Pregabalin in Experimental Spinal Cord Injury: Oxidative Stress, Antioxidant Defence and Neuroprotective Mechanisms

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Preface

Spinal cord injuries are one of the most complex and multifaceted clinical conditions in neurosurgery. Oxidative stress, excitotoxicity, inflammation, and mitochondrial dysfunction, which play a significant role in the damage cascade that develops and is activated after primary mechanical injury, can lead to the deepening of neuronal loss. Understanding and targeting secondary damage at the molecular level remains a fundamental area of research in the development of neuroprotective treatment approaches.

This book is derived from my medical specialization thesis, completed in 2012 at the Department of Neurosurgery, Faculty of Medicine, Yüzüncü Yıl University, with YÖK thesis number: 324949, entitled "Neuroprotective effect of Pregabalin in experimental spinal cord injury: Investigation of antioxidant enzymes in blood and neural tissue in terms of oxidative stress." This thesis has been comprehensively reviewed, restructured, and transformed into an academic monograph format in light of current literature.

This work evaluates the neuroprotective potential of pregabalin in an experimental spinal cord injury model, primarily through endogenous antioxidant systems such as superoxide dismutase and glutathione peroxidase. The findings point to the potential regulatory effects of pregabalin on secondary damage processes and offer new research perspectives in the field of translational neuroprotection.

This book aims not only to present the results of an experimental model but also to discuss the biological and pharmacological foundations and clinical implications of the neuroprotection paradigm in spinal cord injury within a holistic framework.

During the preparation of the text, AI-based language editing tools were used to improve grammatical accuracy and clarity of expression and to detect

errors. However, I bear full academic responsibility for all scientific content, data analysis, interpretation, and conclusions presented in this book.

I would also like to express my deepest gratitude to my family for their patience and support throughout my scientific journey.

Burhan Oral Güdü, MD

Assistant Professor of Neurosurgery

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CHAPTER 1

Mechanistic and Biological Foundations of Spinal Cord Injury

Spinal cord injury is a significant health problem with a high mortality rate and a long-term risk of permanent disability; it leads to a multidimensional clinical picture that significantly affects an individual's physical functionality, psychosocial integrity, and quality of life, while also creating a serious socioeconomic burden(1–4). Spinal cord trauma can create a wide spectrum of injuries ranging from mild and transient neurological deficits to complete damage, depending on its severity. Although the true incidence of spinal cord injuries is not precisely known, it varies from country to country. The annual incidence of spinal cord injury is estimated to be around 15–40 per 1,000,000 (5–7). In Turkey, an average of 1,600–2,000 cases of severe acute spinal cord injury are reported annually(8). The most common cause of spinal cord injuries is traffic accidents, followed by traumatic causes such as falls, violence, and sports injuries, as well as many degenerative, vascular, infectious, and neoplastic diseases(9). Most people who suffer spinal cord injuries are young men. In the last 20 years, studies on the treatment of spinal cord injuries have increased, but none of them have become the standard treatment for use in humans.

Acute spinal cord injury occurs through two mechanisms. The first is primary injury, which is a mechanical injury caused by the trauma at the time of the injury and is largely unavoidable. The second is secondary injury, which develops as a result of the activation of endogenous cell death pathways triggered by the primary injury and cascades that are still not fully understood. Halting or attenuating this secondary damage cascade that begins after the injury is the main goal of clinical treatment. Experimental studies on the

progression and prevention of secondary injury are still ongoing. In the secondary damage process, free radicals, lipid peroxidation, glutamatergic, cholinergic and catecholaminergic neurotransmission systems, the inflammatory cascade, calcium and other ion channels, activation of the apoptotic cascade, and regeneration play an important role (10–13).

Free radicals are molecules that contain one or more unpaired electrons in their outer orbitals. Reactive oxygen species (ROS) and their products cause progressive tissue damage in spinal cord injury. Antioxidant mechanisms in the tissue decrease rapidly after trauma, and the free radicals formed react with lipids, proteins, and nucleic acids, causing tissue damage. Another mechanism responsible for secondary damage in spinal cord injury is ion channels and glutamate excitotoxicity. Understanding the pathophysiology of spinal trauma guides the development of treatment strategies.

Although methylprednisolone, commonly used in spinal cord injury, has been shown to be a useful treatment agent, its benefit is debatable(14). Although many experimental studies have reported the effectiveness of several pharmacological agents in treating spinal cord injury, this has not been conclusively demonstrated in clinical trials.

Pregabalin (PGB) is commonly used in the treatment of epilepsy and neuropathic pain. PGB exerts its effects by potently and selectively binding to the alpha-2-delta (α2δ) subunit of voltage-gated calcium channels (VGCCs). PGB reduces glutamate release from hyperexcited neurons and modulates calcium influx in the presynaptic area. PGB has been shown to have neuroprotective effects in the spinal cord and brain(15). Studies on the effect of PGB on oxidative stress in blood and spinal cord tissue are very limited in the literature. PGB is thought to have a neuroprotective effect because it modulates calcium influx in the presynaptic area, reduces excitatory neurotransmitters such as glutamate and norepinephrine, and has anti-inflammatory effects. Within experimental contusion paradigms, PGB has been investigated as a potential modulator of oxidative stress, particularly in relation to the SOD–GPx axis and its association with histological integrity and neurological recovery.

1.1. Epidemiology and History of Spinal Cord Trauma

Acute spinal cord trauma is a significant health problem affecting individuals physically, psychosocially, and economically. Spinal cord injury is seen at an average of 15–40 per million annually(16). The main causes of spinal cord trauma include motor vehicle accidents, falls, workplace accidents, and exposure to violence (17). Young men are more exposed to trauma. According to the National Center for Spinal Cord Injury Statistics (NSCISC), the incidence

of traumatic spinal cord injury in the USA is reported as approximately 40/1,000,000 cases, or 12,000 new cases annually. According to NSCISC data from 2010, the average age of injury was 33.9, and 80% of those injured were male. Spinal cord injuries, including complete tetraplegia and paraplegia, decreased from 25.3% and 27.7% in 2005 to 15.3% and 20.7% in 2010 respectively(17,18). A study conducted in Turkey in 1994 estimated the incidence to be 16.9 per million, with a male-female ratio of 5.8/1 and an average age of 31(8). The first mention of spinal cord injury is found in the Edwin Smith Papyrus, written by Egyptian surgeons between 3000-2500 BC(19,20). Hippocrates defined paralysis in 400 BC (13). In the 15th century, Şerafettin Sabuncuoğlu described traction treatment for a patient with spinal trauma with an illustration(21).

1.2. Spinal Cord Embryology and Anatomy

During the third embryonic week, the ectoderm located in the dorsal midline of the embryo begins to thicken and forms the neural plate. The spinal cord is formed from the ectoderm, and the surrounding spinal canal from the mesoderm. The brain develops from the rostral 2/3 of the neural plate and neural tube, and the spinal cord from the caudal one-third. While the spinal cord and vertebral column are the same length in the 3rd month of fetal life, the vertebrae grow rapidly, creating a length difference so that in adulthood the lumbar 1st vertebra remains below the spinal cord and is located in the upper 2/3 of the vertebral canal(22).

The spinal cord is covered by membranes called the pia, arachnoid, and dura. Cerebrospinal fluid is found in the subarachnoid space. Thirty-one pairs of nerves emerge from the spinal cord, formed by the anterior (motor) and posterior (sensory) roots (8 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 1 coccygeal spinal nerves)(23).

The vertebral column consists of 33 vertebrae: 7 cervical, 12 thoracic, 5 lumbar, 5 sacral, and 4 coccygeal. Due to its segmented structure, the vertebral column is a flexible structure composed of vertebrae, joints, and fibrous cartilage cushions called intervertebral discs. The spinal canal is formed by the vertebral bodies and arches and provides very strong protection to the spinal cord against trauma(24).

The spinal cord is roughly cylindrical in shape, beginning at the end of the medulla oblongata at the foramen magnum above and ending at approximately the level of the first lumbar vertebra(24). The spinal cord expands in two places, the cervical region and the lower thoracolumbar region, and plexuses arise from these areas. Below, it forms the conus medullaris and ends by

attaching to the posterior surface of the coccyx with the filum terminale. The deep longitudinal groove extending along the midline on the anterior surface of the spinal cord is called the fissura mediana anterior, and the shallower groove extending along its posterior surface is called the sulcus medianus posterior(25).

The inner part of the spinal cord consists of gray matter (substantia grisea) and the surrounding white matter (substantia alba).

Gray Matter (Substantia Grisea): This is an H-shaped structure formed by the anterior and posterior horns, connected by the commissura grisea surrounding the central canal. The amount of gray matter is directly proportional to the number of muscles it innervates. The amount of gray matter is greater in cervical and lumbosacral expansions. Gray matter consists of nerve cells, their extensions, neuroglia, and blood vessels. Most of the cells in the anterior column are large and multipolar. Their axons exit the anterior column as alpha efferents innervating skeletal muscles. Smaller nerve cells exit as gamma efferents innervating intrafusal muscle fibers. The posterior column has four groups of nerve cells; the substantia gelatinosa extends along the spinal cord and is located at the apex of the posterior column. It contains a large number of Golgi type 2 neurons and receives afferent fibers from the posterior root related to pain, temperature, and touch. The nucleus proprius (nucleus proprius) is located anterior to the gelatinous substantia, extending throughout the spinal cord and receiving afferent fibers related to position, movement sensation, two-point discrimination, and vibration sensation. The nucleus dorsalis (Clarke's column) is located at the base of the posterior column. It extends from the 8th cervical segment to the L3–L4 segments and is involved in proprioceptive endings. The visceral afferent nucleus is located laterally to the nucleus dorsalis. It extends from the 1st thoracic segment to the 3rd lumbar segment and is involved in visceral efferent information reception. The commissure grisea and canalis centralis (central canal) are connected on either side of the spinal cord by a commissure grisea. The canalis centralis is located in the center of the commissure grisea. The central canal continues above with the 4th ventricle, expands below in a fusiform form within the conus medullaris as the terminal ventricle, and ends below in the root of the filum terminale. The central canal is filled with cerebrospinal fluid. Therefore, the central canal is closed below and opens into the 4th ventricle above(25).

White Matter (Substantia Alba): The white matter consists of three funiculi. The anterior funiculus is located between the anterior median fissure and the exit points of the anterior nerve roots, the lateral funiculus is located between the exit points of the anterior nerve roots and the exit points of the posterior

nerve roots, and the posterior funiculus is located between the entry points of the posterior nerve roots and the posterior median sulcus. The white matter is composed of nerve fibers, neuroglia, and blood vessels. The white matter surrounds the gray matter and appears off-white due to myelinated nerve fibers. The spinal cord receives its arterial supply from three arteries: two posterior spinal arteries and one anterior spinal artery. The posterior spinal artery arises from the vertebral arteries or the posterior inferior cerebellar artery (PICA) and supplies the posterior third of the spinal cord. Each artery extends downward from the posterior surface of the spinal cord near the posterior spinal roots and gives off branches that enter the spinal cord. In the upper thoracic region, they are small, and the upper thoracic segments are particularly susceptible to ischemia caused by occlusions(26,27).

Anterior spinal artery: It is formed by the confluence of branches arising from each vertebral artery. It extends downwards within the anterior median fissure. It supplies the anterior 2/3 of the spinal cord. It is relatively small in the upper and lower thoracic segments and, in the event of an occlusion, may result in ischemic necrosis, particularly in the fourth thoracic and first lumbar segments(28).

Radicular arteries arise from adjacent arteries at the level of each vertebral segment. Many radicular arteries enter through the intervertebral foramen and head medially. They primarily supply the nerve root(28,29).

Additional segmental arteries originate from the thoracic and abdominal aorta, enter through the intervertebral foramen, and anastomose with the anterior and posterior spinal arteries entering through the vertebral canal. The large and important nourishing artery, the great anterior segmental medullary artery (artery of Adamkiewicz), originates from the aorta at the level of the lower thoracic and upper lumbar vertebrae, is unilateral, and in most individuals enters the spinal cord from the left side. It is the principal blood supply to the lower two-thirds of the spinal cord. The veins of the spinal cord have a similar distribution to the spinal arteries. The anteromedian and anterolateral veins drain into 6–11 anterior radicular veins along the anterior surface of the spinal cord and which subsequently drain into the internal vertebral (epidural) venous plexus(30).

1.3. Mechanisms of Damage in Spinal Cord Injuries

In the current literature, the pathophysiology of spinal cord injury is described as a three-phase process: primary, secondary, and chronic phases. Mechanical tissue damage occurring at the moment of trauma constitutes the primary injury, followed by a secondary damage process mediated

by biochemical and cellular events such as oxidative stress, excitotoxicity, neuroinflammation, and apoptotic cell death, which begins within minutes and hours. The chronic phase, which can last from days to years, is characterized by glial scar formation, persistent neuroinflammation, demyelination, and progressive neurodegeneration(11,31)

1.3.1. Primary Injury Mechanisms

Primary injury is usually caused by mechanical forces such as flexion, extension, rotation, distraction, and axial loading. At the time of trauma, the primary injury consists of damage to vascular and neuronal structures. Trauma can affect the spinal cord itself or the surrounding vertebral column. Primary injury refers to the initial damage caused by fractured bone fragments or the traumatic force itself, resulting in mechanical disruption of neurons, axons, neuroglial structures, and blood vessels(12,31). Fractures, dislocations, acute disc ruptures, and penetrating injuries, in addition to causing neuronal damage, may interrupt blood flow and lead to hypoxia, ischemia, and local infarction. Four characteristic mechanisms of primary injury have been described (**Table 1.1**)(12,32–34).

Table 1.1. Major Primary Mechanical Injury Mechanisms in Spinal Cord Trauma

Mechanical Force	Typical Injury Mechanism / Pattern
Axial compression (impact + sustained compression)	Burst fracture, vertebral body compression fracture
Flexion–compression	Wedge fracture, anterior column failure
Flexion–distraction	Chance fracture, posterior ligamentous complex injury
Hyperextension	Posterior element fracture, central cord syndrome
Rotation (torsion)	Fracture–dislocation, facet dislocation
Penetrating force	Laceration or transection (e.g., gunshot wound, sharp trauma)

The most common primary injury mechanism is permanent compression of the spinal cord following trauma. Burst fractures and fracture-dislocations are common causative factors. The third mechanism is injury caused by distraction forces resulting from flexion, extension, rotation, or dislocation in the axial plane, leading to stretching of the spinal column and rupture of the spinal cord and/or blood vessels. This mechanism is more common in children due to incomplete development of the vertebral bodies, musculature, and ligamentous laxity, and it may not be detectable radiologically(35). In cases of laceration

and transection, gunshot wounds, sharp instrument injuries, sharp bone fragments, or severe distraction may cause extensive damage ranging from minor damage to complete transection. The crushing and contusion caused by mechanical impact lead to a reduction in blood flow necessary for spinal cord perfusion. Compression, rupture, petechial hemorrhage, and contusion of arteries and veins can cause diffuse neuronal damage(13,36).

1.3.2. Secondary Damage Mechanisms

The pathophysiological events triggered by primary mechanical trauma continue with a prolonged secondary damage phase. Secondary damage is a chain of pathophysiological events that occurs minutes to hours after the primary event, and its exact mechanisms are not fully understood. pathophysiological events that occur minutes or hours after the primary event, the mechanism of which is not fully understood. Following primary injury, pathological mechanisms are activated due to changes in vascular, electrolyte, biochemical, and energy metabolism resulting from edema, ischemia, membrane damage, increased intracellular calcium, and the emergence of excitatory amino acids and free radicals. These events, which develop after the primary injury, have both direct and indirect effects and collectively constitute secondary damage. The primary aim in the treatment of spinal cord injury is to prevent or limit secondary damage(13,37–39). After acute injury, histopathological changes such as hemorrhage, edema, and necrosis of neurons and axons occur within the spinal cord. Secondary damage occurs under the influence of both systemic and local factors. Neurogenic shock and respiratory failure play a significant role among systemic effects(36,40). Impaired spinal cord autoregulation and systemic hypotension after trauma increase post-traumatic ischemia. Local effects include changes due to vascular damage. Vascular damage leads to hemorrhagic and ischemic injury (35). Primary injury cannot be prevented; however, secondary damage may be prevented or attenuated. In the development of secondary damage, impaired spinal cord blood flow, the release of neurotoxic substances due to membrane disruption, activation of the injury cascade, and disruption of the intracellular and extracellular environment all contribute to further tissue injury(36,41). In the early post-traumatic period, impaired blood supply and energy deficiency in the spinal cord lead to necrosis. In spinal cord injury, damage is not limited to the lesion site; neurons in descending pathways are also affected, leading to apoptosis and necrosis.

Secondary damage typically begins in the gray matter and subsequently progresses to the white matter (**Table 1.2**)(42,43)

Table 1.2. Secondary Injury Cascade: Systemic and Local Pathophysiological Components

Category	Pathophysiological Components
Systemic Effects (Neurogenic Shock)	Transient tachycardia followed by prolonged bradycardia; Brief elevation in BP followed by sustained hypotension; Decreased peripheral vascular resistance; Reduced cardiac output.
Local Vascular Injury	Mechanical disruption of capillaries; Hemorrhage (gray matter); Microcirculatory failure (thrombosis, vasospasm).
Biochemical Alterations	Glutamate-mediated excitotoxicity; Accumulation of catecholamines; Generation of ROS; Lipid peroxidation; Eicosanoid and cytokine production.
Electrolyte Shifts	Increased intracellular Ca 2+ and Na + ; Elevated extracellular K + .
Inflammatory Response	Macrophage infiltration; Glial cell activation; Axonal degeneration; Release of myelin debris; Apoptosis.
Metabolic Impairment	Reduced ATP production; Failure of energy-dependent ion pumps.

1.3.3. Systemic Effects

The systemic effects of acute spinal cord injury include bradycardia, hypotension, decreased peripheral vascular resistance, an increase followed by a decrease in catecholamines, and decreased cardiac output(44). Neurogenic shock causes decreased cardiac output, loss of vasomotor tone, hypotension, and bradycardia due to decreased peripheral resistance. Neurogenic shock may exacerbate neural tissue damage(35). Heart rate increases briefly after trauma and is followed by a prolonged decrease, while blood pressure shows a similar initial rise followed by a sustained decrease(45,46).

1.3.4. Local Vascular Effects

Contributors to the vascular mechanism include ischemia/reperfusion injury, impaired autoregulation, systemic hypotension (neurogenic shock), hemorrhage (especially in the gray matter), and microcirculatory disturbances(47). After trauma, a decrease or cessation of blood flow occurs, leading to the progression of ischemia within a few hours. Hemorrhagic and ischemic injury develops as a result of vascular damage. The exact mechanism underlying ischemia remains unclear(43,48,49). Vasospasm or the release of vasoactive amines secondary to mechanical damage may be responsible. Hemorrhage may contribute to the development of ischemia, and platelet aggregation can lead to thrombosis. Damage to venules and capillaries, particularly at the site of trauma, may

progress both caudally and rostrally. Petechial hemorrhages occur especially in the gray matter following trauma. Protein leakage from vascular structures due to impaired microcirculation leads to spinal cord edema, resulting in increased intramedullary pressure and reduced blood flow (43,49–51). Impaired spinal cord autoregulation following trauma may lead to systemic hypotension, thereby exacerbating ischemia. Endothelial damage further impairs spinal cord perfusion. Ischemia promotes local spinal cord edema. Edema reduces spinal cord blood flow at the site of trauma, increases interstitial pressure, and causes fluid accumulation in the damaged area, thereby contributing to further cord injury. Loss of microcirculation due to impaired autoregulation leads to small-vessel damage and hemorrhage, while glutamate-mediated excitotoxicity and ischemia, particularly in a dose-dependent manner, exacerbate tissue injury within hours(13,50,52,53).

1.3.5. Glutamate Excitotoxicity

Glutamate is the most abundant excitatory neurotransmitter in the central nervous system, exerting its effects by stimulating ionotropic and metabotropic glutamate receptors. Glutamate receptors have been identified in motor and nociceptive pathways, including the corticospinal and rubrospinal tracts, as well as in both the anterior and posterior horns of the spinal cord. The first evidence of excitatory amino acid–induced neurotoxicity was reported in 1957 when Lucas and Newhouse observed that systemic administration of glutamate damaged the inner neural layers of the mouse retina(11,13,54).

A deficiency of glutamate disrupts normal neuronal excitation, whereas excessive glutamate impairs calcium homeostasis, leading to excitotoxicity and cell death. Glutamate and related amino acids have been shown to cause acute neuronal and glial swelling, followed by delayed neuronal degeneration. Extracellular glutamate levels increase at the site of injury after trauma. Glutamate levels rise both directly due to traumatic cellular damage and indirectly as a result of microcirculatory impairment, reactive oxygen and nitrogen species production, and secondary ischemia(41,52,53).

Calcium influx into the cell induces excitotoxic cell death, leading to both necrosis and apoptosis. Neurons and oligodendrocytes are particularly vulnerable to glutamate because of their high density of glutamate receptors. Excitotoxic damage results in neuronal loss, axonal demyelination, and significant impairment or complete interruption of axonal conduction, ultimately leading to motor and sensory deficits (43,55,56).

Glutamate receptor activation increases intracellular sodium levels, followed by water influx, resulting in cytotoxic edema, intracellular acidosis, and

membrane lysis. Disruption of the Na^+/K^+ pump and sustained elevation of intracellular calcium levels represent key final pathways leading to neuronal death(57–59). Following trauma, membrane depolarization triggers excessive glutamate release from synaptic vesicles; however, due to energy depletion, glutamate reuptake mechanisms fail, thereby exacerbating excitotoxicity(59,60).

The glutamate-induced increase in intracellular calcium leads to ATP depletion, disruption of neurofilament and microtubule integrity, impairment of mitochondrial oxidative phosphorylation, axonal degeneration, and activation of lytic enzymes such as proteases, phosphatases, and endonucleases. Increased phospholipase activity promotes the release of free arachidonic acid, activation of the cyclooxygenase pathway, and subsequent initiation of apoptotic pathways(61,62)

1.3.6. Apoptotic Cell Death

In acute spinal cord injury, necrosis develops as a result of cell death caused by mechanical trauma, leading to cellular inflammation and membrane rupture. Within hours after injury, apoptosis becomes a central mechanism of cell death. Unlike necrosis, apoptosis is not an inflammatory event; rather, it is a programmed, energy-dependent process requiring active protein synthesis(63–66).

Programmed apoptotic pathways are implicated in spinal cord injury, as well as in many other neurological disorders. Neurons, oligodendrocytes, microglia, and astrocytes participate in this apoptotic cascade. Calcium influx into the cell is considered a major trigger of apoptosis. Elevated intracellular calcium activates key enzymes, including caspases and calpains, leading to the degradation of intracellular proteins, neurofilaments, microtubules, and membrane components, ultimately resulting in cell death(67–70).

Numerous extracellular and intracellular stimuli, such as ischemia, oxidative stress, and inflammatory cytokines, can initiate apoptosis. Modulation of caspase activity and the signaling pathways that regulate them may have therapeutic potential in preventing apoptotic cell death. DNA and protein degradation during apoptosis is calcium (Ca^{2+})-dependent(71–75).

1.3.7. Increase in Calcium and Other Electrolytes

An increase in intracellular calcium is considered the final common pathway in cell death. The concentration of calcium ions is approximately 1,000 times higher in the extracellular space than in the intracellular space. Elevated intracellular calcium levels play a central role in secondary injury. Calcium enters

the cell through damaged cell membranes, voltage-gated calcium channels, and glutamate-activated receptor channels.

Increased intracellular calcium activates lytic enzymes such as proteases, phospholipases, endonucleases, and phosphatases. Calcium also interacts with mitochondrial enzymes, disrupting the electron transport chain and promoting free radical formation(55,72,76,77). Calcium (Ca^{2+}) influx and free radical generation occur simultaneously and may exert synergistic effects.

Glucocorticoids inhibit phospholipase A_2 activity by inducing the synthesis of lipocortin, a regulatory protein. Calcium acts as an excitotoxic ion in neurons. Intracellular Ca^{2+} activates calcium-dependent proteases, leading to further cellular damage. Calcium also plays an important role in Na^{+} and K^{+} channel regulation, as well as in the storage and release of certain neurotransmitters. Excessive activation of Ca^{2+} channels in oligodendrocytes leads to cellular injury and subsequent demyelination(57,78–81).

1.3.8. Lipid Peroxidation

The oxidation of free fatty acids by free radicals is referred to as lipid peroxidation. Oxidative stress and free radicals cause cellular damage, particularly to lipids, which are highly susceptible to oxidative attack. Lipid peroxidation begins when a free radical generated by trauma, hypoxia, or ischemia abstracts a hydrogen (H^{+}) atom from the methylene group of polyunsaturated fatty acids (PUFAs). The removal of this hydrogen atom leaves behind a lipid radical containing unpaired electrons(82–84).

This lipid radical is unstable and undergoes intramolecular rearrangement, forming conjugated dienes. These conjugated dienes readily react with molecular oxygen to form lipid peroxyl radicals. Lipid peroxyl radicals can interact with adjacent membrane lipids or proteins. The primary mechanism sustaining the chain reaction is the abstraction of a hydrogen atom from a neighboring lipid molecule by a lipid peroxyl radical, resulting in the formation of lipid hydroperoxide. This propagation process amplifies membrane damage.

The chain reaction terminates when two lipid radicals or peroxyl radicals combine, eliminating unpaired electrons. Final breakdown products of lipid peroxidation include hydrocarbon gases such as ethane and pentane, as well as short-chain fatty acids(85–87). Bioactive aldehydes are also generated during the decomposition of lipid hydroperoxides, most notably malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These reactive aldehydes may be metabolized intracellularly or diffuse into surrounding tissues, causing secondary cellular damage(88,89).

Oxygen-derived free radicals generated during metabolic processes or under pathological conditions induce oxidation of lipids, particularly PUFAs present in cell membranes and lipoproteins. Following trauma, ischemia, or hypoxia, free radicals initiate lipid peroxidation in neuronal, glial, vascular endothelial, and myelin membranes. Lipid peroxidation disrupts not only membrane lipids but also associated membrane proteins.

The nervous system is particularly vulnerable to lipid peroxidation for several reasons. First, neuronal membranes are rich in polyunsaturated fatty acids. Second, the antioxidant capacity of neural tissue is relatively limited compared to other tissues. Additionally, the nervous system contains high levels of monoamine neurotransmitters such as dopamine, epinephrine, and norepinephrine, which generate hydrogen peroxide during oxidative deamination by monoamine oxidase(82,90–93)

1.3.9. Cellular Damage Caused by Free Radicals

Free radical (FR)–induced damage involves multiple mechanisms; however, its principal effects include lipid peroxidation, disulfide bond formation in proteins, and DNA damage. Lipids are among the molecules most susceptible to free radical attack. FRs target membrane lipids, disrupting membrane integrity and stability, which leads to rapid cellular and tissue injury(55,72,94)

Disulfide bond formation occurs when thiol groups, such as those in glutathione (GSH) and cysteine residues, are oxidized, resulting in the formation of disulfide bridges. Although thiol radicals are less reactive than hydroxyl radicals, they can still exert significant biological effects. These sulfur-centered radicals promote homolytic cleavage reactions within proteins, leading to abnormal disulfide bond formation. Such alterations disrupt protein conformation and impair their biological and metabolic functions(70,95–97).

Free radicals can also induce mutations by damaging DNA structure, including base modifications and strand breaks. Furthermore, FRs alter protein function by modifying their secondary and tertiary structures (**Table 1.3**) (55,77)

Table 1.3. Target Tissues of Free Radical–Induced Damage, Types of Injury, and Their Biological Consequences

Target Structure	Damage	Consequence
DNA/RNA	Deoxyribose ring cleavage, base damage, strand breaks	Mutations, translation errors, inhibition of protein synthesis
Protein	Aggregation and cross-linking, fragmentation and cleavage, thiol group modification	Altered ion transport, increased intracellular calcium influx, changes in enzyme activities
Polyunsaturated Fatty Acids	Formation of lipid peroxidation products such as MDA and 4-hydroxy-2,3-transnonenal	Altered membrane fluidity, changes in membrane permeability, impairment of membrane-bound enzymes

1.4. Oxidative Stress

In living organisms, the rate of free radical production and the rate of their elimination are normally maintained in balance, a state referred to as redox homeostasis. As long as this redox equilibrium is preserved, cells are protected from the harmful effects of free radicals. However, an increase in free radical production or a decrease in the efficiency of antioxidant defense mechanisms disrupts this balance, resulting in oxidative stress.

All major cellular components, including lipids, proteins, nucleic acids, and carbohydrates, are susceptible to oxidative damage. The extent of oxidative injury depends on the concentration of reactive oxygen species (ROS), the duration of exposure, and various intracellular and extracellular factors(57,78)

1.4.1. Free Radicals

The term *free radical* refers to an atom or molecule that possesses one or more unpaired electrons in its outer orbital. Atoms consist of a nucleus surrounded by electrons arranged in specific energy levels known as orbitals. Each orbital can accommodate two electrons with opposite spins, a configuration that confers stability. In contrast, radicals are unstable and highly reactive due to the presence of unpaired electrons.

Unpaired electrons increase the chemical reactivity of an atom or molecule, making it more likely to participate in redox reactions. Free radicals may be organic or inorganic and can carry a positive charge, negative charge, or be electrically neutral(79–82,98–101).

During oxidative metabolism, when oxygen is reduced to water for energy production, a small proportion of oxygen is converted into reactive

intermediates known as free radicals. Because of their unpaired electrons, free radicals readily donate or accept electrons from other molecules, thereby altering molecular structure and function and potentially causing cellular damage in various tissues. The most common source of biologically relevant free radicals is the partial reduction of molecular oxygen. Reactive oxygen species (ROS) are highly unstable and short-lived; they may exist as positively charged, negatively charged, or neutral species. Radicals are conventionally denoted by a superscript dot (•), indicating the presence of an unpaired electron. Under physiological conditions, approximately 1–2% of molecular oxygen is partially reduced to form superoxide ($O_2^{\bullet -}$) and, subsequently, hydroxyl radicals (•OH) (77,94,95).

Due to their high reactivity, free radicals interact with oxidizable cellular components, particularly lipids, proteins, carbohydrates, and DNA, leading to structural and functional alterations. Superoxide radicals are primarily generated as a result of incomplete electron transfer within the mitochondrial electron transport chain.

Under physiological conditions, free radicals are neutralized by various defense mechanisms that protect tissues from oxidative injury. Reactive oxygen species can damage the vascular endothelium and contribute to disruption of the blood–spinal cord barrier. Under physiological conditions, reactive oxygen species are neutralized by enzymatic antioxidant defenses—primarily superoxide dismutases (SOD), catalase, and glutathione peroxidases (GPx)—in concert with non-enzymatic antioxidants such as tocopherols, carotenoids, glutathione, ascorbic acid, urate, bilirubin, and albumin. Metal-binding proteins such as ceruloplasmin, transferrin, and lactoferrin also contribute to antioxidant defense by limiting transition metal availability (99,103–106).

The central nervous system is particularly vulnerable to oxidative damage due to its relatively limited antioxidant capacity. In addition, the high content of polyunsaturated fatty acids, cholesterol, ascorbic acid, and iron in the central nervous system increases its susceptibility to free radical–mediated injury (Table 1.4 and 1.5)(90–93,107).

Table 1.4. Classification of reactive oxygen, nitrogen, and lipid-derived species involved in oxidative stress

Category	Species	Full Name	Type
Reactive Oxygen Species (ROS)	$O_2^{\bullet -}$	Superoxide anion	Radical
	H_2O_2	Hydrogen peroxide	Non-radical
	•OH	Hydroxyl radical	Radical
	$^1O_2$	Singlet oxygen	Non-radical
	O_3	Ozone	Non-radical
	HO_2•	Hydroperoxyl radical	Radical
	HOCl	Hypochlorous acid	Non-radical
Lipid-Derived Species	LOO•	Lipid peroxyl radical	Radical
	LO•	Lipid alkoxyl radical	Radical
	LOOH	Lipid hydroperoxide	Non-radical
Reactive Nitrogen Species (RNS)	NO•	Nitric oxide	Radical
	$ONOO^-$	Peroxynitrite	Non-radical

Table 1.5. Major reactive species and their biological targets and cytotoxic effects

Species	Primary Source	Major Targets	Biological Effects
$O_2^{\bullet -}$	Mitochondria, NADPH oxidase	Proteins, enzymes	Initiates oxidative cascade
H_2O_2	Dismutation of $O_2^{\bullet -}$	Lipids, DNA	Membrane damage, signaling
•OH	Fenton reaction	DNA, lipids, proteins	Severe cellular damage, apoptosis
$^1O_2$	Photochemical reactions	Lipids, proteins	Lipid peroxidation
LOO•	Lipid peroxidation chain reaction	Cell membranes	Membrane destabilization
NO•	Nitric oxide synthase	Proteins, DNA	Nitrosative stress
$ONOO^-$	Reaction of NO• with $O_2^{\bullet -}$	DNA, mitochondria	Protein nitration, cell death

1.4.1.1. Superoxide Radical

In aerobic cells, the first product formed during the one-electron reduction of molecular oxygen is the superoxide radical ($O_2^{\bullet -}$). Although it is a relatively weak oxidant, it acts as a strong reducing agent and plays a significant role in oxygen toxicity. The enzyme superoxide dismutase (SOD) protects cells against superoxide-mediated damage(108,109).

The biological importance of the superoxide anion lies in its role as a precursor of hydrogen peroxide (H_2O_2) and in its ability to reduce transition metal ions. At low pH values, superoxide can be protonated to form the perhydroxyl radical ($HO_2\bullet$), which is more reactive and membrane-permeable.

Superoxide undergoes a dismutation reaction in which one molecule is oxidized and another is reduced, producing molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). This reaction may occur spontaneously or be catalyzed by SOD(110–114).

Superoxide can react with thiol groups, leading to glutathione (GSH) depletion and increased oxidative stress. It may also interact with thiol groups in enzymes and other cellular proteins, resulting in their functional inactivation. Furthermore, superoxide participates in reactions that generate more reactive species.(108,115–117)

In biological systems, hydroxyl radical (•OH) formation does not occur efficiently through the classical Haber–Weiss reaction alone, as this reaction proceeds at negligible rates under physiological conditions. Instead, superoxide contributes indirectly by reducing ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), thereby facilitating the Fenton reaction. This iron-catalyzed redox cycling—often referred to as the iron-dependent Haber–Weiss cycle—represents a major mechanism of hydroxyl radical generation during oxidative stress(112,118).

1.4.1.2. Hydrogen Peroxide (H_2O_2)

Hydrogen peroxide is formed during the two-electron reduction of molecular oxygen or through the dismutation of superoxide ($O_2\bullet^-$). In biological systems, the primary source of hydrogen peroxide is the dismutation of superoxide. In this reaction, two superoxide anions react with two protons to form hydrogen peroxide and molecular oxygen:

$$2\ O_2\bullet^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

This process is termed a dismutation reaction because it yields non-radical products. It may occur spontaneously or be catalyzed by the enzyme superoxide dismutase (SOD)(119–121).

Hydrogen peroxide is a relatively weak oxidant but serves important physiological and signaling functions. It readily diffuses across cellular membranes. Under physiological pH and temperature, and in the absence of transition metal ions, H_2O_2 is relatively stable. However, in the presence of ferrous iron (Fe^{2+}), hydrogen peroxide participates in the Fenton reaction, generating highly reactive hydroxyl radicals (•OH).

In the Fenton reaction, Fe^{2+} reacts with H_2O_2 to produce Fe^{3+}, hydroxyl radicals, and hydroxide ions. The resulting reactive species possess strong oxidizing properties and can initiate lipid peroxidation and other forms of oxidative damage in cellular membranes.

Because of its potential to generate highly reactive radicals, hydrogen peroxide must be tightly regulated and rapidly removed in biological systems. This detoxification is primarily mediated by antioxidant enzymes such as catalase and glutathione peroxidase (GPx)(122–127)

1.4.1.3. Hydroxyl Radical (•OH)

The hydroxyl radical (•OH) is the most reactive and toxic radical species in biological systems. It can be generated through several mechanisms. One major pathway is the Fenton reaction, in which hydrogen peroxide reacts with ferrous iron (Fe^{2+}) or other transition metal ions to produce hydroxyl radicals.

In addition, the Haber–Weiss reaction involves the interaction of superoxide ($O_2^{\bullet -}$) and hydrogen peroxide, typically catalyzed by iron or copper, resulting in the generation of hydroxyl radicals.

The hydroxyl radical exerts its most significant biological effects on lipids, proteins, cytochromes, and nucleic acids. It abstracts a hydrogen atom from various biomolecules—including fatty acids, nucleic acids, carbohydrates, and proteins—leading to the formation of carbon-centered radicals. The attack of hydroxyl radicals on polyunsaturated fatty acids within membrane phospholipids is a major initiating event in lipid peroxidation and cellular oxidative injury.

The hydroxyl radical can also induce mutagenic effects by attacking the deoxyribose backbone of DNA. Furthermore, it reacts with purine and pyrimidine bases in DNA and RNA, generating additional radical species and oxidative base modifications. When oxidative damage exceeds cellular repair capacity, mutations and ultimately cell death may occur(53,122,128–130)

1.4.1.4. Nitric Oxide (NO)

Nitric oxide (NO) exerts protective effects at the cellular level under physiological conditions. However, under oxidative stress, NO reacts with superoxide ($O_2^{\bullet -}$) to form peroxynitrite ($ONOO^-$), a highly potent oxidant. NO was first identified as an endothelium-derived relaxing factor (EDRF). Although it plays a crucial role in maintaining homeostasis in biological systems, it may exert cytotoxic effects under pathological conditions.

Nitric oxide is a gaseous free radical synthesized in smooth muscle cells, endothelial cells, neurons, and various other mammalian cells through the oxidation of the guanidino nitrogen of L-arginine by nitric oxide synthase (NOS) (131–134) NO participates in neurotransmission, neuroplasticity, and, under certain conditions, neurotoxicity within both the central and peripheral nervous systems. In the spinal cord, it functions as a secondary messenger with neurotransmitter-like properties.

Unlike classical neurotransmitters, NO diffuses freely across cell membranes and is not stored in synaptic vesicles. It does not act through conventional membrane-bound receptors but instead exerts its effects via intracellular signaling pathways after diffusing into adjacent cells.

NO is synthesized by three isoforms of nitric oxide synthase: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (154–156). Low concentrations of NO produced by nNOS and eNOS serve as intracellular and extracellular signaling molecules in neural and vascular tissues. In contrast, iNOS is typically induced during inflammatory responses and produces larger amounts of NO.

In nNOS and eNOS, NO synthesis is calcium/calmodulin-dependent. Therefore, agonists that elevate intracellular calcium levels enhance NO production. In contrast, iNOS activation is largely calcium-independent and is induced by endotoxins and pro-inflammatory cytokines.

Increased NO production contributes to metabolic alterations in spinal cord neurons, primarily through activation of soluble guanylate cyclase and subsequent elevation of cyclic guanosine monophosphate (cGMP). Following spinal cord injury, tissue NO levels rise rapidly. Because NO is lipophilic and diffusible, it readily migrates from endothelial cells to adjacent smooth muscle cells, where it binds to the heme moiety of soluble guanylate cyclase. Activation of this enzyme increases intracellular cGMP levels, leading to vascular smooth muscle relaxation and inhibition of platelet adhesion and aggregation(135–137)

Nitric oxide (NO) reacts with the superoxide anion ($O_2^{\bullet -}$) to form peroxynitrite ($ONOO^-$), a highly potent oxidizing and nitrating species. Peroxynitrite induces tissue damage through several mechanisms:

1. Upon protonation, peroxynitrite (ONOOH) can decompose to generate highly reactive species, including hydroxyl radicals ($\bullet OH$) and nitrogen dioxide ($\bullet NO_2$).
2. It directly and rapidly oxidizes sulfhydryl (–SH) groups, lipids, proteins, and DNA.

3. It can form reactive intermediates such as the nitronium ion (NO_2^+), leading to nitration of aromatic amino acids—particularly tyrosine residues—in proteins.

In the presence of superoxide dismutase (SOD), superoxide anions are rapidly converted to hydrogen peroxide, thereby limiting peroxynitrite formation. Consequently, NO-mediated neurotoxicity associated with peroxynitrite is reduced(138).

1.5. Antioxidants

Various defense mechanisms, collectively referred to as antioxidant defense systems, have evolved in living organisms to counteract the harmful effects of free radicals. When the balance between free radicals and antioxidants is disrupted, numerous pathological changes, including cellular injury, may occur.

Antioxidants inhibit lipid peroxidation through both enzymatic and non-enzymatic mechanisms. Enzymatic antioxidants maintain redox balance by facilitating the reduction of reactive species, interrupting the lipid peroxidation chain reaction, scavenging reactive oxygen species (ROS), and inactivating transition metal ions. Non-enzymatic antioxidants act primarily by directly neutralizing free radicals and other reactive intermediates (Table 1.6) (85,122,139–141).

Table 1.6. Major Antioxidant Systems and Their Cellular Localization

Category	Antioxidant	Primary Localization	Functional Note
I. Enzymatic Antioxidants			
	SOD1 (Cu/Zn-SOD)	Cytosol, nucleus	Superoxide detoxification in cytoplasm
	SOD2 (Mn-SOD)	Mitochondrial matrix	Primary mitochondrial ROS defense
	SOD3 (EC-SOD)	Extracellular space, plasma	Extracellular superoxide scavenging
	Glutathione Peroxidase (GPx1–4)	Cytosol, mitochondria, nucleus	Reduces H_2O_2 and lipid peroxides
	Glutathione Reductase (GR)	Cytosol, mitochondria	Regenerates reduced glutathione (GSH)
	Catalase	Peroxisomes	Decomposes hydrogen peroxide
	Peroxiredoxins (Prx)	Cytosol, mitochondria, nucleus	Fine regulation of peroxide signaling

Category	Antioxidant	Primary Localization	Functional Note
	Thioredoxin System (Trx, TrxR)	Cytosol and mitochondria	Redox signaling and protein repair
II. Non-Enzymatic Antioxidants			
A. Lipid-Soluble			
	Vitamin E (α-Tocopherol)	Cellular membranes, plasma lipoproteins	Lipid peroxidation chain breaker
	Vitamin A (Retinoids)	Membrane-associated	Secondary antioxidant role
	Ubiquinol (CoQ10)	Inner mitochondrial membrane, other membranes	Electron transport & lipid antioxidant
B. Water-Soluble			
	Vitamin C (Ascorbate)	Cytosol, extracellular fluid, plasma	Regenerates vitamin E; ROS scavenger
	Glutathione (GSH)	Cytosol, mitochondria, nucleus	Central intracellular redox buffer
	Uric Acid	Plasma	Major circulating antioxidant in humans
	Bilirubin (Albumin-bound)	Plasma	Peroxyl radical scavenging
C. Metal-Binding Proteins			
	Albumin	Plasma	Binds transition metals; antioxidant buffering
	Transferrin	Plasma	Iron sequestration
	Ceruloplasmin	Plasma	Copper transport; ferroxidase activity
	Ferritin	Cytosol	Intracellular iron storage and detoxification
	Metallothioneins	Cytosol	Heavy metal binding and redox regulation

1.5.1. Enzymatic Antioxidants

1.5.1.1. Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) catalyzes the conversion of the superoxide anion ($O_2^{\bullet -}$), generated during aerobic metabolism, into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). It is a key enzyme in protecting cells against oxidative damage. Although cells continuously generate superoxide radicals during normal metabolic processes, SOD maintains intracellular superoxide levels at low concentrations.

Due to its high reactivity, the superoxide radical exerts direct cytotoxic effects. Several isoenzymes of superoxide dismutase have been identified. The cytosolic isoform, copper–zinc superoxide dismutase (Cu/Zn-SOD or SOD1), is primarily localized in the cytoplasm. The mitochondrial isoform, manganese superoxide dismutase (Mn-SOD or SOD2), is located in the mitochondrial matrix. Given that mitochondria are a major source of reactive oxygen species through the electron transport chain, Mn-SOD plays a crucial role in antioxidant defense.

Cytosolic SOD is particularly abundant in erythrocytes, where it neutralizes superoxide generated by the auto-oxidation of hemoglobin. A third isoform, extracellular superoxide dismutase (EC-SOD or SOD3), is present in extracellular fluids such as plasma and synovial fluid and is also a copper–zinc–containing enzyme(108,142–144).

1.5.1.2. Glutathione Peroxidase (GPx)

Glutathione peroxidase (GPx) catalyzes the reduction of hydrogen peroxide (H_2O_2) and lipid hydroperoxides to water and their corresponding alcohols, respectively. GPx is primarily localized in the cytosol and is a selenium-containing enzyme with a tetrameric structure, each subunit incorporating one selenocysteine residue.

In humans, several isoforms of GPx have been identified, including cytosolic GPx (GPx1), gastrointestinal GPx (GPx2), plasma or extracellular GPx (GPx3), and phospholipid hydroperoxide GPx (GPx4).

GPx catalyzes the oxidation of reduced glutathione (GSH) to oxidized glutathione (GSSG, glutathione disulfide) while simultaneously reducing hydrogen peroxide or lipid hydroperoxides. Through this reaction, H_2O_2 is converted into water and detoxified, thereby contributing to the maintenance of cellular redox homeostasis(145–149).

1.5.1.3. Catalase

Catalase is an antioxidant enzyme that catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water (H_2O) and molecular oxygen (O_2). Glutathione peroxidase (GPx) also reduces hydrogen peroxide; however, these two enzymes differ in their kinetic properties and substrate affinities.

At low concentrations of H_2O_2, GPx plays a predominant role in detoxification due to its higher affinity for hydrogen peroxide. In contrast, at higher H_2O_2 concentrations, catalase becomes more significant because of its high catalytic capacity.

Catalase is a tetrameric heme-containing enzyme, with each subunit containing a ferriprotoporphyrin (heme) group. Similar to superoxide dismutase (SOD), catalase activity varies among tissues. Although catalase is present in most tissues, it is particularly abundant in the liver and erythrocytes (150–153).

1.5.1.4. Mitochondrial Cytochrome c Oxidase

Cytochrome c oxidase (Complex IV) is a copper-containing hemoprotein complex and the terminal component of the mitochondrial electron transport chain. It catalyzes the transfer of electrons from cytochrome c to molecular oxygen, the final electron acceptor, reducing oxygen to water.

During electron transport, partial reduction of oxygen may result in the formation of superoxide ($O_2^{\bullet -}$). Although cytochrome c oxidase contributes to efficient electron transfer and minimizes electron leakage, superoxide production can exceed the capacity of this system under pathological conditions. In such cases, reactive oxygen species are detoxified by antioxidant enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx)(154–157).

1.5.1.5. Glutathione S-Transferases (GSTs)

Glutathione S-transferases (GSTs) are a family of detoxification enzymes composed of two protein subunits (dimers). They are broadly classified into cytosolic, mitochondrial, and microsomal forms. GSTs protect cells by catalyzing the conjugation of reduced glutathione (GSH) to electrophilic compounds, including reactive intermediates and strong alkylating agents, thereby facilitating their detoxification and elimination(158–161)

1.5.2. Non-Enzymatic Antioxidants

Numerous molecules contribute to antioxidant defense. The most important non-enzymatic antioxidants include glutathione (GSH), vitamins A, E, and C, melatonin, flavonoids, uric acid, cysteine, ceruloplasmin, ubiquinone (coenzyme Q), oxypurinol, bilirubin, mannitol, lipoic acid, haptoglobin, and hemopexin(161–164).

1.6. Pathology of Spinal Cord Injury

Histopathological changes following spinal cord trauma are classically categorized into acute, subacute, and chronic phases. After the initial hemorrhage and rapid necrosis, astrocytes and microglia become reactive, and inflammatory cells infiltrate the lesion site. In the subsequent weeks, glial scar formation and cavitation occur, and various stages of Wallerian degeneration are observed in the white matter tracts (11–13,165,166).

1.6.1. Acute Phase (Hemorrhagic Necrosis)

The earliest change following injury involves disruption of the microvasculature within the gray matter of the spinal cord. Multifocal petechial hemorrhages, particularly around the central canal and in the anterior horns, tend to spread radially within hours. The presence of microthrombi and blood extravasation becomes evident within the first 24 hours. Endothelial tight junctions begin to separate within minutes after injury.

As central petechial hemorrhages expand, glial activation and neuronal degeneration become more pronounced. The earliest morphological signs of neuronal necrosis may appear within the first hour. Histopathological features include cytoplasmic eosinophilia, neuronal "ghost cells," loss of Nissl substance, neuronal shrinkage, hyperchromasia, and irregular cellular contours.

Necrotic changes in the gray matter intensify during the first few hours and begin to extend into the white matter after approximately 8 hours. Necrosis is typically observed earlier in anterior horn motor neurons than in posterior horn neurons. Both neurons and glial cells undergo cell death within a similar temporal window.

Elevated intracellular calcium contributes to cellular injury through activation of nucleases, proteases, kinases, phospholipases, and nitric oxide synthase, ultimately resulting in necrotic and apoptotic cell death. Axonal–myelin separation occurring within hours after trauma is primarily due to edema and the formation of intramyelinic vacuoles. The progression of these myelin alterations imparts a spongiform appearance to the white matter under

light microscopy. These axonal changes radiate outward from the epicenter of injury and may extend toward the pial surface over several days(13,48,166–169).

1.6.2. Subacute Phase

During the subacute phase, activated microglia and astrocytes contribute to the development of reactive gliosis. In the presence of neuronal degeneration, microglia differentiate into phagocytic macrophage-like cells under the influence of inflammatory mediators. These cells primarily accumulate at the lesion core and rarely extend into distant tissue. Whether they selectively eliminate damaged cells or also contribute to secondary injury of partially viable cells remains unclear.

In response to injury, astrocytes undergo hypertrophy and proliferation. Compared with the uninjured spinal cord, reactive astrocytes exhibit enlarged cell bodies and increased numbers of processes, characterized by upregulation of glial fibrillary acidic protein (GFAP) within intermediate filaments. Oxidative and lysosomal enzyme activity also increases. Reactive astrocytes begin to accumulate around the lesion during the first week after injury. The astrocytic response peaks around day 14 and may persist until approximately day 28.

Disruption of the blood–spinal cord barrier leads to edema formation within hours after injury. Edema progresses both radially and longitudinally and becomes prominent within the first 24 hours. It may persist for up to 8 days and can be classified as vasogenic or cytotoxic in nature(170–172). Because of the limited elasticity of the pia mater, edema contributes to increased vascular resistance and subsequent reduction in spinal cord blood flow.

Inflammatory cell infiltration occurs in two waves. In the first wave, polymorphonuclear leukocytes infiltrate the lesion site within hours after injury and may contribute to neuronal damage through cytotoxic mechanisms. Their numbers peak within 24 hours and decline by approximately day 3(173–175). In the second wave, monocytes and macrophages accumulate at the lesion site and phagocytose cellular debris.

Additional peripheral cells, including Schwann cells, meningeal cells, and fibroblasts, migrate to the lesion site. Schwann cells contribute to axonal regeneration by remyelinating injured axons and secreting neurotrophic factors. Although the precise role of meningeal cells remains incompletely understood, they may participate in the formation of the glial limitans. Fibroblasts, likely derived from the meninges, contribute to fibrotic scar formation. Increased release of basic fibroblast growth factor (bFGF) following trauma may promote fibroblast proliferation and neovascularization(160,176–180).

1.6.3. Chronic Phase

In the weeks and months following injury, as acute and subacute events subside, cystic cavities filled with cerebrospinal fluid (CSF) may develop, occasionally communicating with the central canal. Wound healing in the spinal cord frequently results in cavitation.

Guízar-Sahagún et al. described three phases in post-traumatic cyst formation(181):

- **Necrotic phase:** Begins on day 1 and lasts for approximately 1–2 weeks after trauma.
- **Repair phase:** Occurs between 2 and 8 weeks after trauma.
- **Stabilization phase:** Extends from approximately 8 weeks up to 1 year following injury.

By the third day after trauma, microcystic cavities begin to appear, accompanied by parenchymal hemorrhage, vascular thrombosis, edema, axonal fragmentation, and inflammatory infiltration. Two to three weeks after contusion injury, macrophages phagocytose necrotic tissue and subsequently leave the lesion core, resulting in the formation of cavities of varying sizes(12,48,181).

Four to five weeks after trauma, the margins of cystic cavities within the trabecular scar tissue become more clearly defined. Myelin loss is also a key feature of the late lesion stage. In moderate injuries, axonal continuity may be preserved, whereas selective demyelination occurs. Demyelination begins within the first 24 hours, peaks during the second week, and remyelination typically begins during the third week.

Although oligodendrocytes contribute to remyelination, migration of Schwann cells from the dorsal root entry zone into the lesion area suggests that these peripheral glial cells also participate in remyelination(181–185).

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CHAPTER 2

Mechanistic Insights into the SOD–GPx Axis and Redox Homeostasis in Spinal Cord Injury

Spinal cord injury triggers an oxidative stress defense response that results in dysfunction of the mitochondrial electron transport chain. Subsequently, increased NADPH oxidase activation and inflammatory cell infiltration significantly increase reactive oxygen species (ROS) production (1–3). The superoxide anion formed in this early phase is a central component of the oxidative burden and is targeted by superoxide dismutase (SOD) in the first step of endogenous antioxidant defense (1,2,4).

SOD plays a role in protecting cellular structures from direct superoxide-induced attack by converting the superoxide anion into hydrogen peroxide, a less reactive intermediate. Although hydrogen peroxide is not a free radical, if it accumulates in tissue, it can be converted into a hydroxyl radical, becoming much more cytotoxic. Therefore, increased SOD activity alone is not an absolute protective response. It is important whether hydrogen peroxide is effectively detoxified by enzymes such as glutathione peroxidase (2,5–8).

GPx is a complementary component of the redox cascade that converts hydrogen peroxide, formed via SOD, back into water and oxygen using reduced glutathione (GSH). This same enzyme system also contributes to the maintenance of axonal membrane integrity and conduction by detoxifying lipid hydroperoxides originating from membrane lipids, particularly in myelin-rich white matter. (8–10) . In cases where GPx activity is insufficient, the conversion of hydrogen peroxide to hydroxyl radical via Fenton reactions can cause irreversible damage to membrane lipids, structural/proteolytic proteins and nucleic acids. Therefore, the SOD– GPx axis is predicted to be one of the

key determinants of oxidative–antioxidative balance in the pathophysiology of spinal cord injury (1,12,13).

Experimental spinal cord injury models are not merely passive biochemical reflections but also an indicator of the cell's adaptive capacity. While early increases in SOD and GPx levels following trauma are interpreted as a compensatory response to oxidative load, depletion or functional suppression of these systems under continued excessive ROS production may contribute to the deepening of secondary damage. (13–15) . In fact, experimental studies using different pharmacological agents have reported that preserving or enhancing SOD and GPx activity attenuates oxidative damage and improves functional recovery after spinal cord injury ((2,4,5,12,17,18).

The GPx response is crucial for preserving mitochondrial integrity by inhibiting hydrogen peroxide accumulation , resulting in stable mitochondrial membrane potential, delayed cytochrome - c release, and suppression of caspase- mediated apoptotic pathways. This can contribute to limiting neuronal and glial cell loss. Conversely, decreased GPx activity, despite the relative preservation of SOD activity, can accelerate cell death by increasing hydrogen peroxide load and indirectly promoting the formation of more aggressive oxidant species (1,2,5,12).

Post-traumatic intracellular calcium loading is a significant factor in disrupting the fragile redox balance. Increased Ca^{2+} influx accelerates superoxide production by increasing intramitochondrial electron runoff. This then creates a self-sustaining cycle between oxidative stress and calcium dyshomeostasis . This can lead to ROS production exceeding the buffering capacity of the SOD– GPx axis. Opening of mitochondrial permeability pores, disruption of ATP synthesis, and necrotic/apoptotic cell death may become inevitable (4,5).

Modulating calcium influx and enhancing the endogenous antioxidant response via transcription factors such as Nrf2 both indirectly reduces ROS production and supports the integrity of SOD, GPx , and the associated antioxidant network. In this context, protecting the antioxidant enzyme system does not only mean scavenging free radicals; it is also considered a core component of a holistic neuroprotective strategy in terms of stabilizing mitochondrial function, limiting the inflammatory response, and slowing the molecular progression of secondary damage (7,19,20).

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CHAPTER 3

Contemporary Pharmacological Strategies in SCI

3.1. Corticosteroids

Corticosteroids have historically been among the most frequently used pharmacological agents in the treatment of acute spinal cord injury (SCI), although their clinical use remains controversial. Their proposed mechanisms of action include attenuation of the inflammatory response and inhibition of lipid peroxidation. In SCI, one of their principal effects is believed to be the reduction of lipid peroxidation. Additional proposed mechanisms include suppression of pro-inflammatory cytokines, inhibition of inflammatory cell infiltration, modulation of calcium influx, and improvement of spinal cord perfusion(1–6).Methylprednisolone sodium succinate has been the most extensively studied corticosteroid in spinal cord trauma. Its effects were evaluated in large prospective, randomized, double-blind, multicenter clinical trials, notably the National Acute Spinal Cord Injury Study (NASCIS) series.

In the NASCIS I trial, methylprednisolone was administered as a 1,000 mg bolus followed by maintenance doses of 250 mg every 6 hours for 10 days; however, no significant neurological benefit was demonstrated. In the NASCIS II trial, a higher-dose methylprednisolone regimen was used. When a 30 mg/kg bolus was administered within the first 8 hours after injury, followed by a maintenance infusion of 5.4 mg/kg/hour for 23 hours, modest but statistically significant improvements in motor and sensory scores were observed at 6 and 12 months.NASCIS III investigated the optimal duration of maintenance therapy. In patients treated between 3 and 8 hours after injury, extending the maintenance infusion to 48 hours was associated with greater motor improvement compared with the 24-hour regimen at follow-up evaluations.

Despite these findings, the routine use of corticosteroids remains debated due to concerns regarding limited clinical benefit and increased risk of adverse effects. High-dose corticosteroid therapy has been associated with complications such as pneumonia, sepsis, pulmonary embolism, and wound infection (6–12)

3.2. Naloxone

Naloxone, an opioid receptor antagonist, was investigated as a potential neuroprotective agent in acute spinal cord injury. It was evaluated as one of the treatment arms in the NASCIS II trial.

In this study, naloxone was administered as a 5.4 mg/kg bolus followed by a continuous infusion of 4.0 mg/kg over 23 hours. Initial analyses suggested possible neurological improvement; however, subsequent evaluations failed to demonstrate consistent or statistically significant benefits.

Due to the lack of reproducible clinical efficacy and absence of a clear consensus regarding its therapeutic value, naloxone was not adopted as a standard treatment for spinal cord injury (11,13,14)

3.3. Gangliosides

Gangliosides are complex acidic glycosphingolipids that are present in high concentrations in the membranes of central nervous system cells. Among them, monosialotetrahexosylganglioside (GM1) is the most extensively studied compound in spinal cord injury research.Experimental studies have demonstrated that GM1 promotes neuronal survival, enhances neurite outgrowth, and exerts anti-apoptotic effects. These findings suggested a potential role in neuroprotection and neural repair. In clinical trials, treatment with GM1 was associated with improvements in motor and sensory recovery, particularly during the first 6 months after injury, as assessed by the ASIA (American Spinal Injury Association) Impairment Scale. A commonly reported regimen consisted of a 600 mg loading dose followed by a maintenance dose of 200 mg/day for 56 days.However, despite encouraging early results, subsequent studies failed to establish definitive long-term clinical benefit, and GM1 has not become a standard therapy in spinal cord injury management(15–18).

3.4. Cyclooxygenase Enzyme Inhibitors

Cyclooxygenase (COX) inhibitors are classic non-steroidal anti-inflammatory drugs (NSAIDs) widely used in clinical practice for their analgesic and anti-inflammatory properties. Experimental studies have suggested potential neuroprotective effects in spinal cord injury, primarily through inhibition of

prostaglandin synthesis and attenuation of inflammation. However, robust, large-scale clinical trials demonstrating consistent neurological benefit in spinal cord injury are lacking (14,19–21)

3.5. Other Pharmacological Agents

To counteract glutamate-mediated excitotoxicity that occurs within minutes after trauma, N-methyl-D-aspartate (NMDA) receptor antagonists such as MK-801 have been investigated in experimental models of spinal cord injury and have shown neuroprotective effects. However, due to significant adverse effects and safety concerns, these agents have not progressed to routine clinical use.Calcium channel blockers have also demonstrated beneficial effects in experimental settings; nevertheless, convincing clinical evidence supporting their routine use in spinal cord injury is lacking (22,23). Similarly, sodium channel blockers have shown neuroprotective potential in preclinical studies but have not been established as standard clinical therapies (24). Minocycline, a tetracycline-derived antibiotic, has been shown to reduce microglial activation and modulate inflammatory responses in experimental models, with some early clinical investigations suggesting possible benefit(25)

Experimental data also indicate that erythropoietin may exert neuroprotective effects through anti-inflammatory and antioxidant mechanisms(26). Immunosuppressive agents such as tacrolimus (FK506) and cyclosporine have demonstrated neuroprotective effects in experimental studies, primarily through modulation of inflammatory and apoptotic pathways; however, their clinical application remains investigational(27,28). ProCord is an autologous macrophage-based therapy consisting of macrophages derived from the patient's peripheral blood and activated ex vivo. These cells are exposed to peripheral nerve segments or skin tissue to induce a reparative phenotype before being injected into the injured spinal cord. Early-phase clinical trials have been initiated to evaluate the safety and feasibility of this approach; however, its efficacy has not yet been conclusively established(29).

3.6. Pregabalin

Pregabalin is a structural analog of gamma-aminobutyric acid (GABA) used in the treatment of epilepsy, generalized anxiety disorder, and neuropathic pain. Chemically, pregabalin is the S-(+)-enantiomer of 3-isobutyl γ-aminobutyric acid and is defined as S-(+)-3-(aminomethyl)-5-methylhexanoic acid. Its molecular formula is $C_8H_{17}NO_2$, and its molecular weight is 159.23 g/mol. Pregabalin is freely soluble in water and in both acidic and basic aqueous solutions(30–35). In vitro studies have demonstrated that pregabalin binds with high affinity to the auxiliary α2δ subunit of voltage-gated calcium

channels (VGCCs) in the central nervous system, displacing radiolabeled (^{3}H)-gabapentin. Experimental evidence suggests that binding to the α2δ subunit is essential for its analgesic and anticonvulsant effects in animal models(36,37). Four isoforms of the α2δ subunit have been identified, with differential tissue expression. The α2δ-1 and α2δ-2 isoforms are highly expressed in small dorsal root ganglion neurons, whereas α2δ-3 is predominantly expressed in large dorsal root ganglion neurons and in the brain. Pregabalin exhibits high affinity for the α2δ-1 and α2δ-2 isoforms but has minimal affinity for α2δ-3 and α2δ-4

Following binding to the α2δ subunit in hyperexcitable neurons, pregabalin reduces depolarization-induced calcium influx, thereby decreasing the presynaptic release of excitatory neurotransmitters such as glutamate, norepinephrine, and substance P. This modulation attenuates postsynaptic excitatory signaling and helps restore neuronal excitability toward physiological levels. Notably, pregabalin preferentially reduces pathological ectopic discharges while largely preserving normal synaptic transmission.Pregabalin does not bind to GABA-A or GABA-B receptors, is not converted metabolically into GABA, and does not inhibit GABA reuptake or degradation. It readily crosses the blood–brain barrier. The oral bioavailability of pregabalin is ≥90% and dose-independent (261–265). Steady-state plasma concentrations are achieved within 24–48 hours following repeated administration. Pregabalin does not significantly bind to plasma proteins and undergoes negligible hepatic metabolism. After administration of radiolabeled pregabalin, approximately 98% of the recovered urinary radioactivity corresponds to unchanged drug. Elimination occurs primarily via renal excretion. The mean elimination half-life is approximately 6.3 hours. Dose adjustment is required in patients with renal impairment or those undergoing hemodialysis (38–42).

Pregabalin is approved in many countries for the treatment of peripheral neuropathic pain and as adjunctive therapy for partial-onset seizures in adults. In several European countries, it is also indicated for central neuropathic pain and generalized anxiety disorder(43–45)Data regarding the use of pregabalin during pregnancy are limited. It is unknown whether pregabalin is excreted in human breast milk, although it has been detected in rat milk; therefore, breastfeeding is generally not recommended during treatment. The most commonly reported adverse effects are dizziness and somnolence. These adverse reactions are typically mild to moderate in severity. In controlled clinical trials, discontinuation due to adverse events occurred in approximately 13% of patients receiving pregabalin, compared with 7% in placebo-treated patients. Other frequently reported adverse effects include increased appetite, euphoria, confusion, decreased libido, irritability, ataxia, impaired concentration, coordination disturbances, memory impairment,

tremor, dysarthria, paresthesia, blurred vision, diplopia, vertigo, dry mouth, constipation, vomiting, flatulence, erectile dysfunction, fatigue, peripheral edema, gait disturbance, and weight gain. In addition to its neuromodulatory properties, pregabalin has demonstrated anti-inflammatory and anti-apoptotic effects in experimental models, as evidenced by histopathological and biochemical analyses (46–49).

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CHAPTER 4

Experimental Models of Spinal Cord Injury

A total of 44 male Sprague–Dawley rats (250–400 g), housed under standard laboratory conditions at the İnönü University Experimental Animal Production and Research Center, were included in the study. All animals were neurologically intact prior to the experiment. Ethical approval was obtained from the Experimental Animal Ethics Committee of İnönü University Faculty of Medicine (approval no: 2011-A108).

The animals were randomly assigned to six experimental groups.

4.1. Experimental Groups

The study consisted of the following six groups:

- **Sham group** (laminectomy only, n = 6)
- **PB-only group** (pregabalin 40 mg/kg, n = 6)
- **SCI group** (spinal cord injury + 0.5 mL saline, n = 8)
- **MP + SCI group** (methylprednisolone 30 mg/kg, n = 8)
- **PB 40 + SCI group** (pregabalin 40 mg/kg, n = 8)
- **PB 80 + SCI group** (pregabalin 80 mg/kg, n = 8)

In all groups except the Sham and PB-only groups, spinal cord injury was induced using the Allen weight-drop method.

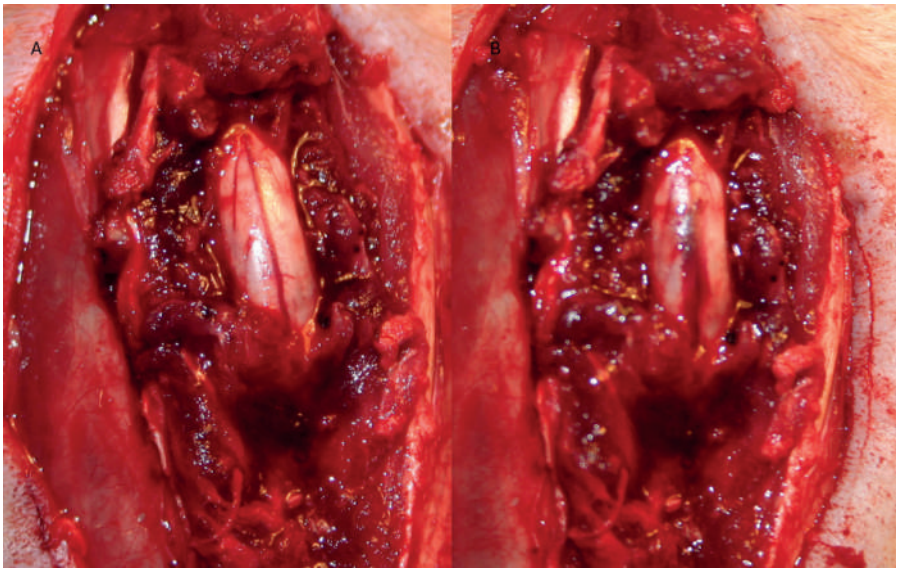
Pharmacological treatments were initiated 30 minutes after injury and administered intraperitoneally at 12, 24, 36, and 48 hours post-injury.

4.2. Anesthesia and Surgical Procedures

General anesthesia was induced in all rats by intraperitoneal administration of ketamine (50 mg/kg) and xylazine (10 mg/kg). After shaving the dorsal thoracolumbar region and performing antisepsis with povidone–iodine solution, a midline skin incision was made extending from T9 to L1. The paravertebral muscles were separated using blunt dissection, and a laminectomy was performed at the T9–T11 vertebral levels. Spinal cord injury was induced at the T10 level using the modified Allen weight-drop method, following exposure of the intact dura mater. A 5-g weight with a 2-mm tip diameter was dropped vertically from a height of 10 cm onto the exposed spinal cord(1–4) **(Figure 1).**

Figure 1. Postoperative laminectomy and induction of spinal cord injury at the T10 level

a,Dorsal view following T9–T11 laminectomy, showing the intact dura mater. b,Contusive spinal cord injury generated at the T10 level via the Allen weight-drop method, demonstrating traumatic disruption of the neural tissue.



4.3. Drug Administration

Pregabalin (PB; Lyrica®, 150 mg capsules) and methylprednisolone (MP; Sopharma) were administered intraperitoneally.Drug administration was initiated 30 minutes after spinal cord injury and repeated at 12, 24, 36, and 48 hours post-injury.For pregabalin preparation, the contents of the capsules were weighed using a precision analytical balance, suspended in sterile 0.9%

sodium chloride (NaCl) solution, and vortexed to ensure homogeneous dispersion. Pregabalin was administered at doses of 40 mg/kg or 80 mg/kg according to group allocation.

4.4. Neurological Assessment

Motor function was evaluated on postoperative days 1–3 by a neurosurgeon who was blinded to group allocation. Neurological assessment was performed using the Tarlov scoring system and the inclined plane test (5–7).

The Tarlov score (0–5) was defined as follows:

0 = no voluntary movement (paraplegia)

1 = minimal movement

2 = ability to sit with assistance

3 = ability to sit independently

4 = ambulation with abnormal gait

5 = normal ambulation

Hindlimb motor performance was additionally evaluated using the inclined plane test. In this test, rats were placed in a prone position on an adjustable inclined platform. The maximum angle at which the animal was able to maintain its position for at least 5 seconds without falling was recorded. Each animal was tested three times, and the mean value was used for statistical analysis.

4.5. Sacrifice and Tissue Collection

At 72 hours of the experiment, rats were re-anesthetized by intraperitoneal administration of xylazine (10 mg/kg) and ketamine (50 mg/kg) and sacrificed by thoracotomy. Approximately 2 mL of blood was collected via cardiac puncture. Blood samples were centrifuged at 3000 rpm for 10 minutes to separate the serum and stored at -30°C.

The spinal cord was carefully removed; an approximately 1 cm segment from the T10 lesion area was separated for histopathological examination, and a 2 cm segment from the caudal region of the lesion was separated for biochemical analysis. Both tissue samples were stored at -30°C.

4.6. Histopathological Evaluation

Spinal cord segments harvested from the T10 level were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at a thickness

of 6 μm. Transverse sections were stained with hematoxylin and eosin (H&E) and examined under a Leica DFC 280 light microscope equipped with digital image analysis software.

The extent of hemorrhage, necrosis, and edema was evaluated semi-quantitatively based on the proportion of the affected area relative to the total cross-sectional area of the spinal cord. Histopathological damage was graded as follows:

- 0 = no damage
- + = <25% of the total area
- ++ = 25–50%
- +++ = 50–75%
- ++++ = >75%

This semi-quantitative scoring system enabled standardized comparison of histopathological injury severity among the experimental groups.

4.7.Biochemical Analysis

Superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were measured in spinal cord tissue homogenates and serum samples. All specimens were stored at −30°C until biochemical analysis.

4.8.Tissue Preparation for Biochemical Analysis

On the day of analysis, spinal cord tissues stored at −30°C were thawed at room temperature, weighed, and homogenized in ice-cold phosphate buffer at a 10% weight/volume (w/v) ratio. Homogenization was performed on ice at 12,000 rpm for 1–2 minutes to prevent enzymatic degradation.The homogenates were then centrifuged at 5,000 rpm for 30 minutes at 4°C. The supernatants were carefully collected and used for enzymatic activity measurements.

4.9.Measurement of Enzyme Activities

Superoxide dismutase (SOD) activity was determined using the method described by Sun et al. (12), based on inhibition of nitro blue tetrazolium (NBT) reduction.

Glutathione peroxidase (GPx) activity was measured according to the method described by Paglia and Valentine, which is based on monitoring the decrease in absorbance at 340 nm due to the oxidation of NADPH

(nicotinamide adenine dinucleotide phosphate) during the enzymatic reaction(8).

4.10.The Effect of Animal Losses on Statistical Power

Although the initial study design included 44 rats, the final sample size was reduced to 35 due to experimental losses, resulting in partial imbalance among the groups. To evaluate the potential impact of this reduction on statistical validity, a post hoc power analysis was conducted. The analysis indicated statistical power exceeding 90% for the primary biochemical outcome measures, supporting the adequacy of the final sample size.

4.11.Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, Version 22.0 (IBM Corp., Armonk, NY, USA).

Given the relatively small group sizes and the non-normal distribution of the data, non-parametric tests were employed. The Kruskal–Wallis test was used for comparisons among multiple groups, and the Mann–Whitney U test was applied for pairwise comparisons.

Effect sizes were calculated as eta-squared based on the Kruskal–Wallis H statistic (η^2H) and as r for the Mann–Whitney U test. Effect size estimates were presented together with 95% confidence intervals.

A two-tailed p value < 0.05 was considered statistically significant.

Post hoc power analysis was performed using G*Power software (version 3.1; Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany), and statistical power for the primary biochemical outcomes was estimated to exceed 90%(9,10).

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CHAPTER 5

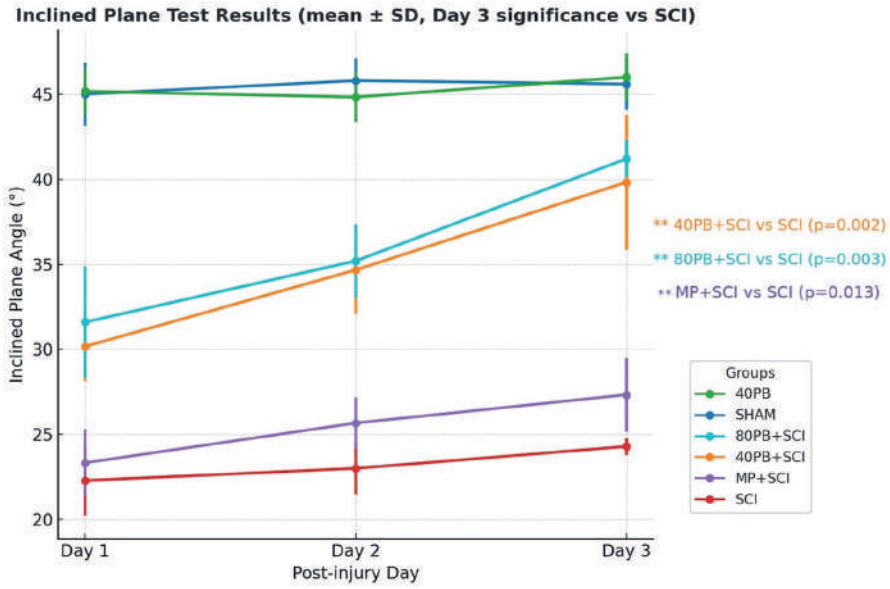
Experimental Outcomes: Functional Recovery and Redox Patterns

Acute spinal cord injury causes significant motor dysfunction in the early stages, while revealing measurable changes in antioxidant enzyme activities at the systemic and tissue levels.

5.1 Early Functional Loss and Recovery Profile

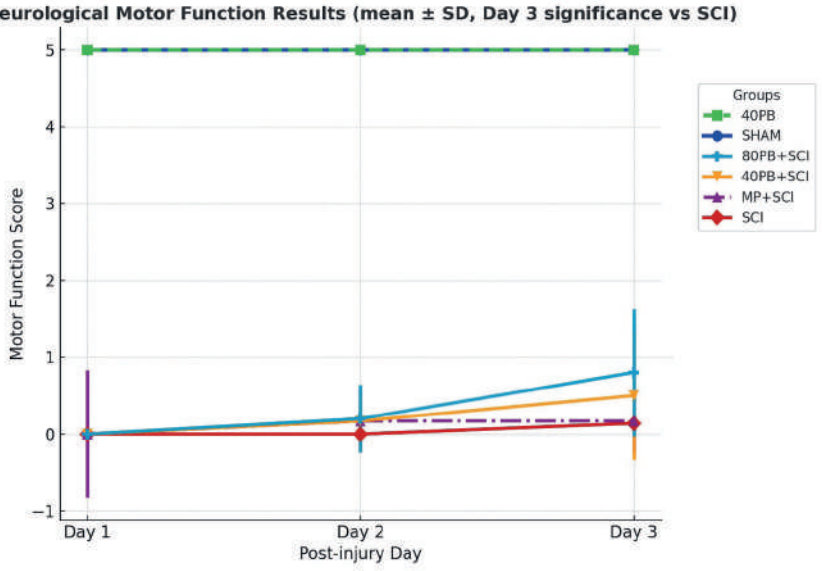
Functional assessments, including inclined plane function and neurological examination, confirmed the successful creation of experimental spinal cord injury. Significant motor loss was observed in all trauma groups on postoperative day 1, severely impacting hind limb control in the acute phase. Over the following 72 hours, a more consistent and pronounced improvement in inclined plane performance was observed in the pregabalin-treated groups. Both pregabalin doses were associated with better functional performance compared to the saline-treated SCI group and the methylprednisolone group. However, a clear dose-response relationship could not be established within the short follow-up period **(Figure 2).**

Figure 2. Inclined plane test outcomes (mean ± SD) and corresponding p-values across experimental groups.



Motor scores showed a similar trend. While motor functions remained within normal limits in the sham and pregabalin-only groups, complete paralysis was observed on the first day in the trauma groups. On the third day, a numerical increase in motor scores was observed in the pregabalin-treated groups, with a more pronounced recovery, especially in the high-dose group. However, this improvement remained limited during the short 72-hour follow-up period and did not reach a level that would create a significant divergence between the trauma subgroups **(Figure 3).** When these findings are evaluated together, it can be said that pregabalin may have a supportive effect on motor performance in the acute phase; however, early functional recovery reflects only the initial stage of the biochemical stabilization process.

Figure 3. Neurological motor function outcomes (mean ± SD) in experimental groups.

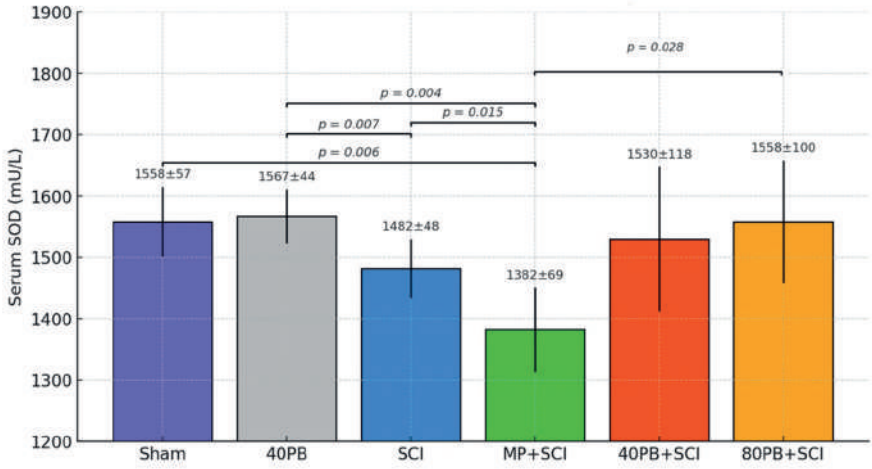


5.2 Systemic Redox Response: Serum-Level Antioxidant Dynamics

5.2.1 Serum SOD

In the experimental spinal cord injury model, serum SOD levels differed significantly between groups (Kruskal–Wallis H=15.40, p=0.009). In the trauma group treated with methylprednisolone (MP+SCI), serum SOD activity was found to be significantly lower than in the sham, SCI alone, and 40 mg/kg PB groups, suggesting that MP suppresses the SOD response at the systemic level (sham vs. p=0.006, SCI vs. p=0.015, 40 mg PB vs. p=0.004). In addition, serum SOD levels were found to be significantly lower in the SCI group compared to the 40 mg/kg PB group, and pregabalin (40 mg/kg) was shown to partially modulate serum SOD activity in a protective/therapeutic manner in the presence of trauma (p=0.007). The fact that serum SOD values were lower in the MP+SCI group compared to the 80 mg/kg PB+SCI group (p=0.028) indicates that high-dose pregabalin alleviates the pressure on SOD compared to MP, but this effect does not show a significant dose-response relationship. Post-hoc power analysis showed that the statistical power for serum SOD data was over 98%, supporting the reliability of the obtained serum findings. **(Figure 4).**

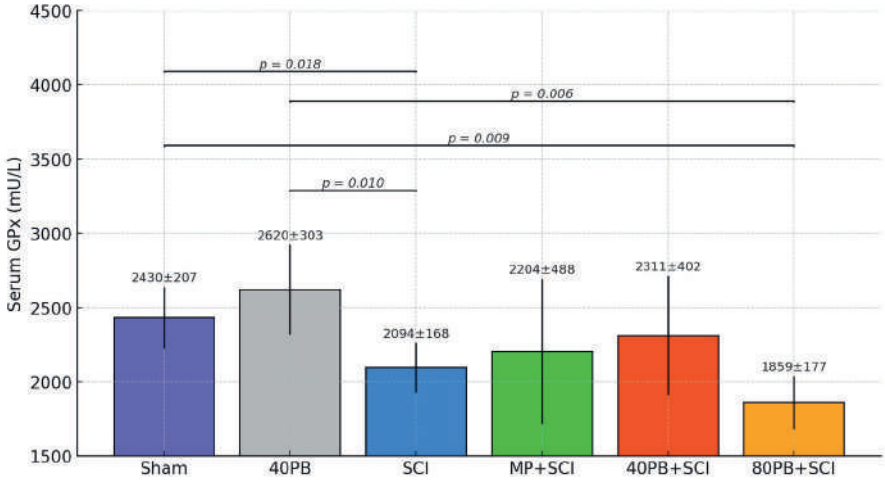
Figure 4. Serum superoxide dismutase (SOD) levels (mean ± SD) with corresponding p-values across experimental groups.



5.2.2 Serum GPx

Serum GPx levels also showed a significant difference between the groups (Kruskal–Wallis H=14.81, p=0.011). In the SCI group that underwent trauma, serum GPx activity was found to be significantly lower compared to the sham and 40 mg/kg PB groups, suggesting that spinal cord contusion suppresses systemic antioxidant capacity (especially the GPx response) in the acute phase (sham vs. p=0.018, 40 mg PB vs. p=0.010). Similarly, in the 80 mg/kg PB+SCI group, serum GPx levels were found to be significantly lower than in the sham and 40 mg/kg PB groups, indicating that high-dose pregabalin could not sufficiently increase serum GPx activity or completely reverse the suppression caused by trauma (sham vs. p=0.009, 40 mg PB vs. p=0.006). In contrast, the higher serum GPx levels in the 40 mg/kg PB group (without trauma) compared to the SCI and 80 mg/kg PB+SCI groups indicate that pregabalin can enhance serum antioxidant defense in non-traumatic conditions, while in the presence of trauma, this effect follows a complex course depending on the tissue and dose. Post-hoc power analysis for serum GPx also revealed statistical power exceeding 97%, thus supporting the biochemical robustness of the serum findings. **(Figure 5).**

Figure 5. Serum glutathione peroxidase (GPx) levels (mean ± SD) with corresponding p-values across experimental groups.

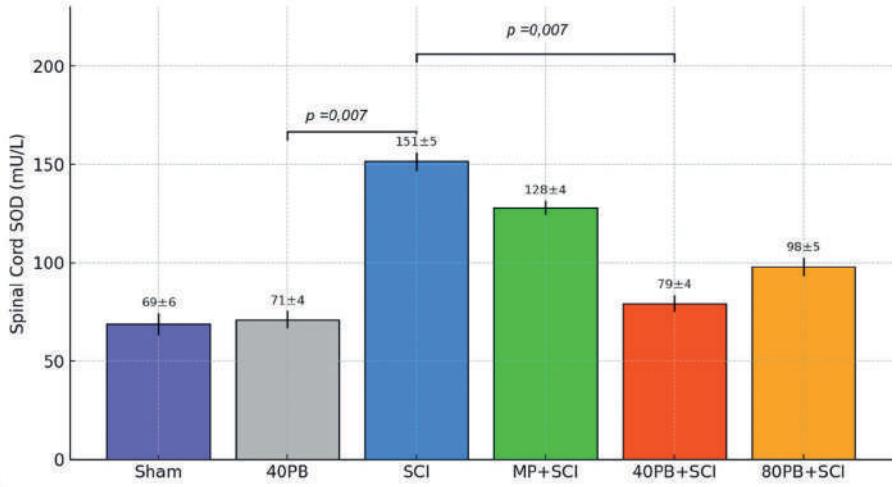


5.3 Antioxidant Response in Spinal Cord Tissue: Compensation or Stabilization?

5.3.1 Spinal Cord SOD

SOD activity in spinal cord tissue showed a significant difference between groups (Kruskal–Wallis H=13.06, p=0.023). In the trauma-treated spinal cord injury group, spinal cord SOD levels were found to be significantly higher compared to both the 40 mg/kg PB group and the 40 mg/kg PB+SCI group, indicating a significant SOD response/induction in the spinal cord after contusion (p=0.007 for both comparisons). The significantly lower SOD activity in the 40 mg/kg PB+SCI group treated with pregabalin compared to the SCI group suggests that pregabalin modulates the oxidative stress response by suppressing SOD activity at the trauma site. However, no additional significant differences were found between other groups (sham, MP+SCI, 80 mg/kg PB+SCI, etc.), suggesting that the effect of pregabalin on SOD is particularly pronounced at the 40 mg/kg dose and in the presence of trauma. The statistical power of spinal cord SOD being over 92% in post-hoc power analysis supports the biochemical reliability of these findings. **(Figure 6).**

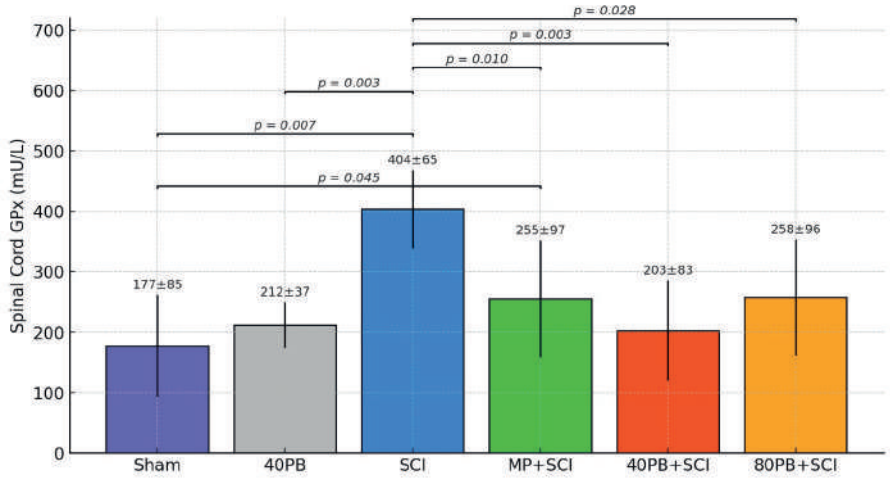
Figure 6. Spinal cord superoxide dismutase (SOD) levels (mean ± SD) with corresponding p-values across experimental groups.



5.3.2 Spinal Cord GPx

Spinal cord GPx levels also showed significant differences between groups (Kruskal–Wallis H=17.75, p=0.003). GPx activity was found to be significantly higher in the SCI group compared to the sham, MP+SCI, 40 mg/kg PB, 40 mg/kg PB+SCI, and 80 mg/kg PB+SCI groups, reflecting a compensatory GPx increase in spinal cord tissue after contusion (sham p=0.007, MP+SCI p=0.010, 40 PB p=0.003, 40 PB+SCI p=0.003, 80 PB+SCI p=0.028). The fact that spinal GPx levels were lower in both pregabalin (40 and 80 mg/kg) and MP-treated trauma groups compared to the SCI group indicates that these agents normalize GPx activity in the local spinal cord tissue, thereby mitigating the trauma-induced oxidative response. The slightly higher GPx activity in the MP+SCI group compared to the sham group (p=0.045) is not entirely consistent with the general trend of oxidative suppression and is interpreted as a temporary compensatory enzyme induction. The post-hoc power analysis for spinal cord GPx exceeding 99% reinforces the statistical robustness of these results. **(Figure 7).**

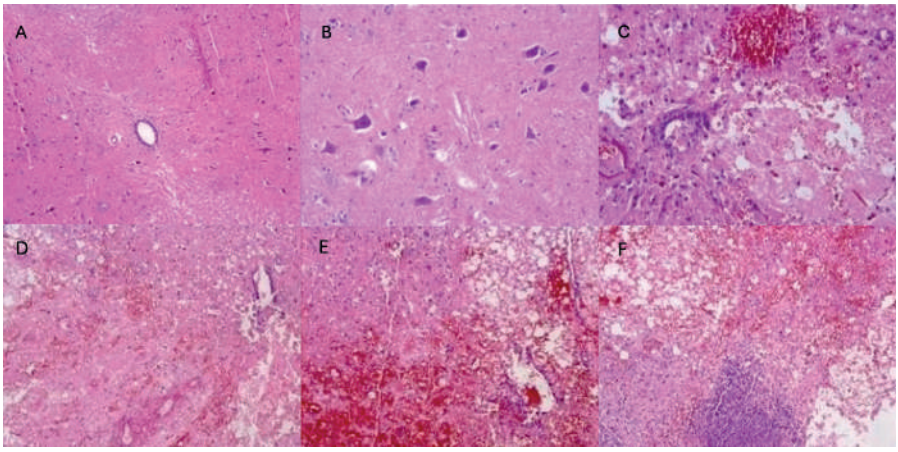
Figure 7. Spinal cord glutathione peroxidase (GPx) levels (mean ± SD) with corresponding p-values across experimental groups.



5.4 Disjunction Between Structural Damage and Biochemical Stabilization

Histopathological examination revealed that spinal cord architecture was preserved in both the sham and pregabalin-only groups, and no hemorrhage areas, necrosis, or significant edema were observed. Moderate to severe parenchymal damage developed in all trauma groups. Hemorrhage areas, neuronal necrosis, and widespread edema were common in the lesion region **(Figure 8).**

Figure 8. Histopathological abnormalities in H&E-stained spinal cord sections from the study groups.



Hematoxylin and eosin staining analyses of all groups (a) Sham: Normal histoarchitecture with intact gray and white matter. (b) 40PB: Preserved neuronal morphology in the substantia grisea. (c) SCI: Extensive hemorrhage and necrosis with disruption of central canal integrity. (d) MP+ SCI: Diffuse hemorrhagic and necrotic areas accompanied by structural degeneration. (e) 40PB+ SCI: Widespread hemorrhage, necrosis, and edema with central canal disruption. (f) 80+SCI: Severe hemorrhage and necrosis with prominent polymorphonuclear cell infiltration

However, it is noteworthy that no significant structural differences were detected between the treatment groups in the early stages. This may be due to the redox modulation observed at the biochemical level, which has not yet translated into significant histological differentiation within the 72-hour period. This situation may indicate two important biological interpretations:

1. Biochemical stabilization may have occurred before morphological recovery.

2. Enzymatic response may not have had sufficient time for structural stabilization in the early stages.

In the acute phase of spinal cord injury, cellular viability is rapidly determined; however, it may take longer for histologically measurable differences to emerge. Therefore, it is conceivable that early modulation in the redox axis could pave the way for more significant structural and functional gains in the following days.Acute spinal cord trauma elicits a significant oxidative stress response at both systemic and tissue levels. The increase in SOD and GPx observed in the trauma group reflects a compensatory defense reflex against increased reactive oxygen species. However, the more balanced response of this enzyme in the pregabalin-treated groups suggests that the oxidative load may have been limited upstream.

The parallelism between the early improvement trend observed in functional tests and biochemical stabilization suggests that redox modulation may create a therapeutic window in the secondary damage process. The fact that histopathological differences are not yet clearly defined suggests that this biochemical effect is a time-dependent process and requires longer monitoring for morphological transformation.This holistic picture reveals that neuroprotection is not merely about reducing structural damage, but a multi-layered process that progresses through balancing the biochemical cascade in the early stages.

CHAPTER 6

Clinical and translational perspective

It is unclear how many of the experimental spinal cord injury models we have been able to translate into viable strategies for patients. Recent studies have shown that oxidative stress, calcium imbalance, and mitochondrial deterioration are central, and therefore antioxidant and mitochondria-focused approaches have become priority targets for translational research (1–5) .

There are significant differences between animal models, cell cultures, and human spinal cord injury. The geometry of the injury, concomitant systemic traumas, the severity of the immune response, the pharmacokinetics of the drugs, and the timing of initiation of immediate and long-term treatment all differ considerably. Experimental studies utilize a controlled lesion model, a homogeneous animal population, and treatments applied within an "ideal" time window. In current clinical practice, most patients present with delayed onset, often accompanied by hypotension, hypoxia, and multiple traumas. Therefore, it is often not possible to directly translate the effect size observed in experimental settings for treatments targeting the same molecular axes into clinical outcomes (1,3,6) .

In preclinical spinal cord injury models, preservation or enhancement of antioxidant enzyme activities, reduction of lipid peroxidation, and improvement of motor scores point to the neuroprotective potential of therapies targeting oxidative stress. Even two patients with the same level of injury can show completely different biochemical responses due to age, comorbidities, systemic inflammation, hemodynamic fluctuations, and time to access treatment, making it difficult to achieve standard treatment (2,3,6,7).

In mild to moderate cases with partial damage, early suppression of oxidative stress contributes more significantly to limiting neuronal and glial cell loss; however, in advanced, complete lesions, it is not realistic to expect significant clinical gains from antioxidant approaches alone. From this perspective, antioxidant treatments should not be applied as a "one-size-fits-all protocol," but rather tailored to the phase of injury and biomarkers. Strategies should be planned as phase-specific, biomarker-guided interventions(1,3,8). In patients who are hemodynamically stabilized and protected from hypoxia in the early stages, therapies targeting redox balance can significantly support functional recovery, especially in incomplete lesions . In severe complete lesions, antioxidant agents should have a more realistic role as a "ground-setting" for regenerative and neurorestorative therapies (1,3) .

The prominence of the Nrf2 pathway in spinal cord injury in recent years has provided a new biological framework for this axis. Nrf2 acts as a transcriptional regulator of numerous antioxidant enzymes and phase II detoxification genes and is considered an important target at both experimental and clinical levels. Animal studies have shown that Nrf2 activation reduces lipid peroxidation, limits demyelination , and promotes functional recovery. Astroglial Nrf2 overexpression can significantly protect spinal cord tissue against oxidative damage (7–9) .

Mitochondria-specific antioxidants are also popular on the translational agenda. Mitochondria-targeted agents such as MitoQ have been shown to increase ATP production, regulate mitochondrial dynamics, reduce reactive oxygen species, and consequently promote angiogenesis, neuronal protection, and functional recovery in both cell culture models and spinal cord injury animal studies. These findings lead us to redefine mitochondria not only as energy-producing organelles but also as a central platform for therapeutic intervention (2, 10) .

Biomarker panels be developed that can be used to divide patients into phenotypic subgroups in acute and subacute phases based on the SOD– GPx axis and Nrf2 activation status? (3,7,9)

If mitochondria-targeted antioxidants are standardized and applied early, can permanent functional gains be achieved, particularly in incomplete lesions, by creating synergy with rehabilitation (2,12)?

Calcium imbalance, excitotoxicity , and oxidative stress; increased intracellular calcium accelerates oxidant production by increasing electron runoff in mitochondria, triggering energy deficiency and cell death in both neuronal and glial cells. Agents that modulate voltage-gated calcium channels,

such as pregabalin , are seen as attractive tools for breaking this cycle. In experimental spinal cord injury models, pregabalin has been shown to reduce oxidative stress markers, modulate antioxidant enzyme response, and promote histological-functional recovery (2) . Pregabalin has been studied more in the clinical field in terms of its effects on neuropathic pain and quality of life , current literature suggests that this drug may be a component of the neuroprotective process in appropriate patient subtypes, rather than just a symptomatic agent. At this point, several concrete hypotheses regarding the neuroprotective use of pregabalin can be raised (1,3)

Pregabalin treatment initiated concurrently with hemodynamic stabilization in the acute phase can delay or reduce the depletion of the SOD– reduce depletion of the SOD–GPx antioxidant axis (2,4).

pregabalin with mitochondria-targeted antioxidants and Nrf2 activators can provide stronger and more sustained functional gains compared to antioxidant therapies given alone (2,3) .

Testing these hypotheses requires biomarker-focused, multicenter clinical trials with long-term follow-up.New generation reviews and original studies indicate that approaches focusing on a single antioxidant molecule in spinal cord injury are insufficient to achieve the desired effect size at the clinical level; therefore, the focus is increasingly shifting to combined strategies. Smart combinations of natural antioxidants, synthetic molecules, mitochondria-targeted agents, Nrf2 modulators, cell-based therapies, and biomaterial-supported delivery systems are being emphasized.

In this context, the fundamental scientific value for the future of antioxidant therapies in spinal cord injury is shifting from being complementary interventions targeting a single objective to becoming central to biomarker-based, personalized, and combined neuroprotective protocols. Oxidative stress, mitochondrial dysfunction, and calcium dishomeostasis have demonstrated strong neuroprotective potential at the experimental level and are being tested with increasingly sophisticated translational models. However, the full realization of this potential in clinical practice depends on the implementation of phase-specific, biomarker- based, multicenter, and long-term follow-up clinical trials. In this context, the SOD– GPx axis, the Nrf2 pathway, mitochondria-specific antioxidants, and calcium modulation require further research (1,3,9).

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CHAPTER 7

Combination Therapy Strategies

The secondary phase following spinal cord injury (SCI) progresses as a dynamic and complex cascade of interdependent processes such as excitotoxicity, calcium dyshomeostasis, mitochondrial dysfunction, oxidative stress, neuroinflammation, and programmed cell death. This multi-layered cascade renders treatments focused on a single biomolecular target ineffective, and a more rational approach involving combination strategies that modulate multiple pathophysiological states should be developed. Current translational studies emphasize that neuroprotective and neuroregenerative agents, hemodynamic optimization, early surgical decompression, and rehabilitation protocols should be used in the treatment of SCI (1–5).

Pregabalin has proven efficacy in neuropathic pain due to spinal cord injury and has FDA approval for this indication. Experimental models show that pregabalin has the potential to reduce excitotoxicity, calcium-mediated mitochondrial stress load, and reactive oxygen species formation; therefore, it is a candidate drug that affects both ionic balance and redox homeostasis in the secondary damage cascade. Clinical and preclinical studies have shown that synergistic or additive effects may occur when pregabalin is combined with different analgesics and neuromodulators; thus, similar clinical benefits may be achieved with may be achieved with lower doses (1,3,6–9).

The oxidative stress cascade plays a central role in the pathophysiology of SCI. Increased reactive oxygen species can accelerate progressive tissue destruction via lipid peroxidation, protein oxidation, and DNA damage. Therefore, pharmacological enhancement of the antioxidant system has become a key component in combination strategies. The application of agents that increase the activity of endogenous enzymes such as SOD-GPx, or directly scavenge reactive oxygen species, in combination with drugs that

modulate calcium currents, can contribute to the preservation of mitochondrial membrane potential and the suppression of cytochrome c release, thereby reducing apoptosis. New approaches offer promising data suggesting that they can create a neuroprotective microenvironment by reducing oxidative stress and increasing antioxidant enzyme activities, making redox modulation even more important for future combination models (1,3,10–12).

The significant impact of neuroinflammation on secondary damage makes anti-inflammatory strategies an integral part of combination therapies. High-dose methylprednisolone protocols have controversial aspects, but randomized studies have shown that when applied in the acute phase, they can provide significant, albeit limited, gains in motor and sensory recovery. Thus, pharmacological suppression of the inflammatory response has been shown to be one of the most evidence-based neuroprotective approaches. Current literature emphasizes that multiple protocols in which corticosteroids, cyclooxygenase inhibitors, cytokine-targeted agents, and microglial activation modulators are evaluated in combination with calcium channel modulators and antioxidant therapies reflect the most realistic translational scenario (1–3,5) .

In combination therapies, timing, dosage, and administration window are parameters as critical as the chosen agents. The secondary damage cascade exhibits different molecular dominances in the acute, subacute, and chronic phases. Interventions targeting excitotoxicity and calcium dyshomeostasis may be more effective in the early phase, while approaches targeting inflammation and glial scar formation may be more effective in the subacute-chronic phase. It has also been shown that oxidative stress modulation is not limited to the first few hours but is associated with mitochondrial biogenesis, axonal regeneration, and long-term functional outcomes; therefore, treatment duration and follow-up periods should be redefined based on biomarkers (1,3–5,10,13,14).

Current consensus suggests that the future of neuroprotection in spinal cord injury lies not in single-agent approaches, but in holistic combination models that target multiple pathophysiological axes simultaneously and incorporate pharmacological and non-pharmacological components. When calcium channel modulation (e.g., pregabalin), antioxidant support (agents that enhance the SOD/GPx axis or directly reduce ROS), and regulation of the inflammatory response are addressed in an integrated manner, a more robust and rational therapeutic paradigm emerges that simultaneously targets redox imbalance, mitochondrial dysfunction, and neuroinflammation. However, for this conceptual framework to be confidently translated into clinical practice, biomarker-focused, multicenter randomized clinical trials comparing different agent combinations, dosing schemes, and timing windows are still needed (1–5,9) .

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CHAPTER 8

Limitations of the Study and Future Research Areas

Experimental spinal cord injury (SCI) models do not accurately represent the biological and clinical spectrum of human spinal cord trauma. While trauma severity and lesion level are standardized in laboratory settings, real-life injuries are multi-level, heterogeneous, and often accompanied by systemic instability. In clinical practice, spinal cord injury follows a dynamic pathophysiological process with accompanying vascular disruption, hemodynamic fluctuations, and comorbid factors, whereas experimental models are inevitably applied in a reduced and controlled manner. Therefore, experimental findings are always approached cautiously in current clinical practice from a methodological perspective regarding their clinical implications. Parameters such as species differences, type of injury (contusion, compression, transection , etc.), dose-response relationships, and follow-up period are critical variables determining translational validity. The clinical significance of the findings is only possible if these limitations are clearly defined and interpreted within a translational context (1–5).

The assessment of oxidative stress is mostly carried out through the activities of specific enzymes such as SOD and GPx; however, redox biology is a dynamic and multidimensional process. Secondary damage cannot be fully understood when the glutathione pool, catalase activity, lipid peroxidation products, indicators of oxidative DNA damage, and mitochondrial function parameters are not considered together. Focusing experiments primarily on the acute phase overlooks redox adaptations, mitochondrial remodeling, and late-stage neurodegenerative changes that occur in the subacute and chronic phases(6–9).

Another significant limitation is that the timing and dosage parameters of pharmacological interventions are optimized under experimental conditions. In a clinical setting, there are difficulties in always initiating treatment in the first hours after trauma, as well as serious systemic hypotension, concomitant organ damage, polypharmacy, and pharmacokinetic differences. Interspecies metabolic and immunological differences also affect dosage. These are additional factors that make it difficult to determine safety limits and predict long-term effects (1–4).

Future studies should investigate the relationship between antioxidant enzyme response and functional neurological recovery with longer follow-up periods and broader biomarker panels. In particular, multiparameter analyses that evaluate indicators of mitochondrial biogenesis, inflammatory cytokine profile, apoptotic pathway markers, and oxidative stress biomarkers together can contribute to a more holistic mapping of the phase-dependent course of secondary damage. In addition, protocols using calcium modulation, anti-inflammatory therapies, antioxidant strategies, cell-based approaches, and biomaterial-supported delivery systems in combination are considered promising research directions for improving translational potential (1,7,8,10,11).

Current findings suggest that maintaining redox balance and preserving mitochondrial integrity in spinal cord injury may hold a significant place in neuroprotective strategies. Upholding cellular energy metabolism is not merely a biochemical detail; it is a central objective in the process. Mitochondrial protection is increasingly advocated for in limiting the spread of damage.

Translating effects that appear significant at the experimental level into clinical practice is often more complex and challenging than anticipated. The difficulty of making strong generalizations without data supported by broader biomarker panels, analyses conducted across different timeframes, and well-designed, human-centered clinical trials, as well as accounting for clinical heterogeneity, must be carefully considered.

Scientific progress is not linear. Observations in the laboratory are applied in the clinic, and clinical uncertainties are fed back into experimental research. Knowledge deepens within this multi-stage cycle and gains meaning for the future (3,4,6,8,9,12).

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CHAPTER 9

Neuroprotection paradigm

The aim of neuroprotection is to preserve salvageable healthy tissue in place of irreversibly damaged tissue. Suppressed, structurally viable cells surrounding the lesion can be protected through multifaceted interventions. Therefore, the modern approach does not rely on a single molecule or a single pathway. Restoring redox balance requires addressing mitochondrial stability and controlling the neuroinflammatory response together. Ultimately, neuroprotection does not aim to completely eliminate damage. However, by limiting secondary biochemical amplification, it can reduce the depth of functional loss. From a clinical translation perspective, the main question is: To what extent does this balance achieved at the molecular level translate into neurological recovery? The answer to this question will determine the direction of future translational research(1–5).

Neuroprotection primarily involves suppressing the inflammatory response or reducing edema . It also encompasses a range of aspects such as maintaining cellular energy metabolism, preserving mitochondrial function, restoring ionic homeostasis, and re-establishing redox modulation. In the secondary damage process, disruption of calcium homeostasis, increased mitochondrial ROS production, and excitotoxicity are key components triggering necrotic and apoptotic cell death programs. Therefore, modulating calcium influx limits glutamate-mediated excitotoxicity. Supporting the endogenous antioxidant enzyme system is a key strategic goal that complements calcium modulation in the current neuroprotective paradigm. Breaking the positive feedback loop between increased intracellular Ca^{2+} and mitochondrial ROS production can slow the progression of secondary injury processes (1,6–9).

Assessing the levels and activities of endogenous antioxidant enzymes such as SOD and GPx is important, as they can serve as key biochemical indicators that highlight the central position of the redox axis within this pathophysiological network. In experimental spinal cord injury models, decreased enzyme activity is associated with increased lipid peroxidation and MDA levels, as well as increased oxidative stress and tissue damage. Protecting or strengthening the antioxidant defense system with drugs not only scavenges free radicals but also preserves mitochondrial integrity and delays apoptotic signals. This supports myelin structure and maintains axonal transmission, increasing the likelihood of functional recovery. Therefore, data obtained from enzymes such as SOD and GPx are critically important for evaluating the potential of protective effects at the biochemical level to translate into neurological and behavioral outcomes (1,2,6–8).

Current studies reveal a shift in neuroprotective approaches from monotherapies focusing on a single molecular target to combination strategies that simultaneously modulate multiple pathophysiological axes. Treatment models that limit oxidative stress, re-regulate the inflammatory response, suppress excitotoxicity , and support regenerative processes (axonal growth, remyelination stem cell-based repair, etc.) offer a multi-layered neuroprotection paradigm better suited to the complex nature of spinal cord injury. In this context, experimental studies should be considered not merely as demonstrating the effects of a specific pharmacological agent, but as a conceptual framework providing biochemical evidence for a broader concept where redox modulation, mitochondrial protection, and ionic homeostasis are targeted together (3,4,6,9,10) .

To effectively prevent secondary damage in spinal cord injuries, we must maintain ion balance. Preserving mitochondrial function requires controlling inflammatory responses and activating antioxidant defense systems. At the heart of this defense mechanism lies redox modulation, which forms the biochemical basis of all neuroprotection aimed at protecting nerve cells. While experimental data increasingly validate this approach, further studies are needed to confidently translate this knowledge into real clinical practice. For the process to become a standard treatment, this holistic strategy needs to be rigorously tested through phase II–III, multicenter, and biomarker-based clinical trials (3,4,9,11) .

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Pregabalin in Experimental Spinal Cord Injury: Oxidative Stress, Antioxidant Defence and Neuroprotective Mechanisms

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