after MTT assay (%). The control was fixed as 100%. Graph comparing the five experimental groups: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm); B) Injured Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours; D) Treatment Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours; E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours. Data are expressed as means ± SEM. *P<0.05 vs H2O2.

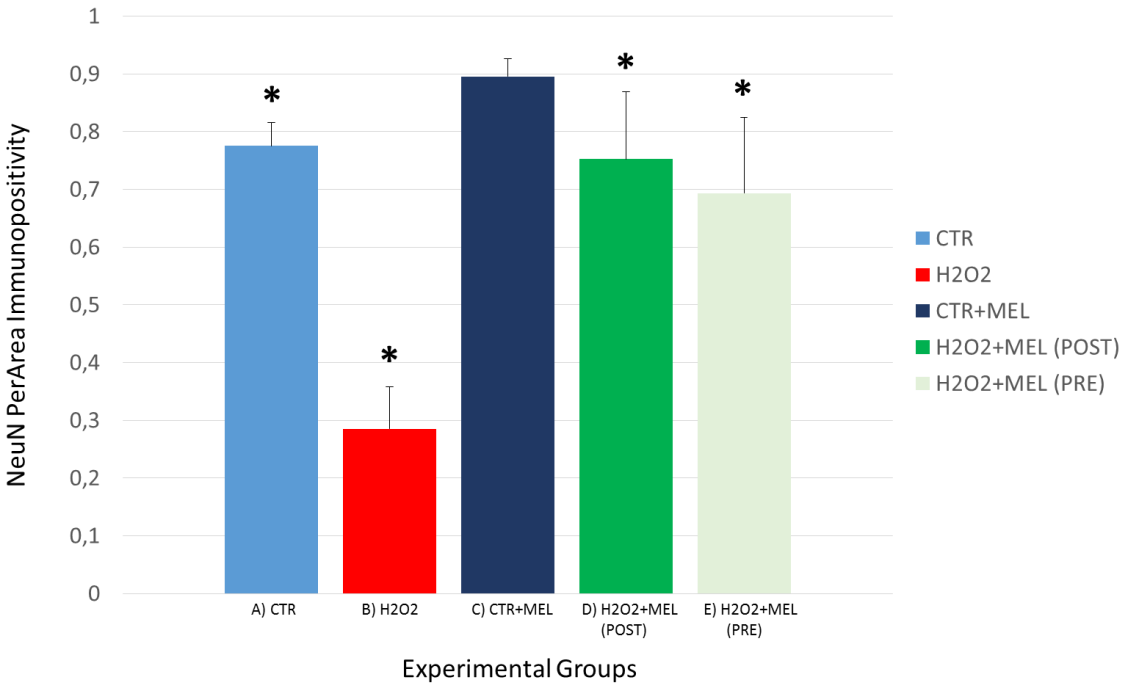

Fig. 4

Percentage analysis of positive immunostaining (PerArea) after immunofluorescence analysis for Neuronal Nuclei (NeuN). Graph comparing the five experimental groups: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm) (0,77±0,04); B) Injured Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) (0,28±0,07); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours (0,89±0,03); D) Treatment Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours (0,75±0,11); E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours (0,69±0,13). Data are expressed as means ± SEM. *P<0.05 vs CTR+MEL.

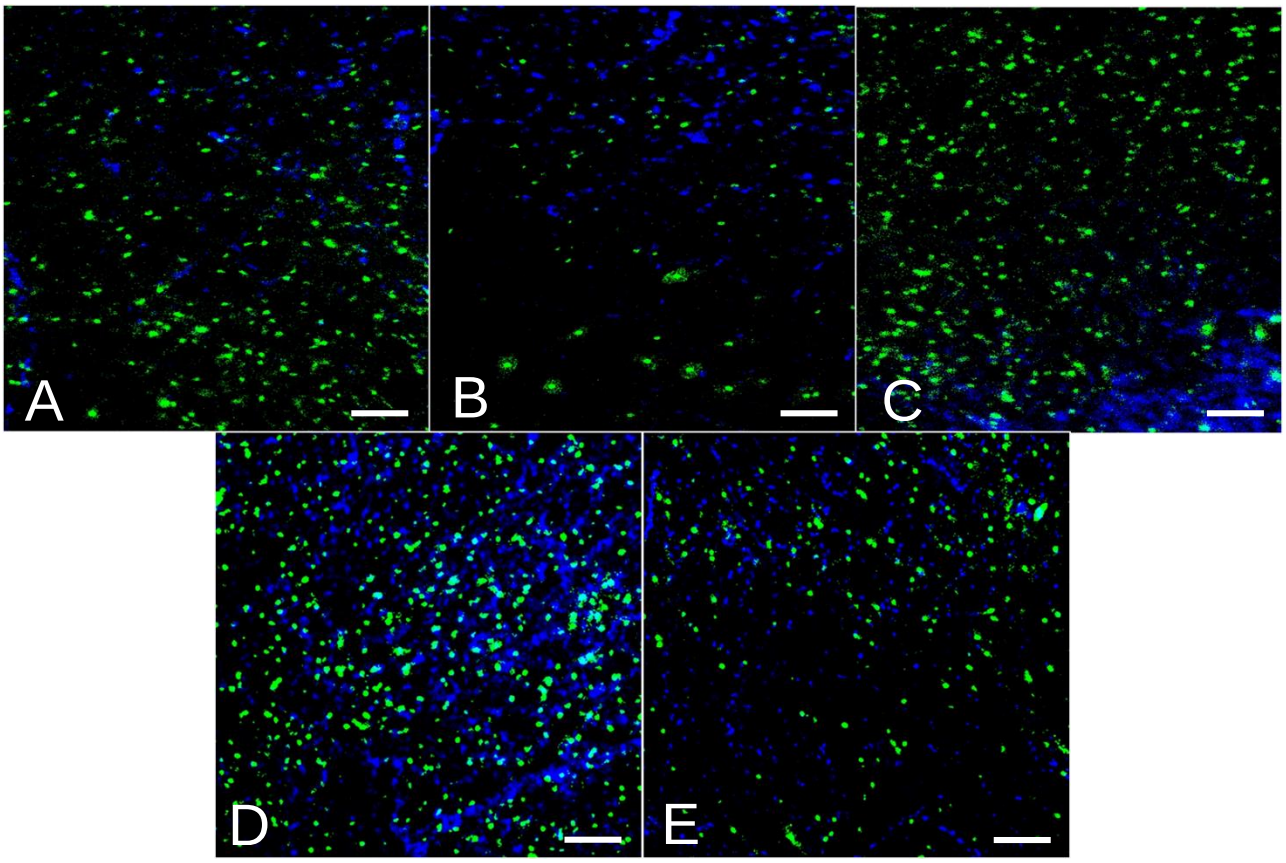

Fig.5

(A-E) Immunofluorescence analyses (100X) of Neuronal Nuclei (NeuN) (green staining) comparing the five experimental groups: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm); B) Injured Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours; D) Treatment Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours; E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours (Bar=100µm).

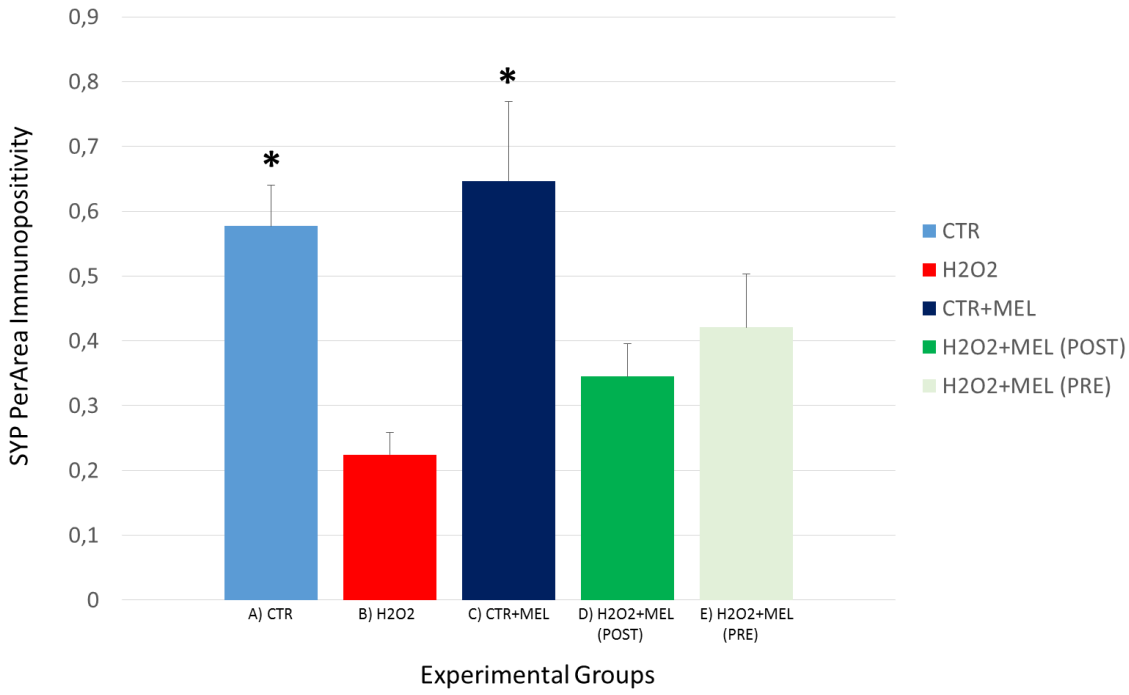

Fig.6

Percentage analysis of positive immunostaining (PerArea) after immunofluorescence analysis for synaptophysin (SYP). Graph comparing the five experimental groups: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm) (0,57±0,06); B) Injured Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) (0,22±0,03); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours (0,64±0,12); D) Treatment Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours (0,34±0,05); E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours (0,42±0,08). Data are expressed as means ± SEM. *P<0.05 vs H2O2.

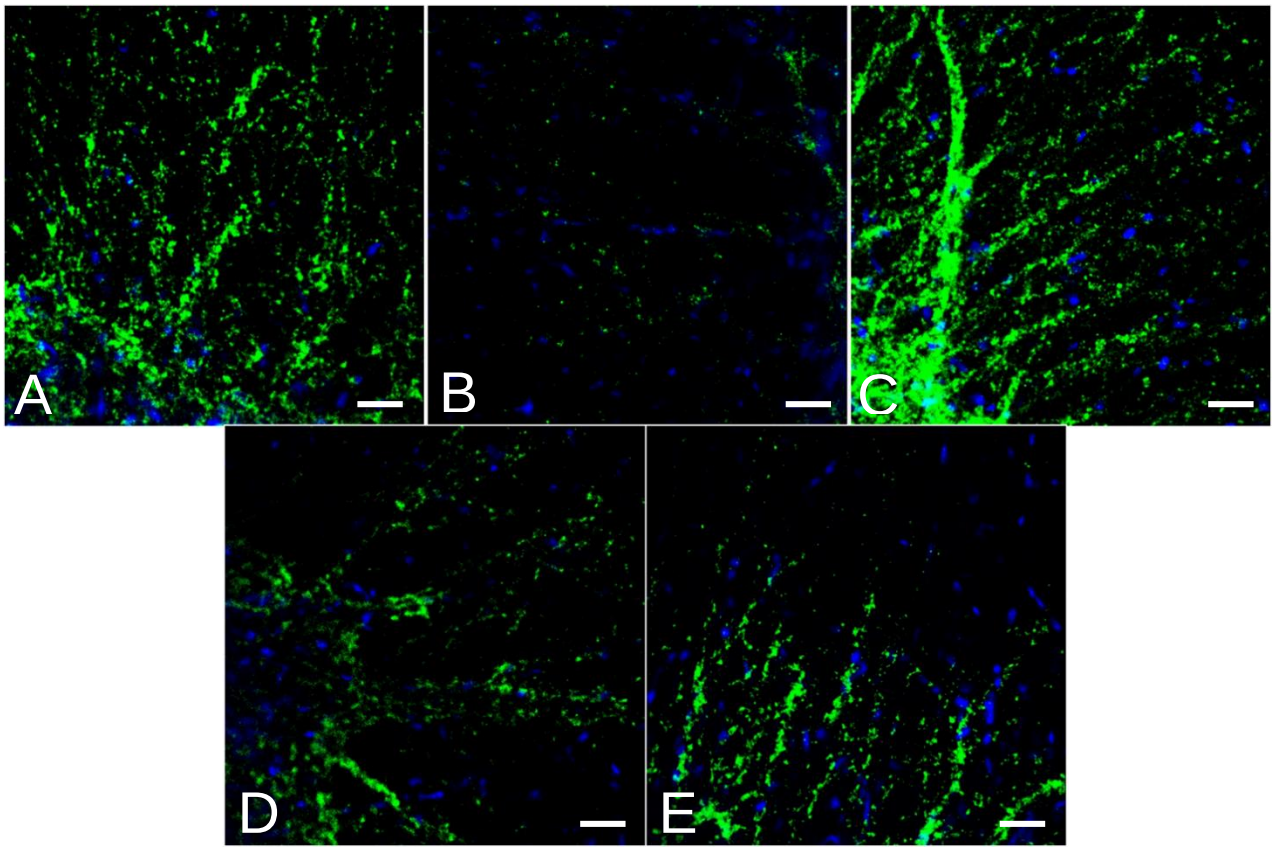

Fig.7

(A-E) Immunofluorescence analyses of synaptophysin (SYP) (green staining) comparing the five experimental groups: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm); B) Injured Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours; D) Treatment Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours; E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours (Bar=15µm).

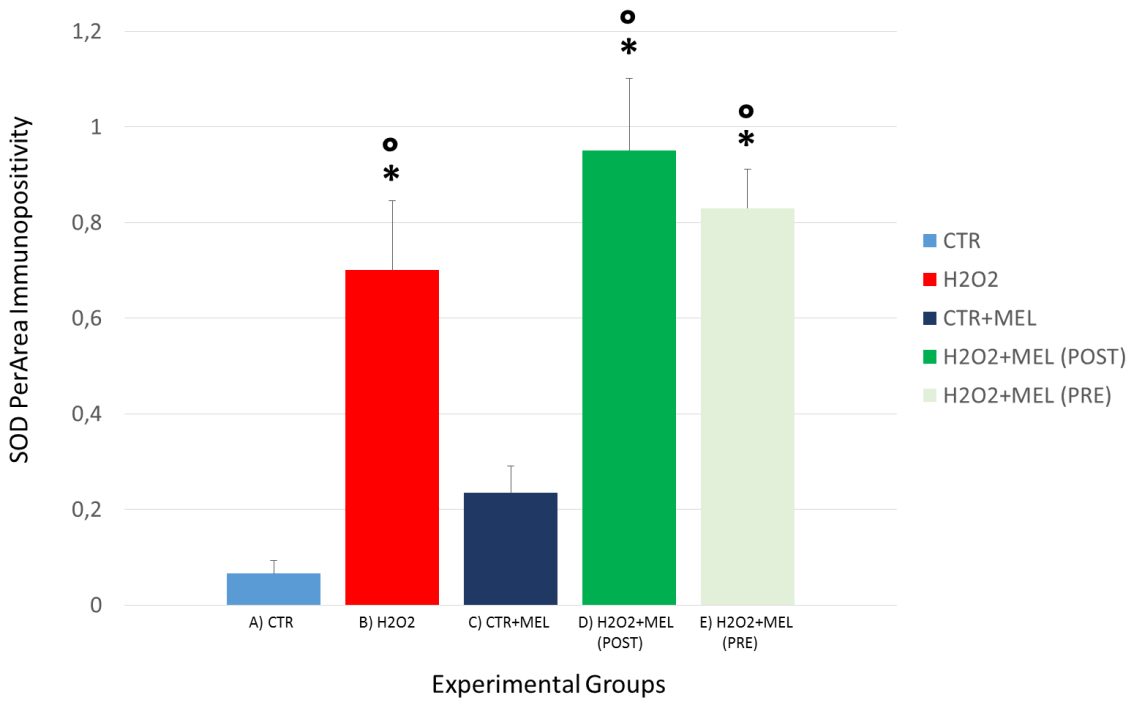

Fig.8

Percentage analysis of positive immunostaining (PerArea) after immunofluorescence analysis for superoxide dismutase (SOD-1). Graph comparing the five experimental groups: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm) (0,06±0,02); B) Injured Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) (0,70±0,14); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours (0,23±0,05); D) Treatment Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours (0,95±0,15); E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours (0,83±0,08). Data are expressed as means ± SEM. *****P<0.05 vs CTR; **°**P<0.05 vs CTR+MEL.

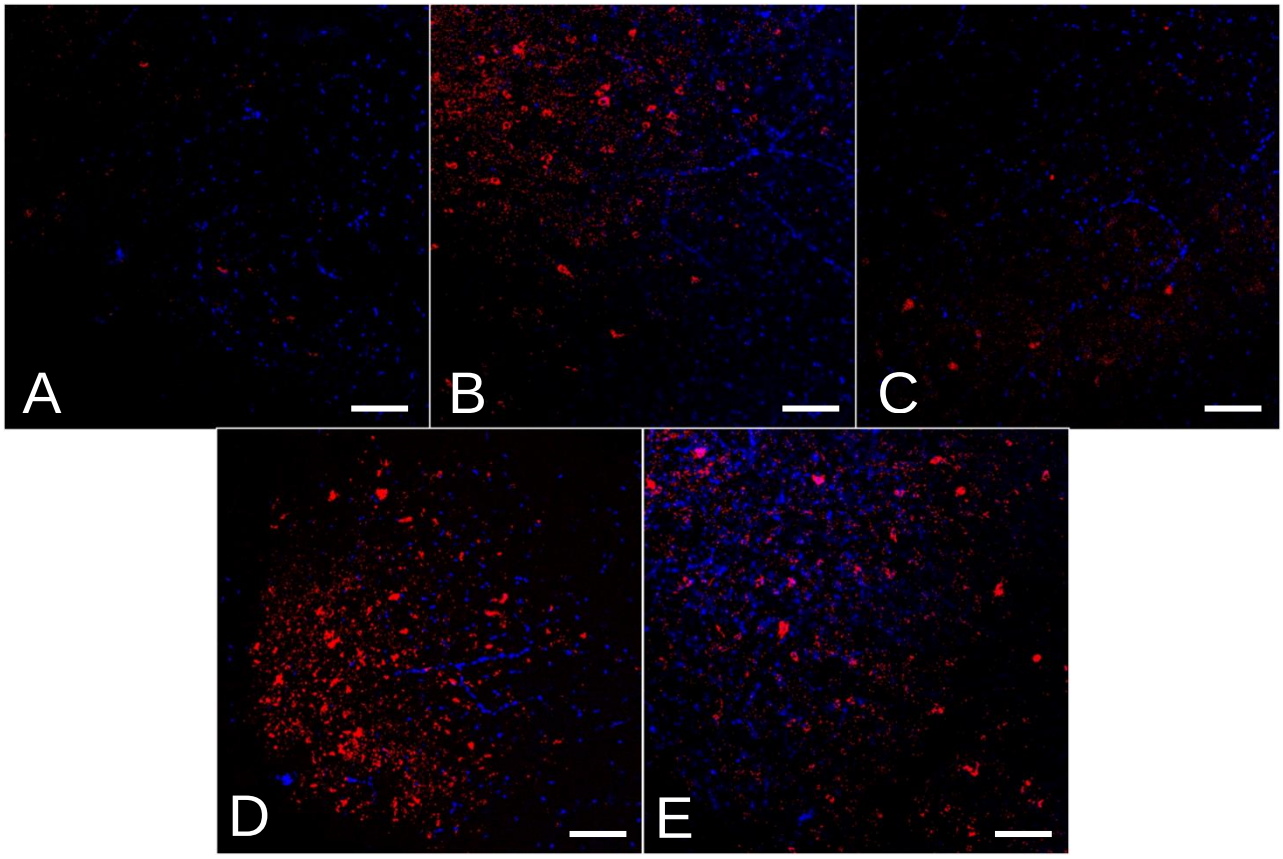

Fig.9

(A-E) Immunofluorescence analyses (100X) of superoxide dismutase (SOD) (red staining) comparing the five experimental groups: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm); B) Injured Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours; D) Treatment Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours; E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours (Bar=100µm).

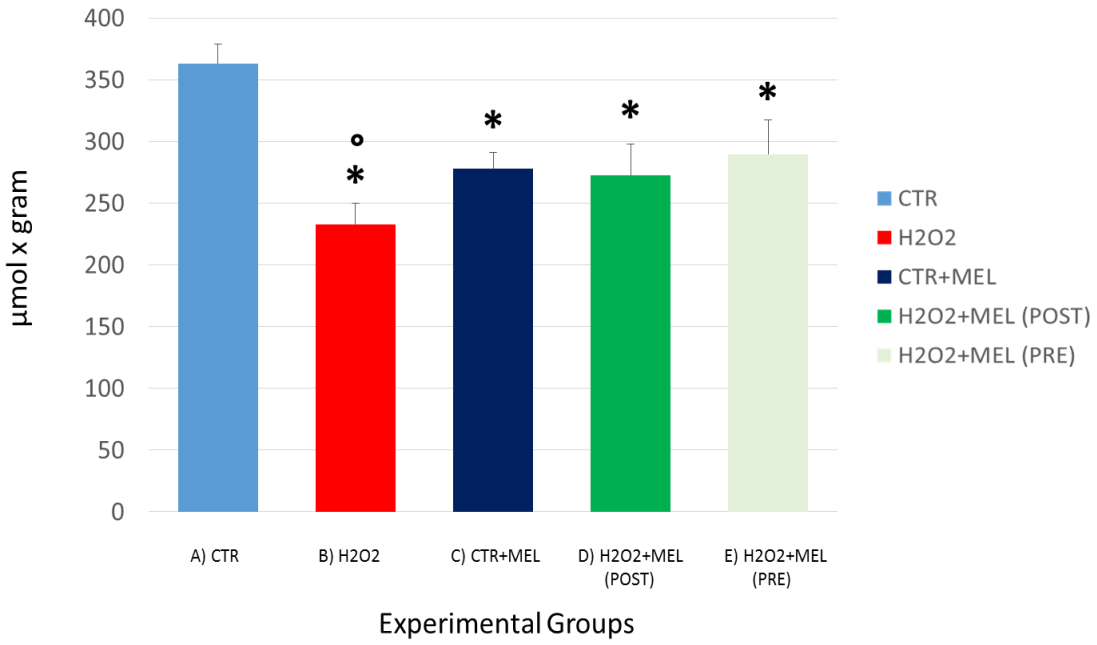

Fig. 10

Analysis of total thiols (totalSH) (µMol x Gram). Graph comparing the five experimental groups: A) Control Group (CTR) – Organotypic spinal cord slice culture (350µm) (363,04±4,75); B) Injured Group (H2O2) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) (233,04±4,74); C) Control Group with a melatonin treatment (10^{-5}M) of 24 hours (CTR+MEL) (277,65±6,6) – Organotypic spinal cord slice culture (350µm) treated with melatonin for 24 hours; D) Treatment Group (H2O2+MEL-POST) – Organotypic spinal cord slice culture (350µm) exposed to H2O2 (50 µM) and treated after 24 hours with melatonin (10^{-5}M) for 24 hours (272,74±8,01); E) Treatment Group (H2O2+MEL-PRE) – Organotypic spinal cord slice culture (350µm) pre-treated with melatonin (10^{-5}M) for 24 hours (50 µM) and exposed to H2O2 for other 24 hours (292,7±7,48). Data are expressed as means ± SEM. *P<0.05 vs CTR; °P<0.05 vs CTR+MEL

5 DISCUSSION

The present study showed that melatonin limits secondary damage in an experimental model of rat spinal cord organotypic slice culture. There are few studies in literature that describe the SCI secondary injury using spinal cord organotypic cultures as experimental model. Organotypic cultures are a valid experimental model to study SCI secondary damages and in particular the oxidative stress related to them. It is a useful system for studying molecules that can alleviate alterations resulting from ROS production or that can have a neuroprotective and neuroregenerative role (Domìnguez-Alonso et al., 2011; Ramirez-Rodriguez et al., 2011); they are viable for several weeks preserving the original organ tridimensional architecture and keeping the synaptic circuitries integrity and functionality (Galimberti et al., 2006; Gahwiler et al., 1997). Organotypic slices are accessible models for drugs testing in different research fields which include cancer (Carranza-Torres et al., 2015), cardiovascular function (Brandenburger et al., 2012) angiogenesis (Staton et al., 2004), thymus (Owens et al., 2005), skin (Botham 2004) and urogenital tissues including kidney and bladder (Akakin et al., 2013). The organotypic cultures of neural tissue explants remain to be the best described and developed. In particular brain slices have been the most used for studies on epilepsies (Morin-Brureau et al., 2013), traumatic injury or neurodegenerative disorders (Noraberg et al., 2005). Spinal cord slice cultures have a great potential for the study of nervous regeneration (Bonnici and Kapfhammer 2008) but also for the study of the secondary damage: it is easy to create with the H2O2 use, the oxidative stress microenvironment characteristic of the SCI secondary phase and study if some molecules are able to decrease it. Although representing a biologically relevant 3D environment that mantains a functional neuronal signaling for long periods and functional links between the various cell types, on the other hand this model has some limitations. It does not possess the biological completeness of animal models and therefore it miss some biological elements that have a key role in the SCI pathophysiology.

Although the etiology and pathogenesis of SCI remain to be fully understood it is well knowkn that secondary deterioration due to inflammation and oxidative stress characterizes the pathophysiology of SCI (Fatima et al., 2015). Today methylprednisolone (MP), a prednisolone derivative belonging to the glucocorticoid group of corticosteroids, is used for the treatment of acute spinal cord injury (SCI) for its anti-inflammatory properties. However, a review of Hurlbert

(Hurlbert et al., 2013) adds pertinent new evidence that there is not consistent or compelling medical evidence of any class justifying the administration of MP for acute SCI. Moreover it is described the association of high-dose MP administration with a variety of complications and deleterious effects: immunosuppression with increased susceptibility to infections, an increased risk of gastrointestinal disturbances (ulcers, bleeding, and ileus), adult respiratory distress syndrome, hyperglycemia, deep venous thrombosis (DVT), and pulmonary embolism and death (Hurlbert et al., 2013). So, the research for an effective neuroprotective strategy to prevent or treat the secondary degeneration during the acute SCI remains a priority for basic scientists. As derived from our results, melatonin administration (10^{-5}M) to spinal cord organotypic slice cultures treated with hydrogen peroxide (H2O2) (50 µM), reduces significantly general cell death in the gray matter of the slice showing a neuroprotective role of the compound. The analysis based on the propidium iodide (PI) uptake measurement, an intercalating agent membrane impermeant and generally excluded from viable cells, which enters cells with damaged cell membranes and binds to nucleic acids of dying ones, showed the significant effect in each of the two groups exposed to H2O2 and treated with melatonin. Both in the pre-treated group than in the post-treated one, the number of dead cells analyzed by the use of PI, is significantly lower than those found in the slice treated with only H2O2. This results confirm the neuroprotective role of the melatonin as already showed in literature. There are some data about the use of the melatonin on the brain and in particular in hippocampal area. Kim (Kim et al., 2014) shows like melatonin reduces KA-induced neuronal injury in organotypic hippocampal slice cultures. Lezoualc'h describes how the preincubation with 1 mM melatonin protects HT22 cells against glutamate-induced cell death and protects organotypic hippocampal slices against H2O2-induced cell death (Lezoualc'h et al., 1996). Also animal models have been useful for confirming that melatonin prevents the cell death and the mitochondrial dysfunction during ischemic-stroke in mice (Yang et al., 2015). But more and more, the neuroprotective implications of the melatonin are studied in the SCI. Melatonin treatment of SCI animals in fact attenuates calpain expression, inflammation, axonal damage and neuronal death (Samantaray et al., 2008). The viability of entire slice was studied with the MTT assay by detecting the OD of formazane dissolved in DMSO (Mozes et al., 2012). Melatonin improves spinal cord slice culture viability with higher values in the post-treated group; however, despite being a high value, from the statystical analysis this finding is not significant as instead the melatonin pre-treated group. The melatonin pre-treated slices vitality is

significantly higher compared to the stressed group. This supports the melatonin protective role in this organotypic model of spinal cord oxidative stress damage. But we can't explain the mechanism of how or where melatonin acts. The MTT assay measures the decreased viability through the indirect evidence of the mitochondrial activity together to the cell death, so the results obtained from this assay are influenced not only by the amount of dead cells but also by the reduction capacity of living cells (Mozes et al., 2012). Literature findings report that, as generally in the entire organism, also in the central nervous system this indoleamine functions at three principal levels to aid the organism in the ability to resist the damage inflicted by radicals. First, melatonin reduces free radical generation at the mitochondrial level: beeing also lipophilic (as well as hydrophilic) (Reiter et al., 1995) it enters readily in neurons and glia and increases the electron transfer efficiency through the mitochondrial respiratory chain thereby reducing electron leakage and free radical generation in a process referred to as radical avoidance and directly scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS) when they are formed. In a second level it works promoting or enhancing the expression of antioxidative enzymes that convert highly toxic species to innocuous products. In a third level it acts inhibiting enzymes that normally produce free radicals as nitric oxide synthase (Reiter et al., 2010; Chang et al., 2008; Pozo et al., 1997; Antolin et al., 1996). The second level described above, was examined in this study evaluating immunopositivity of superoxide dismutase (SOD). There are two elements to consider: in the control treated with melatonin the SOD level is slightly higher compared to the normal control. This suggests that melatonin subministration could enhance slightly SOD antioxidative enzyme levels, also at basal conditions. The second element to consider is that both groups formed by slices exposed to H2O2 and treated with melatonin show an elevated SOD immunopositivity compared to the other groups. Even if not statistically significant, SOD immunopositivity is considerably higher in the post-treated group compared to the exposed group only. This indicates a melatonin stimulation of antioxidative enzymes like SOD. In trauma brain injury, melatonin has been evaluated to be effective significantly increasing SOD activity (Naseem et al., 2014). In experimental animal models with ischemic spinal cord injury following occlusion of the thoraco-abdominal aorta, melatonin treatment increases SOD enzyme activity to levels approximating of the sham control group (Erten et al., 2003). This activity is essential for maintaining cell integrity suggesting a defensive mechanism against ROS attacks. But SOD represents only one of the several endogenous antioxidant defenses that detoxify the organism. Among all the antioxidants available, thiols,

organic compounds containing a sulphydryl group (-SH) constitute the major portion of the whole compart of antioxidants and play a significant role against reactive oxygen species (Prakash et al., 2009). Thiols have a significant role in a countless number of physiological functions among which signal transduction, apoptosis, detoxification and a defense aginst free radicals. The total thiols are composed of both intracellular and extracellular thiols, either in the free form as the oxidized or reduced glutathione, and the thiols bound to proteins. Thiols are defined by Balcerczyk as the major determinants of the total antioxidant capacity (Balcerczyk et al., 2003). A wide array of oxidative modifications can occur on the thiol functional group of the amino-acid cysteine (Cys). Under oxidative stress conditions, oxidation of Cys residues can lead to the reversible formation of mixed disulfides between protein thiol groups and low–molecular-mass thiols by reaction of S-thiolation. This process plays a regulatory and an antioxidant role, since it protects protein–SH groups against irreversible oxidation to –SO2H and –SO3H (Prakash et al., 2009; Padgett and Whorton 1998; Mills and Lang 1996;). In our study the administration of H2O2 to the organotypic spinal cord slice cultures determines in the second group the lowest level of total SH. Compared to the control and to the control with melatonin this is significantly lower. It could be caused by a disulfide formation leading to a minor link of the Ellman's reagent [5'5-dithiobis-(2-nitrobenzoic acid)] (DTNB). In the groups pre-treated and post-treated with melatonin, the total SH levels were most elevated than in the second group, even if not significantly. Their levels move towords the control and substantially have the same values of the control treated with melatonin. These results show that melatonin permits to preserve the total antioxidant capacity in spinal cord slices subjected to an oxidative stress. Collecting these mechanisms melatonin appears to have a protective role in stressed spinal cord slices and this confirms literature evidences describing melatonin to enter nervous cells to protect them from molecular damage induced by ROS/RNS (Reiter et al., 2010). NeuN immunopositivity shows that melatonin protects neurons. The second group, composed by slices exposed to H2O2 has the lowest NeuN immunopositivity compared to the other groups. The third group, composed by control slices treated with melatonin has significantly the highest NeuN immunopositivity. If we consider the groups exposed to H2O2, pre and post-treated with melatonin, the levels of NeuN immunopositivity are very elevated even if not significantly with respect to the second group. Melatonin appears to counteract the action of H2O2 on neurons. Also in another model of nervous central injury, an ischemic neural damage, melatonin restablishes NeuN immunoreactivity with levels very similar to the control

(Lee et al., 2010). Literature evidences show that melatonin could act not only preserving neurons from oxidative stress but also promoting cells replacement by stimulating neuronal progenitor cell proliferation (Reiter et al., 2010; Manda et al. 2009; Kim et al. 2004). Manda et al. (2009) in fact, reports that neural precursors are able to respond to melatonin with elevated proliferative capacity. Moreover, other findings show melatonin role in adult neurogenesis. Kim et al. (2004) describes the melatonin positive modulation of cellular proliferating activity determined in the dentate gyrus in early postnatal rats. It is also reported that proliferation and differentiation of embryonic neural stem cells and differentiation of rat midbrain neural stem cells are influenced by the melatonin (Kong et al., 2008; Moriya et al., 2007). Benitez-King et al. (2004, 2006) shows how melatonin preserves the integrity of cytoskeletan organization and improve neuronal functions including also neurite outgrowth. And a strong neuritic arborization is visible around the gray matter of our control slices treated with melatonin after immunolabeling with a synaptophysin (Syp) antibody, a marker which recognizes the presynaptic protein synaptophysin. Synaptophysin is a transmembrane protein known as a neuronal marker of synaptogenesis and is known to be involved in synaptic vesicle formation and exocytosis. In our experiments, control slices treated with melatonin express the highest and significant Syp immunopositivity compared with the second group of slices exposed to H2O2. The fourth and the fifth groups show a higher but not statistically significant Syp immunopositivity than the second group. In particular the pre-treated group has a higher Syp level with respect to the post-treated group. This results point out that melatonin attenuates H2O2-induced Syp reduction in spinal cord organotypic slices. Melatonin's efficacy in regulating Syp expression positively, is well shown in an experimental animal model of methamphetamine-induced neurotoxicity where Syp levels in the dorsal striatum and prefrontal cortex are significantly increased (Kaewsuk et al., 2009). Moreover, it was found that melatonin increases dendrite maturation and complexity in new neurons formed in the dentate gyrus of mice and increases dendritogenesis in the hilus of hippocampal organotypic cultures (Domìnguez-Alonso et al., 2012). Overall, these findings suggest that melatonin may exert a potentially beneficial effect upon the progression of SCI secondary damage, protecting the tissue from a further degeneration. These elements make melatonin a powerful tool to consider, for acting on the secondary damage typic of SCI.

6 REFERENCES

Akakin D, Kiran D, Ozkan N, Ershakin M¸ Ozdemir-Kumral ZN, Yegen B, Sener G. Protective effects of melatonin against spinal cord injury induced oxidative damage in rat kidney: a morphological and biochemical study. *Acta Histochemica* 115 827– 834. (2013)

Antolin I, Rodriguez C, Sainz RM, Mayo JC, Uria H, Kotler MR, Rodriguez-Colunga MJ, Tolivia D, Menendez-Pelaez A. Neurohormone melatonin prevents cell damage: effect on gene expression for antioxidant enzymes. FASEB J., 10, 882- 890 (1996)

Balcerczyka A, Grzelaka A, Janaszewskaa A, Jakubowskib W, Koziolc S, Marszaleka M, Rychlika B, Soszynskia M, Bilinskic T, Bartosza G. Thiols as major determinants of the total antioxidant capacity.

Barnabé-Heider F and Frisén J. Stem cells for spinal cord repair. Cell Stem Cell. 3;3(1):16-24. doi: 10.1016/j.stem.2008.06.011. (2008)

Benitez-King G, Ramirez-Rodriguez G, Ortiz L, Meza I. The neuronal cytoskeleton as a potential therapeutical target in neurodegenerative diseases and schizophrenia. Curr Drug. Targets CNS Neurol Disord. 3: 515–533. (2004)

Benitez-King G. Melatonin as a cytoskeletal modulator: implications for cell physiology and disease. J Pineal Res. 40: 1–9. (2006) .

Bonnici B, Kapfhammer JP. Spontaneous regeneration of intrinsic spinal cord axons in a novel spinal cord slice culture model. Eur J Neurosci.27(10):2483-92. doi: 10.1111/j.1460-9568.2008.06227.x. (2008)

Botham, P.A. The validation of in vitro methods for skin irritation. Toxicol. Lett. 149, 387–390 (2004)

Brandenburger M, Wenzel J, Bogdan R, Richardt D, Nguemo F, Reppel M, Hescheler J, Terlau H, Dendorfer A. Organotypic slice culture from human adult ventricular myocardium. Cardiovasc Res. 1;93(1):50-9. (2012) doi: 10.1093/cvr/cvr259.

Carranza-Torres IE, Guzmán-Delgado NE, Coronado-Martínez C, Bañuelos-García JI, Viveros-Valdez E, Morán-Martínez J, Carranza-Rosales P. Organotypic culture of breast tumor explants as a multicellular system for the screening of natural compounds with antineoplastic potential. Biomed Res Int. 2015;2015:618021. doi: 10.1155/2015/618021. (2015)

Chang, HM, Huang, YL, Lan CT, Wu UI, Hu ME, Youn SC Melatonin preserves superoxide dismutase activity in hypoglossal motoneurons of adult rats following peripheral nerve injury. J. Pineal Res. 44, 172-180. (2008)

Chiu WT, Lin HC, Lam C, Chu SF, Chiang YH, Tsai SH. Review paper: epidemiology of traumatic spinal cord injury: comparisons between developed and developing countries. Asia Pac J Public Health 22(1):9-18. doi: 10.1177/1010539509355470 (2010)

Connelly CA, Chen LC, Colquhoun SD. Metabolic activity of cultured rat brainstem, hippocampal and spinal cord slices. *Journal of Neuroscience Methods* 99 1–7 (2000)

Cramer G and Darby s. Clinical Anatomy of the Spine, Spinal Cord, and ANS, 3rd Edition. Mosby (2013)

Domínguez-Alonso A, Ramírez-Rodríguez G, Benítez-King G. Melatonin increases dendritogenesis in the hilus of hippocampal organotypic cultures. J Pineal Res. 52(4):427-36. (2012) doi: 10.1111/j.1600-079X.2011.00957.x.

Dubocovich ML, Markowska M. Functional MT1 and MT2 melatonin receptors in mammals. Endocrine. 27(2):101-10 (2005)

Ellman GL, Tissue sulfhydryl groups. Arch Biochem Biophys. 82:70–7. (1959)

Erten SF, Kocak A, Ozdemir I, Aydemir S, Colak A, Reeder BS. Protective effect of melatonin on experimental spinal cord ischemia. Spinal Cord. 41(10):533-8 (2003)

Erten SF, Kocak a, Ozdemir I. Protective effect of melatonin on experimental spinal cord ischemia. BioFactors 17 75–82 75. (2003)

Esposito E. Cuzzocrea S. Antiinflammatory activity of melatonin in central nervous system. Current Neuropharmacology. 8 (3) 228–242 (2010)

Estevez AG, Spear N, Manuel SM, Radi R, Henderson CE, Barbeito L et al. Nitric oxide and superoxide contribute to motor neuron apoptosis induced by trophic factor deprivation. J Neurosci. 18: 923–931 (1998)

Eyre JA, G. J. Clowry GJ. Brain. A journal of Neurology. Development of the human spinal spinal cord. DOI: http://dx.doi.org/10.1093/brain/awf197 2134-2136 (2002)

Fatima G, Sharma VP, Das SK, Mahdi AA. Oxidative stress and antioxidative parameters in patients with spinal cord injury: implications in the pathogenesis of disease. *Spinal Cord* 53, 3–6 (2015)

Favero G, Rodella LF, Reiter RJ, Rezzani R. Melatonin and its atheroprotective effects: a review. Mol Cell Endocrinol. 15;382(2):926-37. doi: 10.1016/j.mce.2013.11.016 (2014)

Gahwiler BH, Capogna M, Debanne D, Mckinney RA, Thompson SM. Organotypic slice cultures: a technique has come of age. Trends Neurosci; 20:471–477 (1997)

Galimberti I, Gogolla N, Alberi S, Santos AF, Muller D, Caroni P. Long-term rearrangements of hippocampal mossy fiber terminal connectivity in the adult regulated by experience. Neuron; 50:749–763 (2006)

Grundy D and Swain A. ABC of Spinal Cord Injury. Published by BMJ Books, London, UK (2002)

Hall ED. Antioxidant therapies for acute spinal cord injury. Neurotherapeutics. 8: 152–167 (2011)

Hardeland R, Tan D-X, Reiter RJ, Kynuramines, metabolites of melatonin and other indoles: the resurrection of an almost forgotten class of biogenic amines," Journal of Pineal Research. 47, 2, 109–126 (2009)

Hulsebosch CE. Recent advances in pathophysiology and treatment of spinal cord injury. Adv Physiol Edu 26: 238–255 (2002)

Hurlbert RJ, Hadley MN, Walters BC, Aarabi B, Dhall SS, Gelb DE, Rozzelle CJ, Ryken TC, Theodore N. Pharmacological therapy for acute spinal cord injury. Neurosurgery. Mar;76 Suppl 1:S71-83. (2015) doi: 10.1227/01.neu.0000462080.04196.f7.

Ji RR, Gereau RW IV, Malcangio M, Strichartz GR. MAP kinase and pain. Brain Research Reviews, (60) 1, 135–148, (2009)

Jinkins JR. Atlas of Neuroradiologic Embryology, Anatomy, and Variants. (2000)

Jun Z, Li Z, Fang W, Fengzhen Y, Puyuan W, Wenwen L, Zhi S, Bondy SC. Melatonin decreases levels of S100β and NFKB, increases levels of synaptophysin in a rat model of Alzheimer's disease. Curr Aging Sci. Jul;6(2):142-9. (2013)

Kaewsuk S, Sae-ung K, Phansuwan-Pujito P, Govitrapong P. Melatonin attenuates methamphetamine-induced reduction of tyrosine hydroxylase, synaptophysin and growth-associated protein-43 levels in the neonatal rat brain. Neurochem Int.. 55(6):397-405. (2009) doi: 10.1016/j.neuint.2009.04.010.

Kao CC, Chang LW, Bloodworth JJ. Axonal regeneration across transected mammalian spinal cords: an electron microscopic study of delayed microsurgical nerve grafting. Exp Neurol 54: 591–615. (1977)

Kim HA, Lee KH, Lee BH. Neuroprotective effect of melatonin against kainic acid-induced oxidative injury in hippocampal slice culture of rats Int. J. Mol. Sci. 15, 5940-5951; (2014) doi:10.3390/ijms15045940

Kirshblum SC, Burns SP, Biering-Sorensen F, Donovan W, Graves DE, Jha A, Johansen M, Jones L, Krassioukov A, Mulcahey MJ, Schmidt-Read M, Waring W. International standards for neurological classification of spinal cord injury (revised 2011). J Spinal Cord Med. 34(6):535-46. doi: 10.1179/204577211X13207446293695 (2011)

Kong X, Li X, Cai Z, Yang N, Liu Y, Shu J, Pan L, Zuo P. Melatonin regulates the viability and differentiation of rat midbrain neural stem cells. Cell Mol Neurobiol. 28(4):569-79. (2008)

Lee CH, Yoo KY, Choi JH, Park OK, Hwang IK, Kwon YG, Kim YM, Won MH. Melatonin's protective action against ischemic neuronal damage is associated with up-regulation of the MT2 melatonin receptor. J Neurosci Res. 88(12):2630-40. (2010) doi: 10.1002/jnr.22430.

Lezoualc'h F, Skutella T, Widmann M, Behl C. Melatonin prevents oxidative stress-induced cell death in hippocampal cells. Neuroreport. 2;7(13):2071-7 (1996).

Manda K, Ueno M, Anzai K . Cranial irradiation-induced inhibition of neurogenesis in hippocampal dentate gyrus of adult mice: attenuation by melatonin pretreatment. J Pineal Res. 46: 71–78. (2009)

Margaritis I, Rousseau AS, Marini JF, Chopard A. Does antioxidant system adaptive response alleviate related oxidative damage with long term bed rest? Clin Biochem 42: 371–379 (2009)

Marino RJ, Barros T, Biering-Sorensen F, Burns SP, Donovan WH, Graves DE, Haak M, Hudson LM, Priebe MM. International standards for neurological classification of spinal cord injury. ASIA Neurological Standards Committee J Spinal Cord Med. 2003 Spring;26 Suppl 1:S50-6 (2003)

Marino RJ, Scivoletto G, Patrick M, Tamburella F, Read MS, Burns AS et al. Walking index for spinal cord injury version 2 (WISCI-II) with repeatability of the 10-m walk time: Inter- and intrarater reliabilities. Am J Phys MedRehabil 89: 7–15 (2010)

Mills BJ, Lang CA. Differential distribution of free and bound glutathione and cyst(e)ine in human blood. Biochem Pharmacol. 52:401–6. (1996)

Morin-Brureau M, De Bock F, Lerner-Natoli M. Organotypic brain slices: a model to study the neurovascular unit micro-environment in epilepsies. Fluids Barriers CNS. 7;10(1):11. (2013) doi: 10.1186/2045-8118-10-11.

Moriya T, Horie N, Mitome M, Shinohara K. Melatonin influences the proliferative and differentiative activity of neural stem cells. J Pineal Res. 42(4):411-8. (2007)

Mozes E, Hunyaa A, Posab A, Penkea B, Datkia Z. A novel method for the rapid determination of beta-amyloid toxicity on acute hippocampal slices using MTT and LDH assays. *Brain Research Bulletin* 87 521– 525 (2012)

Naseem M and Parvez S. Role of Melatonin in Traumatic Brain Injury and Spinal Cord Injury. Hindawi Publishing Corporation. The Scientific World Journal. (2014) ID 586270, http://dx.doi.org/10.1155/2014/586270

Naseem M and Parvez S. Role of Melatonin in Traumatic Brain Injury and Spinal Cord Injury ScientificWorldJournal. 2014: 586270. doi: 10.1155/2014/586270 (2014)

Noraberg J, Poulsen FR, Blaabjerg M, Kristensen BW, Bonde C, Montero M, Meyer M, Gramsbergen JB, Zimmer J. Organotypic hippocampal slice cultures for studies of brain damage, neuroprotection and neurorepair. Curr Drug Targets CNS Neurol Disord. 2005 Aug;4(4):435-52.

NSCISC National Spinal Cord Injury Statistical Center Annual Report. Spinal Cord Injury Model Systems. Complete Public Version (2014)

Oliveira VA, Oliveira CS, PortoIneua R, Moraes-SilvaL, de Siqueira LF, Pereira ME. Lactating and non-lactating rats differ in sensitivity to HgCl2: protective effect of ZnCl2. Journal ofTraceElementsinMedicineandBiology28 240–246 (2014)

Owens BM, Hawley RG, Spain LM. Retroviral transduction in fetal thymic organ culture. Methods Mol. Med. 105, 311–322 (2005)

Oyinbo CA. Secondary injury mechanisms in traumatic spinal cord injury: a nugget of this multiply cascade. Acta Neurobiol Exp (Wars). 71(2):281-99 (2011)

Padgett CM, Whorton AR. Cellular responses to nitric oxide: role of protein S-thiolation/ dethiolation. Arch Biochem Biophys. 358:232–42. (1998)

Partridge CR, Johnson CD, Ramos KS. In vitro models to evaluate acute and chronic injury to the heart and vascular systems. Toxicol. In Vitro 19, 631–644. (2005)

Pozo D, Reiter RJ, Calvo JR, Guerrero JM. Inhibition of cerebellar nitric oxide synthase and cyclic GMP production by melatonin via complex formation with calmodulin. J. Cell. Biochem. 65, 430-442. (1997)

Prakash M, Shetty MS, Tilak P, Anwar N. Total Thiols: biomedical importance and their alteration in various disorders. Ihas. 8(2). (2009)

Purves D, Augustine GJ, Fitzpatrick D, et al., editors. Neuroscience. 2nd edition. Sunderland (MA): Sinauer Associates; 2001. The Internal Anatomy of the Spinal Cord. Available from: http://www.ncbi.nlm.nih.gov/books/NBK11008/

Raisman G. Olfactory ensheathing cells - another miracle cure for spinal cord injury? Nat Rev Neurosci. May;2(5):369-75 (2001)

Ramer LM, Ramer MS, Steeves JD. Setting the stage for functional repair of spinal cord injuries: A cast of thousands. Spinal Cord 43: 134–161 (2005)

Ramirez-Rodriguez G1, Ortíz-López L, Domínguez-Alonso A, Benítez-King GA, Kempermann G. Chronic treatment with melatonin stimulates dendrite maturation and complexity in adult hippocampal neurogenesis of mice. J Pineal Res. 50(1):29-37. (2011) doi: 10.1111/j.1600-079X.2010.00802.x.

Reiter RJ, Manchester LC, Tan D-X. Neurotoxins: free radical mechanisms and melatonin protection. Current Neuropharmacology (8) 3, 194–210 (2010)

Reiter RJ, Manchester LC, Tan DX. Neurotoxins: free radicalmechanisms and melatonin protection. Curr Neuropharmacol; 8:194–210. (2010)

Reiter RJ, Melchiorri D, Sewerynek E, Poeggeler B, Barlow-Walden L,Chuang J, et al. A review of the evidence supporting melatonin'srole as an antioxidant. J Pineal Res;18: –11 (1995)

Reiter RJ, Reyes-Gonzales M, Fuentes-Broto L, Tan DX. Melatonin Reduces Oxidative Catastrophe in Neurons and Glia Activitas Nervosa Superior Rediviva 52 (2) (2010)

Reiter RJ, Reyes-Gonzales M, Fuentes-Broto L, Tan DX. Melatonin Reduces Oxidative Catastrophe in Neurons and Glia. Activitas Nervosa Superior Rediviva Volume 52 No. 2 (2010)

Reiter RJ, Tan D-X, Terron MP, Flores LJ, Czarnocki Z. Melatonin and its metabolites: new findings regarding their production and their radical scavenging actions. Acta Biochimica Polonica 54 (1) 1–9 (2007)

Reiter RJ. Functional pleiotropy of the neurohormone melatonin: antioxidant protection and neuroendocrine regulation. Front Neuroendocrinol.16(4):383-415 (1995)

Reiter RJ. Functional pleiotropy of the neurohormone melatonin: antioxidant protection and neuroendocrine regulation. Front Neuroendocrinol. 16(4):383-415 (1995)

Rezzani R, Buffoli B, Rodella L, Stacchiotti A, Bianchi R. Protective role of melatonin in cyclosporine A-induced oxidative stress in rat liver. Int Immunopharmacol. Aug;5(9):1397-405 (2005)

Rezzani R, Rodella LF, Favero G, Damiani G, Paganelli C, Reiter RJ. Attenuation of ultraviolet A-induced alterations in NIH3T3 dermal fibroblasts by melatonin. British Journal of Dermatology 170, 382–391 (2014)

Ritz MF, Hausmann ON. Effect of 17β-estradiol on functional outcome, release of cytokines, astrocyte reactivity and inflammatory spreading after spinal cord injury in male rats. Brain Research, (1203), 177–188, (2008)

Rowland JW, Hawryluk GWJ, Kwon B, Fehlings MG. Current Status of Acute Spinal Cord Injury Pathophysiology and Emerging Therapies: Promise on the Horizon. Neurosurg Focus. 25(5):E2 (2008)

Samantaray S, Sribnick EA, Das A, Knaryan VH, Matzelle DD, Yallapragada AV, Reiter RJ, Ray SK, Banik NL. Melatonin attenuates calpain upregulation, axonal damage and neuronal death in spinal cord injury in rats. J Pineal Res. 44(4):348-57. (2008)

Sarnowska A . Application of organotypic hippocampal culture for study of selective neuronal death. Folia Neuropathol. Vol. 40, No. 2, 101–106 (2002)

Scheff SW, Ansari MA, Roberts KN, Neuroprotective effect of Pycnogenol following traumatic brain injury. Experimental Neurology (239) 1, 183–191, (2013)

Schiaveto-de-Souza A, da-Silva CA, Defino HLA Del Bel EA. Effect of melatonin on the functional recovery from experimental traumatic compression of the spinal cord. Brazilian Journal of Medical and Biological Research. 46: 348-358. (2013) http://dx.doi.org/10.1590/1414-431X20132322

Schiaveto-de-Souza A, da-Silva CA, Defino HLA, Del Bel EA. Effect of melatonin on the functional recovery from experimental traumatic compression of the spinal cord. Brazilian Journal of Medical and Biological Research 46: 348-358. (2013) http://dx.doi.org/10.1590/1414-431X20132322

Singh A, Tetreault L, Kalsi-Ryan S, Nouri A, Fehlings MG. Global prevalence and incidence of traumatic spinal cord injury. Clin Epidemiol. 23;6:309-31. doi: 10.2147/CLEP.S68889. (2014)

Standring S. Gray's Anatomy, 40st Edition. The Anatomical Basis of Clinical Practice. (2012)

Staton CA, Stribbling SM, Tazzyman S, Hughes R, Brown NJ, Lewis CE. Current methods for assaying angiogenesis in vitro and in vivo. Int. J. Exp. Pathol. 85, 233–248 (2004)

Steinhilber D, Brungs M, Werz O, Wiesenberg I, Danielsson C, Kahlen JP, Nayeri S, Schräder M, Carlberg C. The nuclear receptor for melatonin represses 5-lipoxygenase gene expression in human B lymphocytes. J Biol Chem. 31;270(13):7037-40. (1995)

Steinhilber D1, Brungs M, Werz O, Wiesenberg I, Danielsson C, Kahlen JP, Nayeri S, Schräder M, Carlberg C The nuclear receptor for melatonin represses 5-lipoxygenase gene expression in human B lymphocytes. J Biol Chem. Mar 31;270(13):7037-40 (1995)

Sugden D, Davidson K, Hough KA, Teh MT. Melatonin, melatonin receptors and melanophores: a moving story. Pigment Cell Res. 17(5):454-60. (2004)

Sullivan PG, Thompson MB, Scheff SW. Cyclosporin A attenuates acute mitochondrial dysfunction following traumatic brain injury. Exp Neurol 160: 226–234 (1999)

Sypecka J, Koniusz S, Kawalec M, Sarnowska A. The organotypic longitudinal spinal cord slice culture for stem cell study. Stem Cells Int. 2015:471216. (2015) doi: 10.1155/2015/471216.

Tan D-X. Melatonin and Brain. Current Neuropharmacology 8(3):161 (2010)

Tator CH, Fehlings MG. Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms. J Neurosurg 75: 15–26 (1991)

Tator CH, Koyanagi I. Vascular mechanisms in the pathophysiology of human spinal cord injury. J Neurosurg 86: 483–492 (1997)

Tator CH, McCormick PC, Piepmeier JM, Benzel EC, Young W Biology of neurological recovery and functional restoration after spinal cord injury. J Neurosurg 42: 696–708 (1998)

Tator CH. Review of treatment trials in Human spinal cord injury: issues, difficulties, and recommendations. J Neurosurg 59: 957–987 (2006)

Toklu HZ, Hakan T, Celik H, Biber N, Erzik C, Ogunc AV et al. Neuroprotective effects of alpha-lipoic acid in experimental spinal cord injury in rats. J Spinal Cord Med 33: 401–409 (2010)

Wiesenberg I, Missbach M, Carlberg C. The potential role of the transcription factor RZR/ROR as a mediator of nuclear melatonin signaling. Restor Neurol Neurosci. 12(2-3):143-50 (1998)

Wilson JR, Forgione N, Fehlings MG. Emerging therapies for acute traumatic spinal cord injury. CMAJ : Canadian Medical Association Journal. 185(6):485-492. doi:10.1503/cmaj.121206 (2013)

Yang Y, Jiang S, Dong Y, Fan C, Zhao L, Yang X, Li J, Di S, Yue L, Liang G, Reiter RJ, Qu Y. Melatonin prevents cell death and mitochondrial dysfunction via a SIRT1-dependent mechanism during ischemic-stroke in mice. J Pineal Res. 58(1):61-70. doi: 10.1111/jpi.12193 (2015)

Yoon JJ, Suri V, Nicholson LFB, Green CR. Improved Immunohistochemical Protocol for Organotypic Brain Slice Cultures Microscopy and analysis. Microscopy and analysis. 143; 5-9 (2011)

Zawilska JB, Skene DJ, Arendt J. Physiology and pharmacology of melatonin in relation to biological rhythms. Pharmacological Reports. 61:3, 383–410 (2009)

Zhang N, Yin Y, Xu SJ, Wu YP, Chen WS. Inflammation & apoptosis in spinal cord injury. Indian J Med Res. March; 135(3): 287–296 (2012)