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Chapter

# Current Developments in Antioxidant Therapies for Spinal Cord Injury

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## Abstract

When spinal cord injury (SCI) occurs, numerous sources of reactive oxygen species and nitrogen species may be active within first minutes or hours and even reactivate few days later. Free radical formation and lipid peroxidation (LP) have been described as an important mechanism in the beginning and accelerated progress in the development of diverse pathologies, importantly in those related to central nervous system. The compromise of molecules and cellular structures due to the oxidative state of microenvironment in SCI may determinate survival or apoptosis of resident and infiltrating cells and polarization toward an inflammatory response, which lead to an extension of damaged tissue and loss of neuronal function, or a regulatory/regenerative response. The investigation of new antioxidant agents and their action at a molecular level begins to reveal mechanisms that, if correctly modulated, promise an improvement in recovery of functions with respect to conventional pharmacological therapies. In this chapter, we will review the general mechanisms of oxidative stress and lipid peroxidation, those antioxidant treatments in experimental development and clinical phase, as well as their achievements and limitations.

**Keywords:** antioxidant therapy, lipid peroxidation, free radicals, spinal cord injury, nitric oxide

## 1. Introduction

Among the different pharmacological strategies for treating spinal cord injury (SCI), it has been observed that the quick intervention after the injury results in a better outcome for the patients [1]. This can be explained by the biochemical processes occurring at a cellular level that develop immediately after the mechanical damage, which define the subsequent physiological chain of events determining the evolution of pathophysiology of the SCI and, therefore, the degree of functional loss or recovery. One of the most important processes participating in the balance between the prevalence of damage or protection of tissue structure and the function in the central nervous system (CNS) is the generation of diverse reactive molecules by oxidative stress that target mainly lipids. This process is known as lipid

peroxidation (LP), and its end products could modify proteins and DNA present in cellular structures, causing cell death and a lower probability of regeneration [2]. SCI is a highly disabling and irreversible condition that causes physiological complications (bowel, cardiac, urinary, respiratory) and it has a social-economic impact in patients. The research of new agents targeting degenerative processes such as oxidative stress and LP is important especially due to the lack of efficacy and safety of conventional therapies on patients with SCI [1]. Here, we review the efforts to discover new compounds aimed to offer an option in antioxidant treatments and the use of some in combination or in an innovative way, both in experimental and in clinical trials. We would like to mention that there is a wide range of antioxidant therapies in study, and we are only briefly mentioning some of them at this time.

#### 2. Acute spinal cord injury mechanisms

The pathophysiology of the SCI has been divided in primary and secondary injury, the latter generally described in acute and chronic phases. The mechanisms involved in the secondary injury include biochemical degenerative processes that exacerbates damage, such as the loss of blood-spinal cord barrier (BSCB) integrity, ischemia/reperfusion, hypoxia, loss of ionic homeostasis, Ca<sup>2+</sup> overload, glutamatergic excitotoxicity, immune cell invasion, inflammation, release of cytokines, free radical (FR) production, LP, and excessive production of nitric oxide (NO<sup>•</sup>). All these events occur in the acute SCI and may be clinically targeted due to their times of action, different from the unexpected primary injury [3] (**Figure 1**). It has also been demonstrated that these mechanisms are related in a way that exacerbates when the levels of oxidative stress and LP molecules are increased and that attenuates its effects when the antioxidant treatment is immediately given after SCI [4].

#### 2.1 Mechanism of oxidative stress and free radical's generation

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are molecules that participate in oxidative stress. They are endogenously produced under physiological conditions, and in low amounts, they are essential for biological and immune process [4]. Oxidative stress could be defined as a disturbance in the pro-/antioxidant equilibrium, for the presence of high levels of ROS and RNS that exceeds the endogenous antioxidative defense mechanisms, and they are associated with damage to a wide range of molecular species, such as lipids, proteins, and nucleic acids, contributing to the pathophysiology of SCI [3].

ROS are oxygen-derived compounds that include radicals (unstable molecules with a single unpaired electron), such as superoxide  $(O_2^{\bullet^-})$ , hydroxyl (HO<sup>•</sup>) and peroxyl (RO<sub>2</sub><sup>•</sup>/HO<sub>2</sub><sup>•</sup>) radicals, and non-radicals such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Within the first minutes and hours post-injury, different sources of O<sub>2</sub><sup>•-</sup> such as arachidonic acid cascade, mitochondrial leak, and enzymes systems [nicotin-amide-adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidases, cyclooxygenase (COX), and xanthine oxidase], present in activated microglia and infiltrating cells (macrophages and neutrophils), may act providing O<sub>2</sub><sup>•-</sup> [5], derived from the reduction of oxygen molecules (O<sub>2</sub>) with a single electron (e<sup>-</sup>). Although O<sub>2</sub><sup>•-</sup> itself is reactive, its direct oxidative reactivity toward biological substrates in aqueous environments is relatively weak, but it distinguishes itself as an active nucleophile and oxidizing agent that can react with hydrogen donors (e.g., ascorbate and tocopherol) [4–6]. On one hand, superoxide dismutase (SOD) rapidly catalyzes the dismutation of O<sub>2</sub><sup>•-</sup> into H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub> (2O<sub>2</sub><sup>•-</sup> + 2H<sup>+</sup> → H<sub>2</sub>O<sub>2</sub> + O<sub>2</sub>), and at low pH, O<sub>2</sub><sup>•-</sup> can dismutate spontaneously.



#### Figure 1.

Progression of the inflammatory response in spinal cord parenchyma. The condition produced by the mechanical injury induces the activation of the damage targeting mechanisms, initially propitiated by the resident microglia, which secretes pro-inflammaory cytokines. Astrocytes and endothelial cells allow the permeabilization of BSCB and express chemoattractants to facilitate the admission of immune cells from the periphery, increasing the response at site. Collateral injuries can occur, largely due to the low antioxidant capacity of neural tissue to counteract the ROS produced by inflammatory cells, spreading the damage to other uninvolved cells. The extent of the initial damage is proportional to the final capacity of the organism to recover its motor and sensory functions [155].

In oxidative stress, this  $H_2O_2$  can react with transition metal cations to form oxidizing species such as HO<sup>•</sup> and hydroxyl anion (HO<sup>-</sup>), and this occurs mainly in the presence of iron (Fe) and cooper (Cu) ions. The central nervous system (CNS) is rich in ferric iron (Fe<sup>3+</sup>), contained in transferrin in plasma, and ferritin intracellularly. This iron can be released from its transporters at pH values of 6 or less, like the one reached in hypoxia and accumulation of lactic acid in SCI, and become catalytic; a second source for Fe comes from the hemoglobin released after mechanical-induced hemorrhage.  $O_2^{\bullet-}$  acts donating an electron to Fe<sup>3+</sup>, and the ferrous iron (Fe<sup>2+</sup>) catalyzes the conversion of H<sub>2</sub>O<sub>2</sub> to HO<sup>•</sup> and HO<sup>-</sup>. Therefore,  $O_2^{\bullet-}$  and H<sub>2</sub>O<sub>2</sub> react in the presence of Fe<sup>3+</sup>/Fe<sup>2+</sup> and promote the formation of HO<sup>•</sup> and HO<sup>-</sup> [2].

On the other hand,  $O_2^{\bullet-}$  can interact with NO<sup>•</sup>, a hydrophobic and mildly reactive radical generated enzymatically from L-arginine by nitric oxide synthase (NOS) isoforms, and give rise to one of the most important RNS, peroxynitrite ONOO<sup>-</sup> (NO<sup>•</sup> +  $O_2^{\bullet-} \rightarrow ONOO^-$ ), a potent oxidizing and nitrating agent in vivo, either for direct oxidation reactions, in which it reacts with targets of low molecular weight and proteins (with thiols and metal centers), and carbon dioxide, or by derived radicals from homolytic cleavage, secondary to the reaction with carbon dioxide or protonation, included in RNS [2, 7]. Under biological conditions, ONOO<sup>-</sup> exists in equilibrium with its acidic form, the peroxynitrous acid (ONOOH), which decays rapidly by homolysis to give place to highly reactive nitrogen dioxide radical (NO<sub>2</sub><sup>•-</sup>) and HO<sup>•</sup> favored by the low pH in SCI [8]. Among the different direct reactions of ONOO<sup>-</sup>, one of the most relevant is this with CO<sub>2</sub> (from bicarbonate buffer system), to form nitrosoperoxocarbonate (ONOOCO<sub>2</sub>), forming by cleavage strong oxidant agents, such as nitrogen dioxide (NO<sub>2</sub><sup>•-</sup>) and carbonate (CO3<sup>•-</sup>) radicals [7, 8].

#### 2.2 Lipid peroxidation (LP)

Lipids are the most susceptible class of biomolecules to undergo oxidation; polyunsaturated fatty acids (PUFAs) are long-chain fatty acids with two or more double bonds in *cis* configuration, each separated by a methylene bridge (–CH<sub>2</sub>–) at their carbon backbone, and the hydrogen attached to the methylene bridge is very easy to remove. The LP is defined as an oxidative degradation and decomposition of lipids in an uncontrolled manner by nonenzymatic pathway and occurs when ROS react with PUFAs, leading to the modification of its physicochemical properties, disrupting the cellular membrane integrity. The enzymatic pathway produces lipid mediators such as prostanoids, leukotrienes, lipoxins, resolvins, and maresins by the action of COX or lipoxygenases (LOX), among others, causing dysregulation of blood flow, BSCB damage, inflammatory response, and programmed cell death pathway [9]. The CNS is particularly vulnerable to LP by various factors: it has high oxidative metabolic activity, PUFA content, and transition metal cations. In contrast, it has low antioxidant defenses and neuron-glia replication [8, 10].

The LP is a chain process that involves the participation of ROS, RNS, PUFAs, and oxidative systems, among others, where therapeutic intervention has been proposed with molecules that can both prevent FR formation and prevent those already formed from reacting with biomolecules. Because the peak of ROS production occurs within the first 24 h after the injury, or during ischemia-reperfusion, the drugs that can be used for this "first FR production" are limited by their time of intervention. However, the phases in which LP develops may persist as long as there are oxidizable substrates, so knowing the reactions involved allows the design of strategies and drugs with a greater therapeutic window [11, 12]. The nonenzymatic peroxidation of PUFAs is the principal pathway of oxidative stress; HO<sup>•</sup> participates as one of the starts of LP due to its solubility and the lack of an enzymatic system to eliminate it. This and other radicals remove an H<sup>•</sup> radical inside a lipid (LH), which provides a lipid radical (L<sup>•</sup>) [11, 12] (**Figure 2**). The resonance stabilization of L<sup>•</sup> produces a conjugated diene that reacts with O<sub>2</sub> to form a lipid peroxyl radical (LOO<sup>•</sup>) and generates a lipid hydroperoxide (LOOH) when it withdraws hydrogen from an adjacent PUFA, producing a second L<sup>•</sup> [2, 12]. The LOOH are regarded as the initial product of LP, but these compounds are unstable and can be discomposed with the participation of Fe<sup>3+</sup> or Fe<sup>2+</sup> again in LOO<sup>•</sup> or



The three steps of nonenzymatic lipia peroxidation of PUFAs. In the initiation step, a hydrogen atom at a bis allylic position is removed using either a radical or a redox active metal to generate a resonance-stabilized alkyl radical. The radical isomerizes to form the more stable conjugated diene, prior to reacting with molecular oxygen. In the propagation step, radicals are able to react with new substrates, forming lipid hydroperoxides (LOOH), which can react with iron creating new radicals. This step repeats until the termination step, where radicals are "quenched" by antioxidants or react with another radical. The decomposition of LOOH generates species such as MDA, HNE, etc. LH, lipid; L\*, alkyl/lipid radical; LOO\*, peroxyl radical; LOOH, lipid hydroperoxide; LO\*, lipid alkoxyl radical; HNE: 4-hydroxy-2-nonenal; MDA: malondialdehyde; HHE: 4-hydroxy-2-hexenal. Modified from Gaschler and Stockwell [12].

alkoxyl (LO<sup>•</sup>) radicals, respectively. Both, the reduction of the LOO<sup>•</sup> to an LO<sup>•</sup> by  $Fe^{2+}$  (LOOH +  $Fe^{2+} \rightarrow LO^{\bullet} + HO^{\bullet} + Fe^{3+}$ ) and its conversion back to LOO<sup>•</sup> (LOOH +  $Fe^{3+} \rightarrow LOO^{\bullet} + Fe^{2+}$ ) reactions, have acidic pH optimal conditions and are more likely to occur in SCI tissue environment [5]. The LO<sup>•</sup> can initiate chain reactions too, such as the LOO<sup>•</sup> reactions describe above. Thus, one HO<sup>•</sup> can generate a high number of LOOH through a series of chain reactions. Finally, termination of chain reactions occurs by the stabilization of the radicals reacting between themselves,

forming a new bond and eliminating the radical, or by donating electrons (generally H<sup>•</sup>) to the radicals by compounds, without turning into radicals. In the case of LOOH provided in the previous LP reactions, these undergo fragmentation in which oxidized PUFAs give rise to short-chain secondary products, such as hydroxy-alkenals (neurotoxic aldehydes) relatively stable like malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal (HHE), and 2-propenal (acrolein), that can diffuse within or even escape from the cell and attack targets far from the site of the original event [13] (**Figure 2**). In general, the LOOH can react in different ways that lead to a cleavage of the C-C bond and formation of hydroxy-alkenals by means of different mechanisms [13].

While the LP compromises the integrity of the cell membrane, the highly reactive secondary products can be covalently bound to proteins and DNA, compromising their structure and function. Regarding the HNE as the most studied product of LP, it must be mentioned that the HNE physiological concentration inside the cell ranges from 0.1 to 3  $\mu$ M. Moreover, under oxidative stress conditions, HNE can accumulate at concentrations that range from 5  $\mu$ M to 10 mM [14]. It has been demonstrated that HNE can play an important role as a signaling molecule, enhancing cellular antioxidant capacity and adaptive response at low concentrations; can promote protein and DNA damage in organelles, leading to the induction of autophagy, senescence, or cell cycle arrest; and finally can induce apoptosis or necrosis programmed cell death at a high or very high level [13, 15, 16].

#### 2.3 Proteins as target of oxidation

The oxidation of proteins for ROS can lead to the hydroxylation of aromatic groups and aliphatic amino acid (aa) side chains, nitration of aromatic aa residues, reversible nitrosylation of sulfhydryl groups, sulfoxidation of Met residues, conversion of some aa residues to carbonyl derivatives, cleavage of the polypeptide chain, and formation of cross-linked protein aggregates. Furthermore, functional groups of proteins can react with products of LP and carbohydrate derivatives (glycation/glycoxidation) to produce inactive derivatives [17], where the irreversible protein oxidation is described by four pathways: peptide bond rupture, carbonylation, formation of protein-protein bonds, and nitration [18]. The initial oxidation can form a carbon-centered radical, which can react with  $O_2$  to form a ROO<sup>•</sup>, to cleave protein backbone by either  $\alpha$ -amidation or diamide pathways.

The cleavage of side chains (glutamyl, aspartyl, and probably prolyl side chains) may occur directly or by metal-catalyzed oxidation (proline [Pro], arginine [Arg], lysine [Lys], and threonine [Thr] residues), yielding carbonyl derivatives [17, 18]. One of the most important of irreversible oxidation processes is by protein carbonylation. It involves the previous protein and aa carbonyl derivatives ,CO<sub>3</sub><sup>--</sup> oxidation (reacting preferentially on tryptophan [Trp], Thr, cysteine [Cys], methionine [Met], and histidine [His] residues), ketones and aldehyde reactions over Cys, Lys, His, and by glycation/glycoxidation of Lys amino groups, etc. [2, 8, 17, 18].

The modification of the protein structure after oxidation can also give rise to intra- or inter-protein cross-linked derivatives by several different mechanisms. For example, the protein-protein bond may be due to the interaction of two carboncentered radicals or two aromatic aa residues radicals, formed by direct attack of ROS [17]. Final products of LP, such as HNE and MDA, can cause cross-linked proteins, as reactions of both MDA aldehyde groups with two different residues in the same protein or two different proteins [17]. Another protein-protein bond is disulfide bridge (RSSR) that results from the oxidation of thiols (RSH) forming sulfenic acid (RSOH) as the last intermediate and reacting with another thiol, forming RSSR. This can be promoted in the presence of OONO<sup>-</sup> or driven by ROS

and RNS, with the possibility of chain reactions [8]. Regarding this, some enzymes containing Cys, in its catalytic site, can act as scavengers, by direct interaction or consuming glutathione (GSH), due to the reversible modification of the RSSR bond [8]. HNE possess three functional groups in its structure (Figure 2), making this electrophilic molecule highly reactive toward nucleophilic groups such as thiol (-SH) and amino ( $-NH_2$ ). Thus, as such as Cys, Lys, His, and Arg are HNE targets, whose modification inhibits the functions of a variety of enzymatic and structural cellular proteins [19]. MDA with enhanced reactivity in low pH and existing as  $\beta$ -hydroxyacrolein is strongly reactive to nucleophiles such as Lys, His, or Arg residues [20]. Protein modifications by RNS act over aromatic, Cys, and Met residues; OONO<sup>-</sup> reacts directly with thiol groups present in a variety of proteins such as GSH, albumin, and metalloproteins (heme, myeloperoxidase, cytochrome P450, SOD isoforms, etc.) forming nitrite (NO<sub>2</sub><sup>-</sup>), nitrate (NO<sub>3</sub><sup>-</sup>), or NO<sub>2</sub><sup>--</sup>[8]. Finally, irreversible protein tyrosine nitration by  $NO_2^{\bullet-}$ , with substitution of a hydrogen in the position 3 of the phenolic ring, produces 3-nitrotyrosine (NT-3) as a specific footprint of induced cellular damage by OONO<sup>-</sup> [2, 8]. From all these modifications, diverse molecules can be identified both in cerebrospinal fluid and blood, both in humans and in animals, and they have been proposed as biomarkers to diagnose the severity of SCI. Some of those biomarkers derived from proteins are neurofilament proteins, glial fibrillary acidic protein (GFAP), tau, neuron-specific enolase, and S100 calcium-binding protein  $\beta$  (S100 $\beta$ ), being part of the components of neurons, oligodendrocytes, and reactive astrocytes. A more detailed list can be found in the works of Lubieniecka et al. and the Hulme et al. review [21, 22].

#### 2.4 DNA damage

The ROS/RNS produced in oxidative stress and LP can damage the nucleic acids of DNA; cause DNA-protein cross-links, strand breaks, and modification of purine and pyridine bases; and lead to DNA mutations. More than 20 DNA adducts have been identified, such as 8-hydroxy-2'-guanosine (8-OHdG), increased in patients in whom the antioxidant systems are suspected to be deficient [23]. MDA is an important contributor to DNA damage and mutations that can react with several nucleosides (deoxyguanosine and cytidine) to form adducts, and the major resulting product is a pyrimido-purinone called M1dG [24]. HNE can also react with deoxyguanosine to form two pairs of diastereomer adducts (4-HNE-dG 1,2 and 3,4) or etheno-DNA adducts in the presence of peroxides that could further induce DNA cross-link or DNA-protein conjugates [25, 26]. Other markers of oxidative damage in DNA, among other biomolecules, were reviewed in [23].

#### 2.5 Enzymatic and nonenzymatic antioxidant systems

The cellular antioxidant systems are composed by antioxidant enzymes and nonenzymatic molecules able to donate electrons to different radical chemical structures. In the CNS, they are present in lower concentrations than the oxidizable substrate and are responsible of maintaining the pro-/antioxidant equilibrium, relieving oxidative stress, and reducing or interrupting uncontrolled LP, DNA mutations, protein oxidation/degradation, as well as other cell damage features. The essential endogenous components of the enzymatic antioxidant defense are SOD, catalase (CAT), glutathione peroxidases (GPx), glutathione reductases (GR), and glutathione S-transferases (GST), while the nonenzymatic antioxidants include GSH, proteins (ferritin, transferrin, ceruloplasmin, metallothionein, thioredoxin (Trx), albumin), vitamins C and E (tocopherol), trace elements, and low molecular weight scavengers, such as uric acid, coenzyme Q,

and lipoic acid [4, 6, 23], which act by depleting molecular  $O_2$  or decreasing its local concentration; removing pro-oxidative metal ions; trapping aggressive ROS, such as  $O_2^{\bullet-}$  or  $H_2O_2$ ; scavenging chain-initiating radicals like HO<sup>•</sup>,  $RO_2^{\bullet}/HO_2^{\bullet}$ , or LO<sup>•</sup>; or breaking the chain of a radical sequence [4]. There are also important exogenous nonenzymatic antioxidants (vitamins A, C, E, flavonoids, carotenoids, phenolics, acetylcysteine, exogenous selenium, zinc), acquired through diet, which are being studied. A table of these enzymatic and nonenzymatic antioxidants important in the CNS was reviewed in [23]. Preventing the formation of ROS, or at least its accumulation, and blocking or capturing those radicals already formed is the first defense against oxidative stress. The  $O_2^{\bullet-}$  generated by various sources can be converted to  $H_2O_2$  by SODs [4]. The  $O_2^{-1}$  intracellularly produced in the mitochondria can be converted into  $H_2O_2$  by MnSOD (SOD3) [18]. Once generated,  $H_2O_2$  (but not other peroxides) is decomposed to water and oxygen  $O_2$  (2H<sub>2</sub>O<sub>2</sub> + 2GHS  $\rightarrow$  H<sub>2</sub>O + O<sub>2</sub>) by the action of CAT, a ferrihemecontaining enzyme. However, small amounts of ROS escape from the antioxidant defense and can be converted to HO<sup>•</sup>, which may be scavenged by low molecular mass nonenzymatic antioxidants, such as ascorbate, tocopherol, GSH, etc. [27].  $H_2O_2$  is also reduced by the action of different peroxidases, such as GPx  $(H_2O_2 + 2GHS \rightarrow H_2O + GSSG)$ , which, additionally, can reduce lipid hydroperoxides (LOOH + 2GSH  $\rightarrow$  LOH + GSSG) [11, 12]. Other enzymes that catalyze this reaction include peroxiredoxin and thioredoxin reductase [4]. Some enzymes that participate in the detoxification of LP products by oxidation, reduction, and glutathione conjugation, the latter being a mechanism also used to reverse the effects of RNS, are aldehyde dehydrogenases (ALDH), alcohol dehydrogenase (ADH), aldo-keto reductase (AKR), and the aforementioned GST, GPx, and GR [28].

In SCI, the primary injury causes disruption of blood flow and vascular insult, such as ischemia-reperfusion, which conducts to the loss of metabolic function of cells in gray matter with decrease of ATP, causing depolarization of membranes due to the inhibition of Na<sup>+</sup>/K<sup>+</sup> and Ca<sup>2+</sup> ATPases function. Ca<sup>2+</sup> overload and glutamate excitotoxicity compromise the function and integrity of mitochondria through the activation of proteases and inactivation of important enzymes. Due to the low ratio of antioxidant systems' oxidizable substrate in acute SCI, the mitochondrial antioxidant reserves decrease and are incapable of restoring the redox equilibrium, giving place to an increase of mitochondrial concentration of O<sub>2</sub><sup>--</sup> and an increase and leak of free radicals formed downstream including ONOO<sup>-</sup>, initiating LP. The damage produced by this excess of radicals or end products of LP over proteins and membranes of the mitochondria and endoplasmic reticulum potentiates the processes of secondary injury mentioned here to the local and adjacent cells to SCI [4].

#### 3. Antioxidant therapy strategies

The early therapeutic intervention for SCI is crucial to improve the chances of maximum possible recovery. This was observed in clinical trials where the current treatment of choice, methylprednisolone sodium succinate (MP or MPSS), was effective only when administered within the first 8 h after injury, at high doses (5.4 mg/kg/h). In 48-h regimens, however, it increases the incidence of complications from infections (severe sepsis and pneumonia), while the 24-h safe regimen is not effective in the long term, at least after 3 h [1]. Being an ineffective treatment, there are no alternative therapeutic treatments that offer safety and certainty regarding the recovery of the motor function. The research of new

pharmacological agents for the treatment of SCI focuses on the processes of secondary injury, being antioxidant therapies the most important. The main goals of drug therapies for SCI can be classified in neuroprotection and neuroregeneration; antioxidant therapies are cataloged within the first. Here we present some of the agents that are in experimental phase and others when mentioned, in clinical trials, either because their efficacy has been demonstrated in animal models or because of their use already approved in other pathologies. Regarding the diverse SCI models, they have been used to simulate SCI with high relevance and validity to preclinical evaluation due to the replication of human traumatic injuries. The rational use of animals is strongly controlled, and the possibility of pain and distress must be considered and minimized by veterinary staff through the appropriate use of analgesics and animal care.

#### 3.1 Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is a pleiotropic compound that works mainly in the regulation of circadian rhythms and sleep. When reacting with ROS, such as HO<sup>-</sup>, H<sub>2</sub>O<sup>+</sup>, and LOO<sup>+</sup>, it is converted to cyclic 3-hydroxymelatonin. It stimulates the expression and activity of SOD, GPx, CAT, and GR and inhibits or decreases the expression of pro-oxidative NOs, different signaling pathways, transcription factors, and pro-inflammatory cytokines [29-31]. Decreased melatonin production has been linked to various CNS disorders, and the neuroprotective activity was detected in rat models of traumatic brain injury ischemic stroke and SCI [29, 30]. To cite only some SCI examples, in a study in Sprague-Dawley rats of 250 g with moderate lesion, 10 mg/kg of melatonin was applied subcutaneously twice a day for 4 weeks, and an increase in motor recovery and decrease in inducible nitric oxide synthase (iNOS) expression were observed. Intravenously, it decreased the synthesis of MDA and increased the synthesis of GSH and angiopoietin 1, and in mice with severe lesion, it decreased the expression of interleukin 1 beta (IL-1 $\beta$ ) and NG-2 (neuron/glial antigen 2) [30, 32, 33]. In a model with lesion with vascular clips, the administration of 30 mg/kg alleviated post-traumatic injury associated with SCI by binding the PPAR $\alpha$ -receptor; the administration of 50 mg/kg in moderate lesion decreased the BSCB permeability modulating the expression of brainderived neurotrophic factor (BDNF), growth-associated protein 43 (GAP-43), and caspase-3 [33–35]. In combination therapy with dexamethasone (10–0.025 mg/kg), it showed significant anti-inflammatory effects, attenuating the synthesis of tumor necrosis factor alpha (TNF- $\alpha$ ) and iNOS and the nitration of tyrosine residues, increasing tissue recovery and motor capacity in an experimental SCI model of mouse [36], while the combination with methylprednisolone favored neurological recovery and decreased LP; its administration with zinc activated the internal antioxidant system and also decreased the LP [37–39].

#### 3.2 Minocycline

Minocycline hydrochloride is an available semisynthetic tetracycline antibiotic with potent anti-inflammatory (regulation of phospholipase A<sub>2</sub> and MAPK/PIK3 pathways) and neuroprotective (protecting against glutamate-induced inflammation) activities; it also inhibits matrix metalloproteinases and mitochondrial Ca<sup>2+</sup> influx. Minocycline has antioxidant and antiapoptotic properties, probably acting at high doses as a direct radical scavenger, like vitamin E, due to its phenolic ring structure [40]. In rats with SCI, minocycline given at oral doses of 3, 30, and 90 mg/kg 1 and 24 h after the lesion reduced MDA concentration and increased

GPx and SOD activity in a dose-dependent manner [41]. Minocycline decreased pro-inflammatory cytokines and the chemokines release from microglia and their activation, including their levels of enzymes that regulate LP and NO production [42]. A recovery difference between treatment and placebo, approaching to statistical significance in patients with cervical injury, was shown in a phase II clinical trial. The trail determined safety and dose optimization, within 12 h of SCI and for 7 days, with steady-state concentrations of 12.7 µg/mL in serum and 2.3 µg/mL in in cerebrospinal fluid (ClinicalTrials.gov number NCT00559494) [43].

### 3.3 Estrogen

Treatment with gonadal steroid hormones (estradiol, testosterone, estrogen) has resulted in motor recovery with a reduction of the lesion volume in animal models. Through its receptors (ER $\alpha$  and ER $\beta$ ), estrogen exerts neuroprotection at physiological concentrations, and it exerts better neuroprotection as an antioxidant at high concentrations. Estrogen modulates gene expression; promotes angiogenesis; inhibits inflammation, blocking microglia from releasing inflammatory molecules such as TNF- $\alpha$ , ROS, prostaglandin E<sub>2</sub>, etc.; regulates the expression of antioxidant enzymes; and induces mitochondrial GSH production [44]. Different low doses and times of administration (between 10 and 100  $\mu$ g/ kg/day/7 days to 4 mg/kg/15 min and 24 h, i.v.) appear to be effective, suggesting that pre-treatment or immediate posttreatment at either physiological or supraphysiological dose could minimize secondary injury in SCI and promote functional recovery, reflected in both acute and chronic stages [44, 45]. Additionally, the development of selective agonists of ER with higher affinity for ER $\alpha$ , ER $\beta$ , or both, such as tamoxifen, looks promising in SCI treatment, when applied in subdermal implants 7 days before, immediately, or 24 h post-injury; with an immediate release of 0.71 mg/day for 21 days, it provided motor recovery and preservation of white matter, dorsal and ventral horn neurons, with a decrease of  $O_2^{\bullet-}$  production [46].

### 3.4 Omega-3 fatty acids

The omega-3 fatty acids:  $\alpha$ -linolenic acid, eicosapentaenoic acid (EPA, with five unsaturated bonds), and docosahexaenoic acid (DHA, with six unsaturated bonds) are part of the triacylglycerols that are consumed in the diet. DHA is a primary structural component of human brain, cerebral cortex, and retina. The lack of DHA may affect the fluidity and integrity of the membrane in synaptosomes; additionally, it affects the architecture of proteins that act as receptors and channels. Several studies have studied the effects of DHA in SCI, with treatments that include intravenous bolus, nutritional supplementation, and the use of transgenic [47]. In SCI in rats, a single application of DHA (250 nmol/kg, i.v., 30 min after injury) showed an improvement in motor recovery, smaller lesion size, greater survival of neurons and oligodendrocytes, and lower oxidation of DNA/RNA in comparison to rats without treatment [48]. More details of the application of DHA in SCI are mentioned in the chapter on Samaddar [47], as well as interesting effects on molecules involved in the repair and conservation of axonal integrity.

### 3.5 Endogenous antioxidants (vitamins C, D, and E and ubiquinol)

Several molecules that already act as endogenous antioxidants have been studied as candidates for application in antioxidant therapies for SCI. Vitamin C, or ascorbic acid, is a small water-soluble molecule that has a double bond and participates in

various metabolic processes as a reducing agent. It is considered nontoxic because it does not accumulate and its concentration declines during SCI. In rats, it decreases tissue inflammation and necrosis and only at high doses (200 mg/kg i.p. 1-h postinjury, daily, until they were sacrificed, 4th week) showed improvements in motor evaluations [49]. Vitamin D (1,25-dihydroxyvitamin D3, VDH, active form) is a molecule with cholesterol skeleton and acts similarly to hormones and steroids on several systems. Its receptor (VDR) is widely distributed in the CNS, and it apparently acts on the same targets as progesterone through similar pathways. Its use in CNS damage models in vivo and in vitro has shown promising results on several aspects. The prolongation or exacerbation of inflammation also gives way to greater damage by oxidative stress; therefore, the effect of VDH in vivo on the inhibition of iNOS and increase of IL-4 and TGF- $\beta$  and in vitro modulating the production of molecules involved in oxidative stress, neurotoxic damage, and axonal growth on various cells are of interest for being use in SCI [50]. Tocopherols are a group of four fat-soluble phenolic compounds designated  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ , which are found in vegetable oils, being alpha ( $\alpha$ -T, considered the classic vitamin E) the one with the highest proportion in blood and tissues. All tocopherols are strong chain breaking antioxidants by effectively scavenging ROS and RNS.  $\alpha$ -T significantly reduces the activity of iNOS and COX-2 [51]; in addition, the effect of extracts or synthetic derivatives has been evaluated, decreasing cell death due to excitotoxicity and oxidative stress in astrocytes [52] and accelerating remyelination of focal demyelinated lesions chemically induced [53]. In rats with SCI, the use of  $\alpha$ -T (600 mg/kg i.m., twice weekly, for 6 weeks) decreased the damage caused by ischemia-reperfusion, improving the levels of motor and sensory recovery and the level of oxidative stress [54]. Ubiquinol (reduced form) or coenzyme Q10 is among the antioxidants that decrease their concentration after SCI. It is a fat-soluble cofactor present in the inner mitochondrial membrane acting as an antioxidant in the respiratory chain. Previously, the effect on ischemia-reperfusion damage in the CNS has been proven, preventing LP and reducing the size of the lesions [55].

#### 3.6 Immunotherapy

The use of antibodies in the treatment of SCI is diverse and is directed to the functions of immune cells involved in inflammation and the pathological process. The initial invasion of leukocytes depends on the interaction of CD11d/ CD18 (cluster of differentiation; CD) integrin with vascular cell adhesion molecule-1 (VCAM-1). In the case of the use of anti-CD11d monoclonal antibody administered in rats to determinate the therapeutic window with 1 mg/kg doses i.v. on groups at different times of application (2, 6, 12, 24, or 48 h post-lesion), it was shown that the treatment beginning even up to 6 h after the lesion resulted in an attenuation of infiltrating leukocytes (neutrophils and macrophages, sources of ROS and RNS), lowered the expression of COX-2 and iNOS, and lowered the amounts of HNE, NT-3, and dinitrophenyl (DNP) (used for the detection of protein carbonylation) therefore acting as an indirect antioxidant. This treatment also showed improvement in motor recovery vs. a control antibody [56]. Another important integrin is the dimer  $\alpha 4\beta 1$  also known as very late antigen 4 (VLA-4), and treatments with anti- $\alpha$ 4 blocking monoclonal antibodies (2.5 mg/kg/2 and 24 h/i.v.) or small molecule blocker BIO5192 (10 mg/kg/2 h/ continuous i.v. infusion for assessment of oxidative damage) showed a decreased influx of neutrophils/macrophages, reduced oxidant activity (COX-2, NO or iNOS, MDA), preserved white and gray matter, improved motor function in different evaluations, and decreased mechanical allodynia after SCI, when compared with the controls [57, 58].

#### 3.7 Antioxidant peptides

#### 3.7.1 A91 peptide

Modified neural peptides are peptide analogs of the myelin basic protein (MBP) epitopes that possess one or more aa substitutions and that have a partial agonist or antagonist action when in contact with the T lymphocyte (TL) receptor [59, 60].

Schwartz and Hauben tested the administration of non-encephalitogenic peptides of different aa sequences associated with MBP, which are named according to the position of the aa substitution that is performed: A96, G91, and A91, among others. A91 showed the best results after a traumatic injury, both in the optic nerve and in spinal cord, without showing clinical signs of autoimmune disease, hypersensitivity, immunosuppression, and controlling the destructive action of autoreactive TL [61, 62].

A91 is a peptide belonging to the aa 87–99 sequence of MBP with the substitution of an aa at position 91 of a lysine (VHFF<u>K</u>NIVTPRTP) by an alanine (VHFF<u>A</u>NIVTPRTP), functioning as a partial agonist peptide and promoting a change of the profile of cytokines produced by TL reactive against the 87–99 sequence of the MBP of a Th1 phenotype (interferon gamma [IFN- $\gamma$ ], TNF, IL-2) to a Th2 (IL-4, IL-10) and decreasing the action and synthesis of the FR, among other effects [63]. A91 allows activating the microglia with a phenotype producing neurotrophic factors, which together with the release of factors produced by other cells such as monocytes (MN) and TL reduce secondary neuronal degeneration [64–66].

The beneficial effect of subcutaneous immunization at the base of the tail has been demonstrated with A91 at a single dose (150–200  $\mu$ g/kg) after SCI due to moderate contusion. This immunization, among various factors and effects, promotes neuroprotection and motor recovery by decreasing the expression of iNOS and production of NO<sup>•</sup>, LP, caspase 3, and pro-inflammatory cytokines and increasing the release of neurotrophic factors such as BDNF and NT-3. The effect of the immunization is preserved in the chronic stage of the lesion and as a prophylactic treatment or up to 72 h after the SCI; however, it diminishes when applied to lesions due to severe contusion or complete medullar cut and is eliminated with a double immunization. It has also been determined that the severity of the lesion determines the profile of genetic expression in the lesion after immunization and that immunization plus the removal of the fibroglial scar and/or the implant of a scaffold as support for mesenchymal stem cells favors a permissive microenvironment for motor recovery and improves the electrophysiological activity in the chronic stage after a complete section of the spinal cord [67–73]. The protective response of A91 is between 4 and 6 days, indicating that it acts on subsequent mechanisms to the acute stage. During this time, the oxidative processes are not completely modulated. Regarding this, it has been shown that the therapeutic combination of A91 peptide with peptides acting at shorter times, such as glutathione monoethyl ester (GSH-MEE) or the monocyte locomotion inhibitory factor (MLIF), reduces FR and LP and induces better motor recovery, neural survival, presence of myelinated axons, and tissue protection. In the same way, it was demonstrated that the combination of A91 with GSH-MEE retains the effect if applied until 72 hrs after the lesion [68, 74, 75].

#### 3.7.2 Monocyte locomotion inhibitory factor (MLIF) peptide

MLIF is a pentapeptide (Met-Gln-Cys-Asn-Ser). In vitro studies showed that MLIF decreases MN locomotion, the production of ROS ( $H_2O_2$ ,  $O_2^{-}$ ,  $HO^{-}$ ), NO<sup>+</sup>, and cGMP, and it induces an increase of microtubules associated to the centriole and the concentration of cAMP [76–78]. The pharmacophore group of the MLIF is integrated

by the Cys-Asn-Ser tripeptide, which retains the same biological activities of the factor [79, 80].

The MLIF favors the Th2 response; modulates the synthesis of pro-/anti-inflammatory cytokines and the expression of genes involved in inflammation, proliferation, angiogenesis, synthesis/degradation of extracellular matrix, angiogenesis, and axonal guidance, among others; and acts mainly through the signaling pathways: NF-kB, MAPKinases, and eEF1A1/endothelial nitric oxide synthase [81–84].

In vivo, the factor retards the arrival of MN in Rebuck windows and inhibits cutaneous delayed hypersensitivity to dinitrochlorobenzene, while in guinea pigs, it lobs down the expression of VLA-4 and VCAM-1 adhesion molecules in postcapillary vascular endothelium and decreases the formation of pericardial adhesions in rats when applied directly to the site of injury after surgery [85].

Studies in cerebral ischemia showed that the penetrating, antioxidant, antiinflammatory, and neuroprotective capacity of the pharmacophore group is favored in analogs when the N-terminal end is modified by adding one of the following aa: Asp, His, Try, or Arg. In the same way, cardioprotective effects have been seen in myocardial ischemia [86, 87]. On the other hand, pharmacokinetic studies are underway to determine the concentration of MLIF in plasma [88].

In base studies of our group, rats were subjected to a moderate SCI, and a dose of 200  $\mu$ g of MLIF was applied directly to the site of the lesion. The animals treated with the factor presented a greater motor recovery than the non-treated, and a decrease in the LP, the concentration of NO<sup>•</sup>, and the expression of the iNOS. An increase in the expression of the IL-10 and TGF- $\beta$  (Transforming Growth Factor beta) genes was observed at 3 h and 7 days post-injury, favoring the survival of the ventral horn neurons [75]. Subsequent studies showed that four doses of the MLIF at the same concentration immediately initiating direct administration at the site of injury and subsequently one dose every 24 h for 3 days by i.p. administration are sufficient to improve motor recovery in rats subjected to SCI. In the same way, therapeutic combinations of MLIF, at different times and doses, have favored the effect of the MLIF in the experimental model of SCI modulating the synthesis of the FR and ROS.

#### 3.7.3 Glutathione (GSH) peptide

GSH (**Figure 3**) is a tripeptide (L- $\gamma$ -glutamyl-L-cysteinyl-glycine), nonprotein thiol. It is synthesized in the cellular cytoplasm by the consecutive action of two enzymes. The first,  $\gamma$ -glutamylcysteinyl ligase, is regulated by the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2), which is sensitive to oxidative stress. This enzyme uses glutamic acid (Glu) and Cys aa, glutamic acid (Glu) and Cys aa, as a substrate to form the  $\gamma$ -glutamylcysteine dipeptide ( $\gamma$ -GluCys), which



Figure 3.

Condensed structural chemical formula of glutathione (IUPAC name: (2S)-2-amino-4-{[(1R)-1-[(carboxymethyl) carbomoyl]-2-sulfanylethyl] carbomyl1} butanoic acid). Modified from Gaucher et al. [89].

is combined with glycine (Gly) in a reaction catalyzed by the second enzyme (glutathione synthase) to form GSH, whose concentration is regulated by the nhibition of  $\gamma$ -GluCys ligase, the cellular content of L-cysteine, and the final concentration of GSH. Thus, the intracellular and extracellular concentrations of GSH are determined by the balance among its synthesis, catabolism, and transport between cytosol and the different organelles [89].

GSH, by itself, is not transported effectively into the cells, and under normal physiological conditions, it is in a reduced form. During its oxidation (where the thiol group of Cys is responsible for the redox reactions) by ROS and RNS, it involves two types of reactions, a nonenzymatic reaction with the NO<sup>•</sup>, HO<sup>-</sup>, and O<sub>2</sub><sup>•-</sup> radicals and an enzymatic one providing an electron for the reduction of peroxides in the reaction, catalyzed by GPx to form the oxidized glutathione GSSG (two GSH molecules bound by the disulfide bridge), which is regenerated by Gr, an enzyme that transfers electrons from NADPH to GSSG by reducing it [90, 91]. Thus, the redox state of GSH activates the activator protein 1 (AP-1) responsible for the expression of cytokine genes, TGF- $\beta$ , and collagenase and AP-2 responsible for the activation of c-Jun-N-terminal kinases (JNK), stress-activated protein kinases (SAPK), protein kinase c (PK-C), and tyrosine kinase, while the decrease in the GSH level stimulates the activation of NF-kB, protein kinase B, c-Jun N-terminal Kinase, and mitogen-activated protein kinase with the subsequent increase in synthesis of pro-inflammatory cytokines and caspases. In suitable concentrations, GSH increases the activation, proliferation, and cellular differentiation and regulates the Ca<sup>2+</sup> homeostasis [91], granting a fundamental role in cellular homeostasis and pathologies related to patient's age and oxidative stress states, such as neurodegenerative, neuroinflammatory, cardiovascular diseases, and cerebral ischemia, among others [92, 93]. To increase the intracellular GSH concentration levels, GSH precursors have been used, without modifying the Cys that is critical for the functioning of the peptide. GSH precursor molecules such as N-acetyl cysteine (NAC) stimulate the biosynthesis of GSH that acts directly on ROS, RNS [89, 93, 94], and glutathione esters, mainly mono- and dimethyl esters such as glutathione monoethyl ester [ $\gamma$ - Glu-Cys-Gly-OEt (GSH-MEE)], where the carboxyl group of Gly is esterified and, due to its high hydrophobicity, increases its permeability to the cell membrane and facilitates its transport in brain-spinal fluid [95–97]. Once GSH-MEE is located in the cellular cytoplasm, it is hydrolyzed by the intracellular esterases to release and cause the intracellular increase in the GSH concentration and react with the FR without enzymatic intervention or it reduces the peroxides by means of GPx through its oxidation to GSSG [89, 91, 98, 99].

GSH-MEE has been used effectively to protect cells from oxidizing agents and various toxic compounds in various cell lines and animal models with neurodegenerative and inflammatory processes [92, 99, 100]. Studies of our group and collaborators have shown that the i.p. administration of 12 mg/kg of GSH-MEE divided into four doses in the first 24 h post-lesion in rats subjected to a moderate SCI contributes to the reduction of oxidative stress, significantly improves motor function and survival of red core neurons, and stabilizes spinal cord blood flow [100], while a therapeutic combination of GSH-MEE (at the same dose and under the same scheme) with intradermal application (i.d.) of the A91 peptide at the base of the tail at a dose of 600  $\mu$ g/kg immediately after the injury promotes a better neurological recovery and morphological preservation. This combination is able to maintain its neuroprotective action even if it starts 72 h after the injury [68, 74]. In the same way, our group has demonstrated that the therapeutic combination of GSH-MEE and MLIF promotes greater motor recovery and maintains several morphological aspects on the site of lesion in rats subjected to moderate SCI.

#### 3.8 Natural extracts as antioxidants

A variety of ingredients and active ingredients derived from herbal extracts, known for their antioxidant and anti-inflammatory activity, have also called the attention to complement SCI treatments. Among all these, ingredients such as curcumin, resveratrol, epigallocatechin gallate, ligustrazine, quercitrin, and puerarin and herbs such as Dashen, *Ginkgo biloba*, Ginseng, Notoginseng, and Astragali Radix are outlined as candidates for various experimental studies. In the review by Zhang et al. [101], the molecular structures, application, and dose are listed, as well as the results found at a molecular level on SCI. In particular, the compound curcumin is a polyphenol substance isolated from the yellow extract from rhizome of *Curcuma longa*, and it has been widely used for medicinal purposes due to its potent effect in inhibiting acute and chronic inflammation. Regarding its antioxidant action, its application in SCI (300 mg/kg in DMSO, single dose i.p. after injury) has shown a decrease in MDA and an increase in SOD at 24 h [102], and at a lower dose, it has also increased the concentration and induction of GSH, GPx, and Nrf2 and decreased the expression of NF- $\kappa$ B, TNF- $\alpha$ , and IL-1 $\beta$  [101].

#### 3.9 Spin trappings

A highly studied antioxidant strategy consists of scavengers of FR that include, among others, thiols (lipoic acid), GSH precursors, NAC, polyphenolic compounds, hydroxyl stilbenes, nitrones, and spin trappings (noncyclic and cyclic nitrones); we will only review the latter. Most spin trappings have a nitronate or nitroxide nucleus and are chemical agents that react with FR, forming stable products (adducts), and were originally developed as a tool to detect and stabilize the FR in chemistry and later in biological oxidation processes [103–105]. The first spin trappings had short half-lives and generated toxic HO<sup>•</sup>. By designing the spin trappings with the inclusion of heterocyclic rings (pyrrolines or phenol, generating Imidazolyl-nitrones, Furil-nitrones, Arylnitrons, and others) toxicity was reduced, improving its neuroprotective, anti-inflammatory, functionality, stability, bioavailability, and trapping different types of FR centered on O<sub>2</sub>, carbon, and sulfur derivatives. In turn, this increases their solubility in high concentrations in a large number of solvents (~0.1M), producing a positive effect when administered in a varied-dose scheme before or after a traumatic event [103, 106]. A basic example of the nitrones is phenyl N-tert-butylnitrone (PBN), an arylnitrone with general formula X-CN = NO-Y, which acts by reacting with  $O_2^{-}$  and/or HO<sup>-</sup> to produce adducts. Once the adduct is formed, the radical is inactivated and unable to damage the cell tissue [104, 107]. The general reaction is that of the formation of adduct, schematized in Figure 4. In general, it is indicated that PBN is not toxic and the suitable concentration to form adducts is 10-15 mg/100 g of weight, while the estimated lethal dose is 10 times higher (100–150 mg/100 g of weight) [108]. The first neuroprotective evidence was in neurodegenerative models administered at low doses after injury and in the



Figure 4.

Basic reaction of a nitrone with FR to produce a stable spin product (adduct). Modified from Refs. [105, 106].

prevention of stroke-induced mortality in models of ischemia in gerbils [103, 104, 109–113]. The pharmacological effects of PBN in animal models are extensive, protecting against death after endotoxic shock, bacterial meningitis, teratogenicity induced by thalidomide, diabetogenesis, hepatocarcinogenesis, etc. Many studies have reported a neuroprotective effect in SCI and the brain (the most studied) decreasing the expression of genes associated with apoptosis, inflammation, and iNOS by decreasing the activation of MAP p-38 NF-κB nitrogen kinase and synthesis of NO<sup>•</sup> [114]. In a process of ischemia or perfusion, PBN reduces the size of the infarct by increasing ischemic reperfusion and decreasing neurodegeneration, excitotoxicity, and the activation of microglia; it also induces neurite growth through indirect activation of the Ras-ERK pathway, increasing animal survival [106, 115–117]. The neuroprotective effect of PBN is attributed to its ability to quickly and easily penetrate the membranes and the blood-brain barrier with a half-life of 3 h in plasma; decrease the levels of oxidized proteins, 8-isoprostane, HNE, IL-1 $\beta$ , TNF- $\alpha$ , IFN- $\gamma$ , c-fos, IL-3, IL-4, IL-5, and H<sub>2</sub>O<sub>2</sub>; and favor an increase of GHS and IL-10, among others [106, 117–119]. In a model of cortical contusion in rats, it was demonstrated that pre-treatment with PBN with a single intravenous dose of 30 mg/ kg 30 min before the injury reduces the cognitive deficit and its volume; it has shown to have a wide therapeutic window in focal ischemia rodent models, reducing the infarct volume when administered up to 12 h after the beginning of the stroke and reducing the loss of tissue when administered by fluid percussion 30 min. After injury in rats [120]. Currently, the nitrones derived from PBN [102] are being widely studied as neuroprotective in different CNS pathologies and in traumatic lesions. For example, 2,4-disulfophenyl-N-tert-butylnitrone (NXY-059) has neuroprotective effects when applied 4–5 hr post-occlusion at equimolar doses to PBN and reduced infarct volume from 37.2 to 12.5% when 30 mg/kg was administered i.v. 1 h after reperfusion in Wistar rats [121–124]. Meanwhile, stilbazulenyl nitrone (STAZN) exerts similar effects at lower doses than the one used for NXY-059; in fact, the tolerability and safety of NXY-059 were studied in patients with acute stroke in clinical trials [103, 124]. Although not all compounds have demonstrated their neuroprotective effect when administered 24 h after the traumatic event, some of them have allowed favoring the therapeutic window at repeated doses [103].

Other derivatives are 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) and diesterified nitrone (EMEPO), which have shown similarities to the action of PBN but with some other advantages, such as being less toxic and increasing the levels of antiapoptotic proteins such as Bcl2 and p-Bad and decreasing the synthesis of pro-apoptotic ones such as caspase 3, p53, and Bax [125–127]. In addition, (2, 2, 6, 6-tetramethylpiperidin-1-yl)oxyl (Tempo) and (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-yl)oxyl (Tempol) have shown antioxidant properties in radiation damage and injury [128, 129]. In a traumatic brain injury mouse model, Tempol reduced post-traumatic LP and oxidative damage induced by protein nitration, decreasing mitochondrial damage, cytoskeletal damage, and neurodegeneration and improving motor function [128, 130, 131].

Despite the results observed with the nitrones and the wide range of studies performed for therapeutic uses at different doses and times, their action is attributed to their ability to form adducts, but not before indicating the possible participation of other mechanisms that favor their neuroprotective activity, thus expanding the information on antioxidant therapy strategies in the clinical area.

#### 3.10 Polyethylene glycol (PEG)-superoxide dismutase (SOD)

Polyethylene glycol (PEG) is a surfactant that due to its hydrophilic nature allows the fusion and fluidity of the cell membrane that reduces the oxidative

effects of the secondary stage and that during the acute phase of SCI, it may inhibit nerve fiber degeneration and create a favorable microenvironment for the regeneration of nerve filaments that can stimulate angiogenesis and reduce glial scar, promoting the regeneration of axonal guidance and motor recovery. PEG has been widely used as a scaffold for a large variety of molecules in treatment for SCI [132–136], while the SOD enzyme has antioxidant properties, as mentioned previously. The combination of SOD with PEG (PEG-SOD) allows an increase of the enzyme intracellularly and its antioxidant activity, and it may have an important role in vascular relaxation by reducing the concentration of O2<sup>•-</sup> and limiting the LP [132]. It has been used in myocardial ischemia and in lung injury models, proposed as a treatment vs. oxidative stress [132].

In a controlled phase II study in patients in a coma who suffered a stroke and received a single i.v. dose of 2000, 5000, or 10,000 IU/kg 4 h after the injury, its recovery was better in comparison to the group that received placebo (44% were in a vegetative state or died); no side effects were observed in this study due to the administration of the drug [137].

In a study in a cerebral ischemia model performed in rats, 10,000 IU/kg of PSG-SOD were i.v. administered, and the group presented a significant reduction in infarct size in comparison to the control group [138]. In other study with Sprague-Dawley male rats (300-350g of weight), an occlusion of the hepatic artery was performed and reperfusion was performed after 90 min to generate liver damage. A group of animals received i.v. 5000 U/kg of PEG-SOD before vascular occlusion and immediately after reperfusion, while the control group only received a saline solution following the same scheme. In the group treated with PEG-SOD, hepatic ischemia and LP were attenuated. Meanwhile, another study examined the effect of PEG-SOD on focal cerebral ischemia/ reperfusion in rats; the results showed that the effect is variable, depending on the dosage [132, 139]. In a dog experiment, thoracic aortic cross-clamping was performed; a dose of 5000 U/kg of PEG-SOD was i.v. administered to one group 15–20 min before clamping, and the other group only received a saline solution. Delayed paraplegia was avoided in the group of dogs that received the conjugate, unlike the groups that did not receive it [140]. Edward et al. conducted an important review of the use of PEG-SOD in phase II and III studies in traumatic brain injury [141].

#### 3.11 Mannitol

When mannitol is used for medical purposes, it is administered intravenously. Mannitol can be found in varying concentrations, dissolved in 100 mL of fluid (5, 20, and 25% mannitol). A common solution is 20% mannitol. Cruz and colleagues described the dose-response effect of preoperative mannitol on acute subdural hematomas in traumatic brain injury in which mannitol therapy has been classically directed, establishing and maintaining an osmotic gradient between the blood and brain [142, 143].

Maintaining an adequate spinal cord perfusion pressure is crucial after SCI. Intramedullary edema within the spinal cord and consecutively raised intrathecal pressure at the injury are important secondary injury mechanisms in the pathobiology after traumatic SCI. Increased intraspinal pressure reduces spinal cord perfusion pressure, which leads to worsen post-traumatic ischemia [144].

Mannitol allows the control of blood flow patterns in the spinal cord; it has been used experimentally in some studies in rats that have suffered a controlled SCI and in dogs/cats that suffer an SCI within the clinical area. Mannitol is recommended to reduce the effect of inflammation and edema, an effect that has been corroborated with microangiographic and electrophysiological studies. One hour after the application of a 3 g/kg dose, an improved intramedullary vascular pattern was detected among the animals treated with mannitol compared to those that were not treated,

and 4 h after the perfusion, many areas of the lateral white matter of the spinal cord were almost normal [145]. In a study in dogs, an SCI was experimentally induced, and it was reported that mannitol alone did not help to reverse the paralysis of these animals [146]; however, another study stated that the i.v. administration of mannitol at a dose of 2 g/kg had a good effect on the white matter of the spinal cord and areas of the brain [147]. In a retrospective study with Sprague-Dawley rats, a group with SCI by compression by means of a clamp, 2 g/kg mannitol were administered immediately after the injury, while the control group was given 0.9% saline solution; all groups underwent structural and electrophysiological studies. The group treated with mannitol obtained excellent results, finding significant improvement in neural structures and protection of the spinal cord after SCI [148]. In a study in dogs to which an edema was induced by severe external spinal cord trauma, 3 g/kg of mannitol was i.v. administered, and they were neurologically evaluated, and a myelography study was performed after 2 h of the treatment, to identify the edema, showing that there was reduction of it [149].

#### 3.12 Combinatory therapies and results in symptoms of SCI

In addition to its independent use, several studies have evaluated the use of one or more antioxidants together by themselves or in addition to other existing therapies for SCI, such as rehabilitation exercise or cell transplantation, expecting a synergism to enhance the recovery. Moreover, some therapies not only aim to improve the immediate treatment of SCI but also improve the effects it has on relieving the most common complications in patients. To mention some, the combination of vitamin C as antioxidant (100 mg/kg/1 h and daily/28d, i.p.) together with the transplantation of bone marrow mesenchymal stem cells (BMMSC)  $(3 \times$ 10<sup>6</sup> cells) induced improvements in motor recovery in rats when compared with methylprednisolone (MP), vitamin C, or BMMSC alone in SCI [150]; simultaneous administration of vitamin D (5  $\mu$ g/kg/twice daily) and progesterone (0.5 mg/kg/ twice daily i.m.) for 5 days demonstrated a higher efficacy in reducing neuroinflammation in comparison to when they were administered separately, and when they were administrated early (first 4 h) in SCI patients receiving MP, there was improvement in the motor and sensory functions 6 months after starting therapy [151]. Applying once a day a combination of low-dose fluoxetine (1 mg/kg/i.p.) and vitamin C (100 mg/kg/i.p.) immediately after the event and for 14 days had a protective effect on the BSCB integrity, improving the functional recovery, showing inhibition of the expression and activation of the matrix metalloproteinase, and decreasing the infiltration of leukocytes and the expression of inflammatory and oxidizing molecules, but not when they were applied separately in rats [152]. In SCI patients, dietary supplementation for 3 months, which included three 750 mg per day of omega-3 fatty acids and antioxidants (400 mg of mixed tocopherols, coenzyme Q10, curcumin, etc.), caused a decrease of inflammatory cytokines with reduction in neuropathic pain [153]; 2 months vitamin E dietary supplementation 765–1020 IU/day in rats before SCI showed accelerated bladder recovery, significant motor improvement, and a high number of oligodendrocytes compared to the controls [154].

## 4. Conclusion

After a primary injury occurs on the spinal cord, destructive biochemical mechanisms are initiated (secondary injury) that play a fundamental role in the pathophysiology of spinal cord injury. Within these, oxidative stress and lipid

peroxidation exacerbate the biochemical mechanisms once initiated and propagate neurodegenerative damage, so the degree of loss of long-term motor and sensory functions depends largely on their intensity. This damage suffered during the acute phase and that may be irreversible requires a timely intervention. To guarantee the antioxidant effect that will render better results, it is important to consider the new agents and therapies in the SCI treatment at the appropriate times. There is no fully restorative therapy for SCI, but strategies for the modulation of this damage contribute to neuroprotection and, although partially, to functional recovery.

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## **Conflict of interest**

The authors declare no competing financial interests.

### Acronyms and abbreviations

aa	amino acids
ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
BSCB	blood-spinal cord barrier
BMMSC	bone marrow mesenchymal stem cells
CAT	catalase
CD	cluster of differentiation
CNS	central nervous system
CO <sub>3</sub> ●-	carbonate radical
COX	cyclooxygenase
DHA	docosahexaenoic acid
DNP	dinitrophenyl
EPA	eicosapentaenoic acid
ER	estrogen receptor
FR	free radicals
GAP-43	growth-associated protein 43
GFAP	glial fibrillary acidic protein
GPx	glutathione peroxidases
GR	glutathione reductases
GSH	glutathione, reduced
GSH-MEE	glutathione monoethyl ester
GSSG	glutathione, oxidized
GST	glutathione S-transferases
HNE	4-hydroxy-2-nonenal
$H_2O_2$	hydrogen peroxide
HO●	hydroxyl radical
HO⁻	hydroxyl anion

IFN-γ	interferon gamma
IL .	interleukin
iNOS	inducible nitric synthase
L•	lipid radical
LH	lipid
LP	lipid peroxidation
LOO•	lipid peroxyl radical
LO●	lipid alkoxyl radical
LOOH	lipid hydroperoxide
MLIF	monocyte locomotion inhibitory factor
MDA	malondialdehyde
MP or MPSS	methylprednisolone sodium succinate
NAC	N-acetyl cysteine
NADPH	nicotinamide-adenine dinucleotide phosphate, reduced
NO <sup>●</sup>	nitric oxide
NO <sub>2</sub> •-	nitrogen dioxide radical
NOS	nitric oxide synthase
NT-3	3-nitrotyrosine
O2 <sup>•-</sup>	superoxide
OONO <sup>-</sup>	peroxynitrite
PBN	phenyl N-tert-butylnitrone
PEG	polyethylene glycol
PEG-SOD	polyethylene glycol-superoxide dismutase
PUFA	polyunsaturated fatty acid
ROS	reactive oxygen species
RNS	reactive nitrogen species
$RO_2^{\bullet}/HO_2^{\bullet}$	peroxyl radical
SCI	spinal cord injury
SOD	superoxide dismutase
TGF-β	transforming growth factor beta
TNF-α	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule-1
VDH	vitamin D: 1,25-dihydroxyvitamin D3
VLA-4	very late antigen 4
XO	xanthine oxidase

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