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**EFFECTS OF MITOTEMPO, A
MITOCHONDRIA TARGETING AGENT, IN
RAT MODEL OF SPINAL CORD INJURY-
INDUCED BLADDER DYSFUNCTION**

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DOCTORAL THESIS

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TRABZON-2025

DECLARATION

I declare that this thesis was carried out and written in accordance with the standards of Karadeniz Technical University Graduate School of Health Sciences Thesis Preparation and Writing Guide, that the thesis is an original scientific research carried out by adhering to academic and ethical rules, that I have cited all the information and comments in the thesis that were not obtained by this thesis study and that the used citations are included in the list of references, and that I have not acted in violation of patents and copyrights during the study and writing of the thesis.

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25/09/2025
Zain KALHA

Dedication

I dedicate my doctoral thesis to my precious family and my backbone, Mom, Dad, Bashar. You have made all sacrifices with your unconditional love to help me reach this day and have always been there for me even in the hardest times. To my husband, my partner, and my supporter, Dr. Anes HAYDER, who always walks an extra mile to help me make it throughout the tough journey. To my second family, my mentors, professors, and colleagues who have always believed in me, given me support and encouragement all the way. With you by my side, it has always felt like home. Finally, my Jordan, and my friends there, this work is the fruit of your support and lovely words.

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LIST of ABBREVIATIONS, SYMBOLS and FORMULAS

APS	Ammonium persulfate
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CA	Cellulose acetate
CNS	Central nervous system
CPA	Cyclopiazonic acid
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DSD	Detrusor sphincter dyssynergia
Drp	Dynamin-related protein
EFS	Electrical field stimulation
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ETC	Electron transport chain
EUS	External urethral sphincter
GPx	Glutathione peroxidase
GTP	Guanosine triphosphate
H&E	Hematoxylin & Eosin
HRP	Horseradish peroxidase
Hz	Hertz
IHC	Immunohistochemistry
IL-1β	Interleukin-1 beta
İLA FAR	İlaç ve Farmasötik Teknoloji Uygulama ve Araştırma Merkezi
IMM	Inner mitochondrial membrane

i.p.	Intraperitoneal
IUS	Internal urethral sphincter
IVC	Individually ventilated cage
kDA	Kilodalton
KTU	Karadeniz Technical University
L	Liter
LUT	Lower urinary tract
M	Molarity
Mfn	Mitofusin protein
mL	Milliliter
µL	Microliter
mM	Milimolar
µM	Micromolar
M	Molar
MW	Molecular weight
MT	MitoTEMPO
N	Normality
Na₂EDTA	Ethylenediaminetetraacetic acid disodium salt
NADH	Nicotinamide adenine dinucleotide hydride
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NaPPi	Sodium pyrophosphate
NaVan	Sodium orthovanadate
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NP	No primary antibody
NS	Normal saline
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation

PAG	Periaqueductal gray matter
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
Phe	Phenylephrine
PLC	Phospholipase C
PMC	Pontine micturition center
PMSF	Phenyl methyl sulphonyl fluoride
PNS	Peripheral nervous system
PVDF	Polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SCI	Spinal cord injury
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
s.c	Subcutaneous
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween 20
TEMED	Tetramethylethylenediamine
TNF-α	Tumor necrosis factor alpha
TPP	Triphenylphosphonium

Symbols

α	Alpha
&	And
β	Beta
$^{\circ}\text{C}$	Celsius degree
δ	Delta
%	Percentage

Formulas

Ca^{2+}	Calcium
CaCO_3	Calcium carbonate
CaCl_2	Calcium chloride
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	Calcium chloride dihydrate
CO_2	Carbon dioxide
dH_2O	Distilled water
ddH_2O	Double distilled water
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
K^+	Potassium
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogen phosphate
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	Magnesium sulfate heptahydrate
Na^+	Sodium
$\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$	Sodium citrate
NaCl	Sodium chloride
NaF	Sodium fluoride
NaHCO_3	Sodium bicarbonate
NaOH	Sodium hydroxide
O_2	Oxygen molecule

ABSTRACT

Effects of Mitotempo, a Mitochondria Targeting Agent, in a Rat Model of Spinal Cord Injury-Induced Bladder Dysfunction

Spinal cord injury (SCI) is an important health problem affecting millions of people worldwide by reducing duration and quality of life of patients and causing economic and work force losses. Besides locomotor dysfunction, SCI induces somatic and autonomic dysfunctions that negatively affect the patient's quality of life. Urinary bladder dysfunction is a SCI-induced problem that leads to bladder overactivity or areflexia depending on lesion location and type of affected neurons. Currently, understanding SCI-induced pathophysiology and effective treatments is still limited. In recent studies, SCI-induced dysfunctions have been associated with mitochondrial damage and oxidative stress-induced cellular destruction in neurons and other cell types (such as smooth muscle, epithelial cells, vascular cells). Therefore, therapeutics targeting mitochondrial mechanisms have attracted great interest, yet the effects of these mechanisms on bladder dysfunction are unknown. "Mitocuticals" are mitochondria-targeting agents that prevent mitochondrial damage and thus prevent/reduce the effect of SCI on organs. MitoTEMPO, is a neuroprotective antioxidant of this group that has shown promising results on mitochondrial function after SCI and oxidative stress injury. In this thesis study, the therapeutic effect of mitoTEMPO was investigated in a rat SCI-induced bladder dysfunction model. SCI-induced bladder dysfunction in rats was induced by compression of the thoracic vertebra at T9 level and 1mg/kg/day mitoTEMPO was administered after the injury. After 3 and 14 days of mitoTEMPO administration, metabolic (body weight, water intake, urine output, fecal output), functional (detrusor muscle contraction and relaxation responses), molecular (Drp1 and Mfn protein expressions) and histopathological (H&E staining; TOM20, Drp1, Mfn2 protein immunohistochemistry) evaluations were performed. In conclusion, functional and cellular improvement was observed in bladder dysfunction developing after SCI with 14-day mitoTEMPO treatment, and partially in 3-day treatment. In this study, mitoTEMPO and similar mitocutical agents were shown to have therapeutic potential in neurogenic bladder dysfunction.

Keywords: Antioxidant, Bladder, Drp1, Mitochondria, Mfn2, Organ Bath, Rat, TOM

1. INTRODUCTION AND AIM

Spinal cord injury (SCI) is a traumatic injury occurring in spinal neurons that provide signal exchange between central and peripheral nervous system (1). It is estimated that 2.5 million people worldwide are exposed to health problems caused by SCI, which often occurs as a result of traffic, sports and work accidents. Depending on the anatomical region and the severity of the injury, SCI can cause long-term complications in many systems, leading to high morbidity and low quality of life. The main source of urinary, gastrointestinal, cardiovascular and respiratory system complications, loss of motor system function, and neuropathic pain, is the degeneration that occurs in efferent and/or afferent neurons of the spinal cord. As a result, it causes functional losses that develop with metabolic dysfunction and histopathological changes in target tissues (2, 3).

Bladder dysfunction is an important understudied morbidity encountered after SCI and for which clinical treatment approaches are limited. The prevalence of bladder dysfunction in individuals with SCI is approximately 60% (4). The clinical picture may manifest itself as underactive bladder, bladder overactivity or incontinence depending on the severity of the trauma, its location on the spine and the type of neurons affected (5). Urinary retention, which develops with decreased detrusor contractions after damage to the sacral levels that provide parasympathetic innervation of the bladder (due to loss of innervation), is defined as the incomplete emptying of the bladder or not emptying at all, and this situation can cause chronic urinary tract infections and renal failure that can be life-threatening in the long term (5, 6). On the other hand, overactive bladder, which develops as a result of damage to the suprasacral region of the spinal cord, is defined as a sudden feeling of urgency with or without urge-type urinary incontinence, usually with increased micturition frequency and nocturia. In some cases, urethral sphincter contractions accompanying increased bladder contractions can cause detrusor sphincter dyssynergia (DSD), which can prevent micturition and trigger hydronephrosis, urosepsis, and urolithiasis (7).

Different pharmacological options can be applied in the clinic depending on the type of bladder dysfunction. Due to their side effects (blurred vision, gastrointestinal system disorders, and cognitive disorders) and the limited efficacy in some patients, the use of these agents can affect the patient's quality of life; it can cause the effective therapeutic dose not to be reached, patient compliance to decrease, and even drug discontinuation (8). In addition, the limited effectiveness and expensiveness of existing treatments reduce the patients' work power and quality of life and duration and create a significant socioeconomic burden (4, 9). Clinical observations indicate that bladder dysfunction due to SCI has been investigated much less than other SCI-related problems and more and reliable effective therapeutic are needed for its restoration (10, 11). It is known that mitochondrial damage and oxidative stress-related deterioration in bladder tissue play an important role in the pathogenesis of SCI-induced bladder dysfunction due to impaired bladder innervation following neuronal damage. In light of this information, pharmacological targeting of mitochondrial oxidative damage in the bladder tissue appear to be an important treatment strategy in order to slow down or stop pathological changes present and/or reverse SCI-induced bladder dysfunctions (12).

The aim of our study is to investigate the time-dependent effects of mitoTEMPO in bladder dysfunction induced by the SCI model at functional, molecular, biochemical, histopathological, and immunohistochemical levels in order to test the hypothesis that “MitoTEMPO, a cytoprotective mitochondria-targeting agent with an inhibitory effect on mitochondrial oxidant mechanisms, prevents bladder dysfunction that develops due to increased mitochondrial oxidative stress and neurodegeneration and cellular/structural changes in the bladder after SCI”. Using a rat model of SCI-induced bladder dysfunction, the specific aims of the study are to investigate the effect of time-dependent mitoTEMPO treatment on:

- a) Change in bladder contractility
- b) Change in mitochondria-related molecules (mitofusin2, Drp1) in bladder
- c) Change in bladder histopathology

The findings obtained from this study will be a new contribution to the literature that will aid to comprehensively understand the SCI-induced bladder dysfunction pathophysiology - which is currently under intensive research due to the need of new treatment approaches - and to provide preliminary data needed for further evaluation of mitoTEMPO in clinical studies.



2. GENERAL INFORMATION

2.1. Spinal Cord Anatomy and Physiology

The spinal cord mainly works as a connecting canal between the brain and the rest of the body. The spinal cord starts at the base of the brain (medulla oblongata), exits the skull from the foramen magnum and tissues and bone structure that protect it from external damage, then terminates in the first and second lumbar vertebra (13). The spinal cord is surrounded by both connective tissue of three membranes known as meninges and for more protection, the spaces between these membranes are filled with cerebrospinal fluid along with adipose and loose fibrous tissues. The spinal cord's protective bone structure is called the spinal or vertebral column. It is the central part of the axial skeleton that is segmented into vertebrae held together by intervertebral discs which are fibrocartilaginous joints. The unique structure of the spinal column provides protection through the vertebral part and via these discs by acting as shock absorbing ligaments. All together provide the needed level of protection to the spinal cord structure and function (14). According to their location, the 33 vertebrae surrounding the spinal cord are divided into different segments; cervical (C1-C7), thoracic (T1-T12), lumbar (L1-L5), sacral (S1-S5), and coccygeal vertebra which varies in number (3 to 5) and fused together as one coccyx (Co1) (Figure 1a) (15).

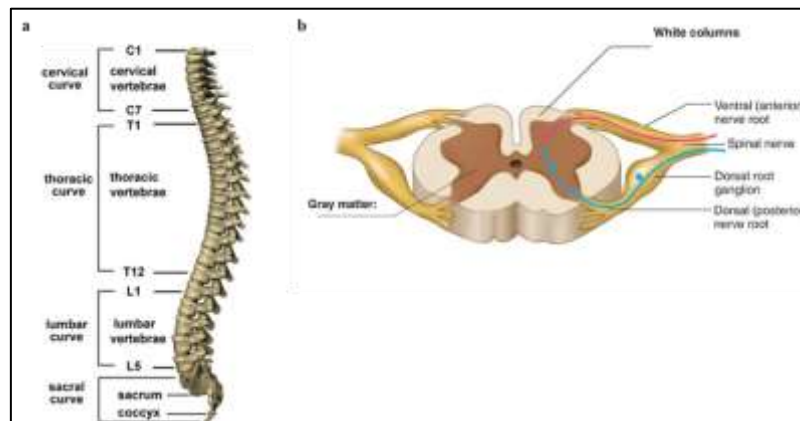


Figure 1. Vertebral column and spinal cord anatomy. **(a)** Lateral view of vertebral column. **(b)** Cross section of spinal cord with nerve roots. (Modified from Moini, 16)

The spinal cord consists of white matter surrounding the central grey matter, an arrangement opposite to that of the brain (Figure 1.B) (16). The white matter is composed of interconnecting ascending and descending tracts which are myelinated sensory and motor axons. Ascending sensory axons transmit input from sensory receptors such as touch, pressure, and proprioception to the upper parts of the brain such as cerebral cortex or hypothalamus. On the contrary, descending motor tracts convey information as output from the CNS to target organs which result in somatic and autonomic actions in the body (17). The neuronal axons are arranged in bundles called nerve roots that enter the spinal cord as dorsal and ventral root that carry sensory input and motor output, respectively. Outside the spinal cord, different dorsal and ventral roots unite together to form spinal nerves from which peripheral nerves emerge to the periphery. The fine arrangement of the spinal cord and its emerging neurons allows every set of spinal nerves to innervate certain area in the body for specific effect. For example, cervical nerves innervate the upper body such as the head, neck, and diaphragm, whereas sacral nerves control sensation and movement of the lower body such as legs and feet. Consequently, the clinical outcome resulting from any injury affecting the spinal cord depends on the type of the injured spinal nerve and the peripheral area innervated by it (18).

2.2. Spinal Cord Injury (SCI)

2.2.1. Definitions and Background

The spinal cord is a tight bundle of nerves and surrounding cells in the shape of a tube within the central nervous system (CNS). The spinal cord's function is to provide the process of sending and receiving signals between the brain and other organs of the body. These signals consist of sensory and motor information that perform in a complex manner to maintain the autonomic and motor functions and reflexes in an optimized manner(2). SCI is a damage or lesion of nerves and cells in the spinal cord which results in partial or complete sensory and/or motor deficit below the level of injury (19).

The resulting dysfunction could be temporary or permanent depending on the severity and location of the lesion. SCI is usually described as traumatic or non-traumatic injury. Non-traumatic SCI results from an internal cause such as tumors, infections, vascular diseases, and others. Traumatic injury originates from an external factor like sports, occupational accidents and falls, or traffic accidents that represent the main cause of SCI with prevalence of 38% of all SCI causes (13, 20).

2.2.2. Epidemiology and Economical Burden of SCI

World Health Organization estimates that over 15 million people suffer from SCI worldwide in 2021 and the number will rise as the incidence and prevalence are increasing each year (21). By gender, the incidence of SCI was shown to be 13.03 and 41.91 case per million in females and males, respectively (22). The threefold increase in SCI incidence in males was attributed to various factors. Among the different causes of occupation-related injuries, SCI has been shown to account for 45% of these causes. Since the majority of workers in professions with high occupational hazards are male, workplace-associated injuries are considered an important factor contributing to the higher incidence of SCI in men (23).

In addition to being recognized as a major cause of disability and poor quality of life for patients, SCI is also identified as a significant economic burden worldwide. Lately, the one-year mean cost of direct medical care for a single patient has ranged between \$33,120 and \$992,144 across different countries and regions, while the lifetime cost is estimated to be between \$700,000 and \$1.1 million and could reach \$2 million in cervical level injuries. The direct costs consist of hospital stay, surgical procedures, medications and other health related services (24). On the other hand, indirect costs include lost wages from missed work days due to disability, early retirement or death, or any payments other than health care expenses such as special transport and adaptation costs. Indirect costs were averaged to \$77,000 for a single individual (25).

2.2.3. SCI Pathophysiology

Injury of the spinal cord occurs in two steps; primary and secondary phases (Figure 2). Primary phase is the initial mechanical injury of the spinal cord whereas secondary phase is a series of cellular and biological events taking place in the body in response to the initial injury. Initial injury causes instant damage of the spinal cord and surrounding tissue and could be in the form of contusion, compression, transection and many others, with compression (persistent contusion) being the most common (26). Hemorrhage, edema and ischemia are considered the hallmarks of the primary phase of SCI that start within seconds up to the first 24 hours after injury. These changes take place because of the tissue damage, disruption of nearby vessels, and hemorrhages that are all followed by clotting and edema. Eventually this ends up in ischemia due to the limited space around the spine and increased local pressure. Despite being localized, the initial trauma may increase blood-spinal cord permeability which allows the entry of vasogenic and cytotoxic substances that exacerbate the damage. This will shift the primary phase into the secondary phase affecting both injured and surviving adjacent cells (27). In the secondary phase, different body systems, such as immune, nervous, and vascular systems, get involved in the pathological cascade following injury.

According to the time after primary injury and the degree of biological changes, secondary phase can be subdivided into acute, subacute and chronic phases. Immediately after the injury, initial high sympathetic activity takes place followed by disruption of the descending sympathetic tracts and uninterrupted parasympathetic activity with spinal and neurogenic shock. Depending on the site of injury, spinal shock can be seen as absence of somatic activity or reflexes and flaccid paralysis while neurogenic shock is manifested with hypothermia, hypotension, and bradycardia (28). Most of the biological changes seen in acute secondary phase occur within the first 3 days of injury and include increased inflammation, excitotoxicity, freeradical production, cellular infiltration, and many others. Cell membrane integrity decline and accumulation of ions and neurotoxic substances like free radicals induce massive destruction of neurons by causing vasogenic edema and lipid peroxidation; respectively. The destruction is most obvious in the mitochondria and cytoskeleton ending up in apoptotic and necrotic events seen in the subacute phase (29).

Free radical production, such as reactive oxygen species (ROS) and the resulting oxidative stress overlaps in both acute and subacute phases of secondary injury. Subacute phase may last for weeks or even months, in which the destructed area enlarges representing progression of the acute phase events. Lipid peroxidation is also seen as it is a result of ROS attack on polyunsaturated fatty acids that starts a chain of progressive damage to cells even in the nearby healthy tissues. This is of great importance as the neural cell membrane is rich with fatty acids making them vulnerable to this type of damage (30). Finally, the immune system also participates in the subacute phase with its inflammatory role that is controversially both neuroprotective and neurotoxic. Different proinflammatory cytokines, mainly Interleukin-1 β and Tumor necrosis factor (TNF- α), are released which activate the process of astrogliosis and glial scar formation. This announces the beginning of chronic phase, the final event of the secondary phase of SCI. Scar tissue may hinder the axonal regeneration by forming a physical barrier and increasing growth inhibitory molecules expression, or it may produce growth promoting substances that aid in the repair of the injury (31). The time line of secondary phase was estimated to be 3 days for acute phase, 14 days for subacute phase, and 38 days for chronic phase during which remodeling and scar formation take place (32).

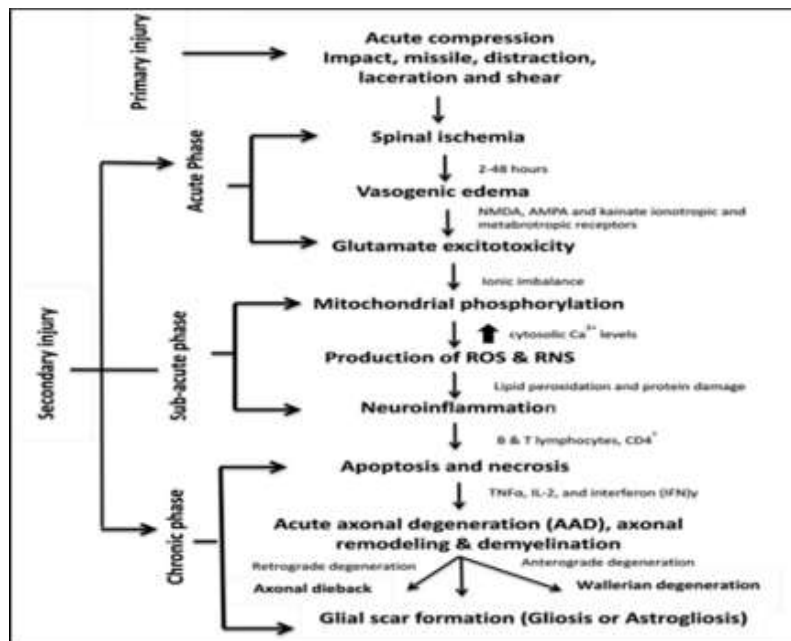


Figure 2. Pathophysiology and stages of SCI (From Anjum, 29)

2.3. SCI-Induced Bladder Dysfunction

2.3.1. Anatomy and Innervation of the Urinary System

The urinary system is divided into the upper urinary tract (UUT) and lower urinary tract (LUT). UUT consists of two kidneys and ureters, whereas the lower part of ureters, bladder, and urethra make up the LUT. Urine is formed by the filtration of blood from waste, water, and electrolytes through the kidney. Ureters drain the formed urine from the kidneys to the urinary bladder where it is stored until it gets excreted (33). The urinary bladder is a hollow muscular organ that lies extraperitoneally in human adults. The empty bladder is of flattened tetrahedral shape with four surfaces named as superior, posterior, and two inferolateral surfaces (Figure 3). The apex or the dome of the urinary bladder is the anterosuperior angle at which the superior surface meets the inferolateral surfaces. The lower angle of the urinary bladder is the bladder neck, which is formed by the meeting of the posterior surface with the inferolateral surfaces, and it is the least mobile part of the urinary bladder as it is fixed to the pelvic floor by a striated muscle (34, 35).

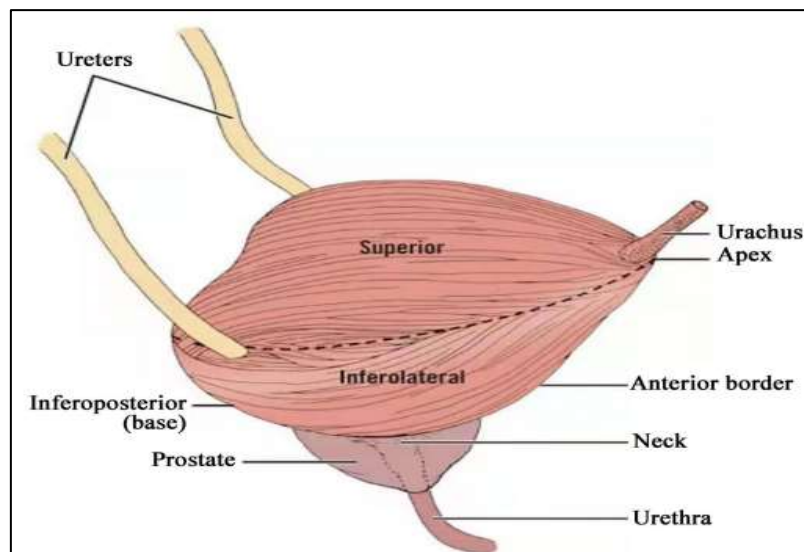


Figure 3. General anatomical structure of the urinary bladder (From Foè, 36)

Two internal urethral orifices are inserted obliquely starting from the bladder neck and terminating in the urethra to form the bladder outlet (Figure 4). The triangular area lined by these orifices is called the trigone (36). The urinary bladder wall is made of the distensible detrusor smooth muscle and lined with the urothelium which is a transitional epithelium tissue. Urothelium itself is surrounded by a thin layer of connective tissue called the lamina propria or submucosa. The mucosal layer of the urinary bladder is loose which gives the bladder wall a rugose (folded) appearance and it is continuous with the linings of ureters and urethra. Unlike the rest of urinary bladder, the trigone consists of two main layers which are the deep trigonal muscle similar to the detrusor muscle layer, and a thin layer of smooth muscle bundles called the superficial muscle. In addition, the trigone lacks the rugae and looks smooth as its lamina propria is more adherent to the underlying muscle layer (37). The contractile nature of the detrusor muscle and the presence of the rugae of the bladder lining allow the bladder to distend upon urine filling and store this urine until it is appropriate to void. On the other hand, the fixed neck and the oblique smooth trigone work as a funnel that direct urine to its way out of the body through the urethra. In the urethra, the internal urethral sphincter (IUS) controls the one-way flow of the urine in an involuntary manner, whereas the external urethral sphincter (EUS) allows urine excretion under developed voluntary control (33). The specific and distinct morphology and structure in the urinary bladder enable it to function both as a reservoir and an excreting organ of urine in response to different signals received by the sophisticated nerve supply from the nervous system (38).

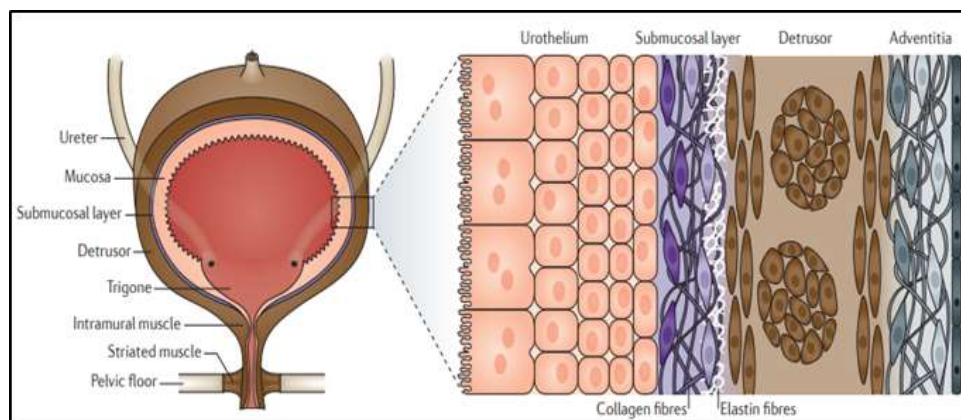


Figure 4. Anatomy and histology of the urinary bladder (From Ajalloueiian, 35)

The function of the LUS is under the fine control of both central and peripheral nervous systems (PNS) (Figure 5). The coordination between CNS and PNS with its both autonomic and somatic parts enable the storage-micturition function in LUS a voluntary process. Afferent nerves transmit sensory information through their free nerve endings from the LUS to secondary neurons in the spinal cord. Neurons of the dorsal root gangli give rise to afferent innervation at the spinal thoracolumbar (T10–L2) and sacral (S2–S4) segmental levels (39). A δ fibers are myelinated axons of afferent nerve fibers that terminate as nerve endings in the urinary bladder detrusor muscle. They are sensitive to stretch and contraction of the detrusor thus also called tension receptors and travel through the pelvic nerves to the sacral spinal cord. On the other hand, the unmyelinated C nerve fibers are sensitive to pain and volume change and they are widespread in bladder tissues like urothelium, detrusor muscle, and trigone. They send their sensory signals to the thoracolumbar spinal segment by the hypogastric nerves (40).

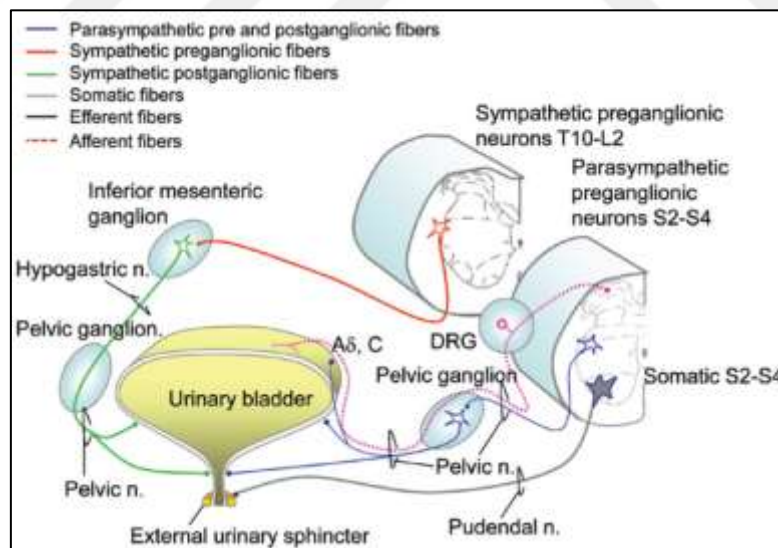


Figure 5. Innervation of the lower urinary tract (From Perez, 33)

The activity of LUS is under the control of the PNS with its sympathetic, parasympathetic, and somatic constituents via the efferent pathways (Figure 6). The sympathetic pathway starts in the thoracolumbar (T10- L2) level of the spinal cord and continues through the hypogastric nerve until it reaches the urinary organs. At the nerve endings, noreadrenaline causes the relaxation of the urinary bladder and the relaxation of the bladder neck and urethra by activating β_3 and α_1 adrenoceptors receptors, respectively (41).

The sacrum segment (S2–S4) of the spinal cord is the origin of the parasympathetic efferent pathway as pelvic nerves that ends up supplying the urinary bladder wall and the urethra. The acetylcholine neurotransmitter is released after parasympathetic activation and causes urinary bladder contraction through the M3 muscarinic receptors. The sacral segment also gives rise to the somatic pathway that pass across the pudendal nerves to act on the nicotinic receptors in nerve endings. As a result, acetylcholine is released causing the contraction the EUS (42).

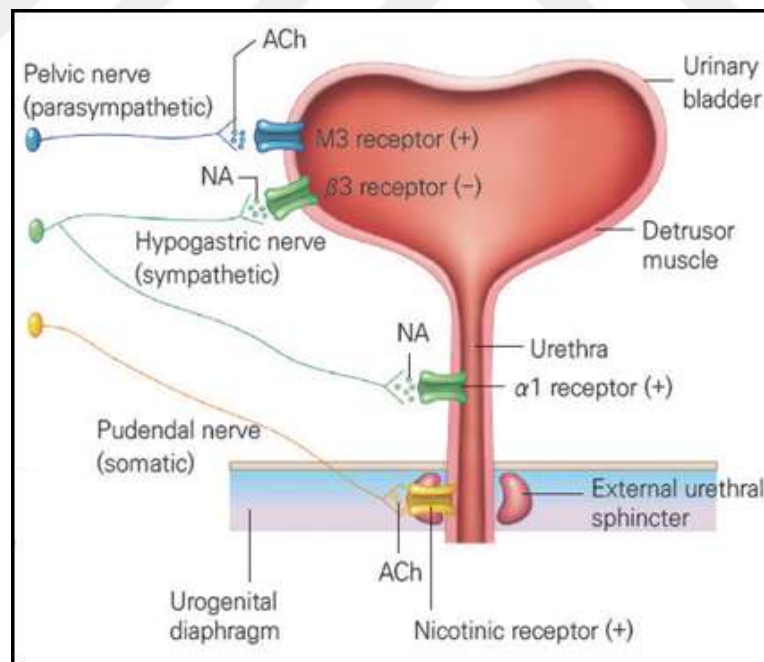


Figure 6. Efferent pathways nerve endings in the lower urinary tract (From Lee, 42)

2.3.2. Physiology of Continence and Micturition Processes

In contrast to visceral organs, the LUT activity is not of a tonic nature, rather it is found either in active or inactive state that resembles on and off activity. In addition, the dependence of LUT on the CNS makes a part of its function under voluntary control. In healthy adults, the coordination between urine storage (continence) and elimination (micturition) in the LUS is achieved by an important neural circuit called the spinobulbospinal pathway (43). This pathway connects the LUS and spinal cord with higher supraspinal brain centers including pontine micturition center (PMC) in pons, periaqueductal gray matter (PAG) in midbrain, and the cerebral cortex. These brain centers along with somatic innervation of the EUS via pudendal nerve make micturition an active and voluntary process that is learned and developed by the maturation of the body. In other words, the decision regarding voiding initiation in the appropriate time and place and the coordination of the process need the involvement higher brain centers in addition to spinal reflex arcs (42). The urethral sphincters and urinary bladder are considered the functional parts of the LUS and they work in a reciprocal way during the continence and micturition phases. When the bladder is empty or the urine amount is minimal, sympathetic activity takes place where the detrusor muscle is relaxed and the internal urethral sphincter is contracted. In contrast, at the time of urine elimination, sympathetic activity disappears and parasympathetic activity ensues resulting in synchronized detrusor muscle contraction and urethral sphincter relaxation.

In normal conditions, the frontal cortex exerts tonic inhibition over the PMC between voluntary episodes of voiding which allows for urine filling and storage until it is appropriate to be eliminated (33). As the bladder starts filling, detrusor muscle's A δ and C fibers send signals slowly to the sacral level of spinal cord which in turn conveys the input to the PMC and from there to the PAG and frontal cortex. Because the urine volume is still lower than the threshold of excretion or if it is not an appropriate time or place to void, PAG transmits inhibition signals from cortex to PMC and the bladder stays in the storage phase. The afferent signals from bladder also result in the contraction of EUS in a process called the guarding reflex. In response to these afferent signals, motor neurons leave the spinal cord and reach the striated EUS muscle via the pudendal nerve causing its contraction.

This reflex occurs tonically between voidings but becomes active in cases of sudden increase in bladder pressure due to events such as coughing or sneezing. In both situation, the guarding reflex is important in preventing the unwanted excretion of urine during the storage phase (44). As the urine volume increases, the signals sent to the sacral spinal cord become faster and reaches the PMC through the same path. From there, PAG receives the input of bladder stretching due to filling and transmits it to the hypothalamus and cortex to determine the shift from storage to micturition processes. If voiding is decided to be appropriate, the frontal cortex removes its inhibition and allows PAG to activate PMC. In turn, PMC initiates the voiding reflex by increasing parasympathetic and decreasing sympathetic activity of the LUS. The contraction of detrusor muscle and the relaxation of the internal (involuntary) and external (voluntary) urethral sphincters enable voiding process to start and the voiding reflex arc remains active until the bladder becomes empty and returns back to the storage state (45, 46). Accordingly, the processes of urine continence and micturition could be considered as spinal reflexes and the switch between them is controlled by the spinobulbospinal neural circuit which develops over time. In infants, only spinal reflexes are responsible of both processes resulting in involuntary voidings. As the CNS develops and neural connections inside and outside the brain start to appear, voiding process becomes a voluntary process owing to the involvement of higher brain centers. However, any damage to this strictly control system such as aging or SCI may interrupt these circuits which makes the involuntary spinal reflexes dominate again resulting in involuntary micturition or many other consequences (47).

2.3.3. SCI-Induced Bladder Dysfunction and Clinical Significance

Dysfunctions and complications secondary to SCI are not limited to motor disability only, but also extend to include problems in various body systems and affect different organs. Among these complications is the dysfunction of the bowel and urinary system, clinically known as neurogenic bowel and bladder that are considered as the most common comorbidities rating near 60% of SCI patients (4). Neurogenic bladder is of a great importance as around 80% of new SCI patients suffer from a certain dysfunction in the bladder and reported it as a crucial reason for distress and hospitalization that exsacerbates economical burdon, social isolation, and low quality of life.

Main clinical outcome of SCI-induced bladder dysfunction is the development of an abnormal micturition pattern along with its subsequent problems. This creates a serious problem as altered continence leads to urinary tract infections, urinary stones, renal impairment, and eventually renal failure (48, 49).

Micturition process is the result of coordination between the brain and spinal cord on one hand, and the bladder and the urethra on the other. Therefore, SCI-induced dysfunction affecting the somatic and autonomic pathways involved in the balance between urine voiding or continence makes the patient in high risk of morbidity and/or mortality. Different locations and severity of SCI may cause different forms of bladder dysfunction (Figure 7a). If the SCI occurs in central (suprasacral or suprapontine) region, the upper motor neurons are lesioned so the communication between PMC and sacral reflex arc is lost although both parts are intact. This means that the sacral micturition reflex is disinhibited causing hyperreflexia of urinary detrusor muscle (Figure 7b) (48). In addition, a condition known as detrusor sphincter dyssynergia (DSD) may take place in which the bladder detrusor muscle is contracting together with the EUS causing urine outflow blocking and resulting in non-voiding contractions with urine retention. If left untreated, DSD may end up in deterioration of the upper urinary tract and renal failure due to high voiding pressure and residual urine volume (49). In spinal (intrapontine-suprasacral) SCI injury, the supraspinal disinhibition may abolish or stay intact due to the injury of sacral roots needed for the micturition reflex (Figure 7c). In both situations, overactivity or areflexia of detrusor muscle and EUS may be seen with the net result of storage dysfunction and urinary incontinence. Injury at the sacral level or below (infrapontine) will cause lower motor neurons damage and voiding reflex arc interruption without affecting the PMC which will result in areflexia of detrusor muscle and EUS flaccidity (Figure 7d) (33).

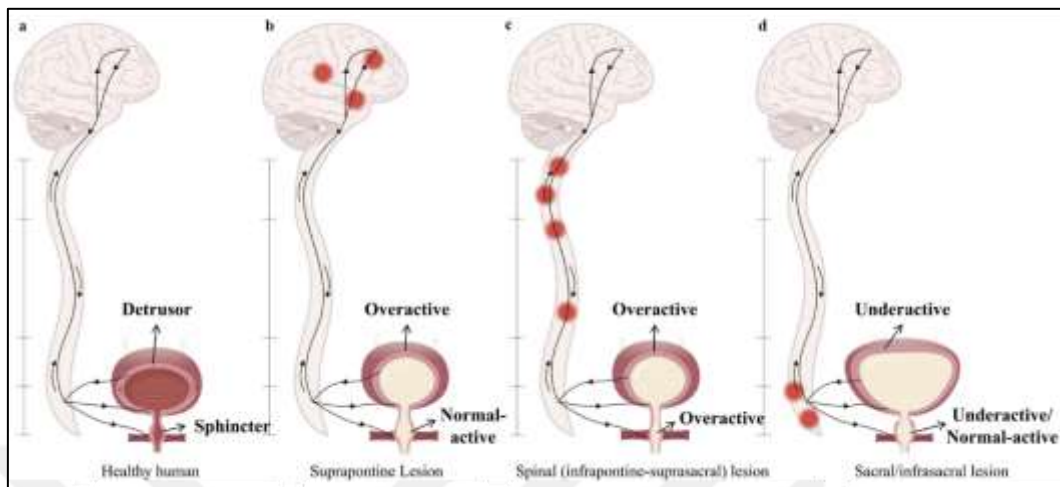


Figure 7. Types of neurogenic bladder according to SCI and lesion level. **(a)** Healthy human, **(b)** Supraspinal, **(c)** Spinal, and **(d)** Sacral lesion. (From Ferreira, 51)

However, the effect of SCI on bladder dysfunction could not be predicted simply due to many factors. First, SCI may occur as multiple lesions at different spinal levels which affect both ascending pathways that relay sensory information of bladder distention, and descending pathways that facilitate bladder emptying resulting in mixed voiding patterns. Second, investigations have revealed a correlation between the extent of injury and the type of bladder dysfunction that was different in patients with complete and incomplete lesions (50). Finally, the spinal shock seen in SCI acute secondary phase causes variable degrees of urinary retention and decreased bladder contractions regardless of lesion location in the spinal cord. This decreased parasympathetic activity may last for weeks before the development of afferent fibres and the emerging of new independent alternative micturition reflex pathways due to neuroplasty which causes involuntary bladder contractions.

The complicated pathophysiology of SCI-induced bladder dysfunction created a challenge in developing appropriate interventions and treatments especially in early stages and the need for more research in this area became warranted (49, 51).

2.3.4. SCI-Induced Bladder Dysfunction Management

Currently, many surgical and non-surgical methods are followed to manage neurogenic bladder with restoring normal LUT function, preventing renal impairment, and improvement of patients' quality of life being the main goals. Interventions used immediately after SCI typically include catheterization and suprapubic percussion to trigger bladder reflex and maintain normal drainage. The disadvantage of these interventions is the high risk of UTI and patient inconvenience which make their use temporary until the stabilization of diuresis (52). The use of pharmacological interventions for bladder dysfunction is a common practice in early phase after SCI. The choice of the pharmacological agent depends on the bladder dysfunction type and targeted receptors. The first-line medication used for neurogenic detrusor overactivity is anticholinergic agents such as oxybutynin that improve incontinence, urgency, and frequency by inhibiting bladder contraction through muscarinic receptors blockage. Overactive bladder is also controlled by the beta-3 adrenergic agonist, mirabegron, which causes detrusor muscle relaxation, pressure reduction, and improvement in bladder capacity. In contrast, bladder neck relaxation and outflow resistance decrease are achieved by the use of alpha-adrenergic antagonists like tamsulosin and terazosin.

Due to their common side effects (blurred vision, gastrointestinal system disorders and cognitive disorders) and the decrease in their effect over time despite their initial success, the use of these agents is less preferred by patients. These side effects can lead to treatment failure, decreased patient compliance and even discontinuation of the drug (8). Commonly used drugs for SCI-induced bladder dysfunctions are summarized in table 1 (33, 53). During the rehabilitation period, the patient is trained to control micturition process by certain exercises and maneuvers. This period includes training of the pelvic floor muscle, regimens for scheduled voiding, and major lifestyle changes such as weight loss and avoiding alcohol, caffeine, and carbonated beverages. Although these methods may help the patient on the long run, they are only useful in incomplete SCI patients who still have some degree of control over voiding process. Finally, surgical procedures may be used after the failure of all non-invasive conservative therapy regimens (52).

Notably, the clinically existing interventions for SCI-induced bladder dysfunction are still of limited effectiveness with high cost and inconvenience to most patients. This renders the research and development of new treatment approaches warranted thus gaining much attention towards more convenient and less invasive interventions to reverse SCI and its secondary outcomes (13).

Table 1. Summary of commonly used drugs for SCI-induced bladder dysfunction

Drug class	Agents	Indication	Effect	Side effects
Anticholinergics	Oxybutynin	Detrusor sphincter dyssynergia	Suppression of detrusor	Dry mouth
	Solifenacin		Efficient voiding	Constipation
	Tolterodine	Overactive bladder	Lower pressure of storage and voiding	Cognitive problems
Beta-3 agonists	Mirabegron	Detrusor hyperreflexia	Detrusor muscle relaxation during filling	Headache
	Vibegron		Urge incontinence prevention	Hypertension
Alpha-blockers	Tamsulosin	Detrusor-sphincter dyssynergia	Smooth muscle relaxation in urethra and bladder neck	Dizziness
	Terazosin		Efficient bladder emptying	Hypotension
Bladder chemo-denervation	Botulinum Toxin A intradetrusor injection	Refractory detrusor hyperreflexia	Reduced detrusor overactivity by inhibiting acetylcholine release	Retrograde ejaculation
Cholinergic agonists	Bethanechol	Detrusor areflexia	Enhanced bladder contractions	Minimally invasive
				Diarrhea
				Bradycardia
				Sweating

2.3.5. SCI in Experimental Animal Models

Experimental studies using animal models are a fundamental approach in research that helps in understanding the pathophysiology of different disorders and to evaluate the effect of new treatment approaches on these disease models. SCI is one of the most studied in depth disease due to its broad effects on patients and health care systems in general all over the world. Since their first conduction in dogs using weight-drop method in 1911, SCI animal models have received large interest in experimental design to optimize the best possible model that mimics human SCI and its related dysfunctions the most (54). To design a well-structured work plan for any experiment, a suitable animal model should be chosen depending on the purpose and main aim of the study. For SCI studies, the chosen SCI model should be feasible and easily applied by the researcher and the available laboratory equipments for better assessment of the parameters and variables of the experiment (55).

The optimization process starts with choosing the suitable animal species since they differ in their anatomy, physiology, genetic background and disease progression pattern. Although non-human primates closely resemble SCI pathology in humans, their housing and care costs and strict ethical/regulatory considerations make rodents more preferred over larger mammals which are rarely used nowadays. Mouse model is preferred in studying basic cellular and molecular changes in SCI. Conversely, injury site in mice is packed with cells which makes the work area small and hard to be efficiently handled. Notably, axonal regeneration is much higher in mice than in other species causing the decrease in lesion size and disappearing of the spinal cysts as time progress which hinder the evaluation of disease progression. On the other hand, rats are the most common used species among rodents due to several factors. The anatomy of rats is well-understood and fits the SCI model better as their corticospinal tract is located dorsally. The lesion in the dorsal part does not create significant disability and since rats are less susceptible to infections after surgery, they show better recovery with less postoperative complications of the procedure (56).

After SCI induction, rats develop apparent pathological changes at the lesion site like the formation of cystic cavities that are large and filled with fluids and unlike mice, their size and cavitation remain stable over time. These features make rats an appropriate model that is relatively comparable to human physiology and their use became common in studies focusing on functional analysis to evaluate the efficacy of novel therapeutic agents (57). It is worth noting that rats differ in some anatomical structures from the human body. Unlike SCI in humans that affects both dorsal and ventral spinal tracts, SCI in rat model causes a lesion in the dorsal spinal cord. However, axonal regeneration and plasticity are suggested to be similar in their molecular mechanisms in rats and humans after SCI as a result of the closely correlated electrophysiological, behavioural, and histological analysis conducted in both species (58). The vertebral column of rats is different from that of humans and consists of 7 cervical, 13 thoracic, 6 lumbar, 4 sacral, and 27caudal vertebrae (Figure 8) (59). These differences should be taken into consideration while performing SCI induction surgery in order to target the correct location of the spinal cord nerves with the intended lesion. Nonetheless, the organization of detrusor interstitial cells is comparable in rat and human with similar immunohistochemical and ultrastructural features. Depending on these findings, rats are considered to be a reliable SCI model and could be used to simulate the changes observed in humans after SCI and bladder-related research (60).

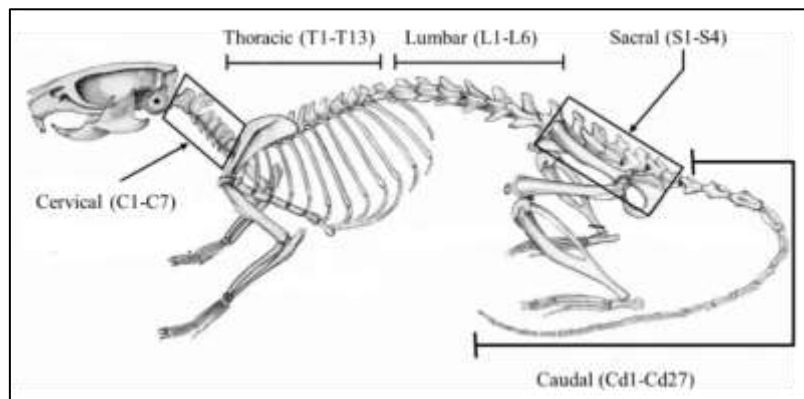


Figure 8. Articulated rat skeleton showing spinal vertevrae (modified from Jolette, 59)

SCI models can be subdivided into mechanical and chemical models where induced injury resembles traumatic and non-traumatic SCI in humans, respectively. Chemical injury models are more used when it is aimed to investigate molecules and pathways in the secondary phase of SCI. Given that SCI of traumatic accidents is the most common type of SCI in humans, mechanical SCI models are used more often in research to evaluate more general post-traumatic changes. Contusion, compression, and transection injuries are widely used as they cause sudden pressure on the spinal cord in a manner consistent with SCI that results due to vertebral damage and herniation onto the spinal cord after a sudden trauma (57, 61). Clinically, SCI resulting from complete transection is rarely seen in humans, yet transection model is used widely in research for the assessment of axonal regeneration and degeneration mainly in tissue engineering and plasticity studies. After laminectomy, thin iridectomy scissors or scalpels are used to induce destruction of targeted conduction pathways in the spinal cord.

Complete transection is an easy to apply reproducible technique but its use is limited only for certain aims as transection SCI is rare in clinical settings. A more reflective model of human SCI is the partial transection model, which could be induced as unilateral selective lesion of spinal cord. This method is more beneficial for comparing functions of organs supplied by healthy fibers with damaged ones in the same animal (62). Contusion was the first SCI model used and alongside with compression is the most preferred model in current research due to their common involvement in human SCI. It is achieved by applying a transient but acute force to induce an injury that is suitable to examine pathophysiological SCI-induced changes. Clips, calibrated forceps, balloons, and impactors like weight-drop apparatus are some of the specialized equipments used to apply a desired force around the target level in the spinal cord to induce the contusion injury (54).

Compression model is similar to contusion but the clip or forceps is placed and left to apply the force for a longer time. In particular, clip compression model outperforms other models in terms of adjusting the degree of injury by controlling closing force and duration. Practically, the applied force is usually between 20-180 g and the clip is removed after 30 seconds to 1 minute (63). This was made possible as SCI animal models showed alterations in numerous molecules like growth factors, receptors, ion channels, and neurotransmitters that mirror the changes seen in humans.

These findings enabled researchers to deeply examine SCI-induced pathophysiological alterations in neuronal pathways and the resulting end-organ dysfunctions. For instance, the observation of bladder afferent fibers hyperexcitability is a major mechanism that underlines LUT dysfunction provided deeper understanding of SCI-induced neurogenic bladder thus bringing research a step closer to manage the common SCI consequences (64).

2.4. Mitochondrial Dynamics and its Role in Bladder Dysfunction

Recently, the new approach in SCI-related research has been oriented to study the subcellular level as it may pave the way to develop new treatments that target cellular and functional survival after SCI. By providing 95% of energy to the cell, mitochondria are called the power house of the cell and started gaining a large interest of this research. Hence, understanding the mitochondrial dynamics and its endogenous defense mechanisms along with its relation to SCI will also aid in utilizing mitochondria as the next intervention that targets SCI-induced dysfunctions (12).

2.4.1. Mitochondrial Physiology and Adaptation to Oxidative Stress

Mitochondria are intracellular organelles with distinct structure and function (Figure 9). Mitochondria consist of the smooth outer mitochondrial membrane (OMM) and the folded inner mitochondrial membrane (IMM) with sandwiched inner membrane space. The OMM is a smooth phospholipid bilayer and contains transport proteins to shuttle different molecules in and out of the mitochondria. IMM is folded into invaginations called cristae to increase its surface area in order to host the electron transport chain (ETC) along with the ATP synthase. ETC is a four-transmembrane protein complex (I-IV) all working as hydrogen carrier that accepts H^+ from metabolites and electron (coming from NADH and $FADH_2$) carrier that reduces O_2 into H_2O which creates an electrochemical gradient. The protein complex, redox reactions, and the resulting gradient work as a complete system to finally produce adenosine triphosphate (ATP), cellular energy form, from adenosine diphosphate ADP via ATP synthase, also known as complex V, in a process called oxidative phosphorylation (OXPHOS). The matrix is the inner mitochondrial compartment containing DNA and ribosomes needed for protein translation in the mitochondria, in addition to many enzymes involved with fatty acid oxidation and Krebs Cycle that produce the substrates needed for energy production via ETC (65, 66).

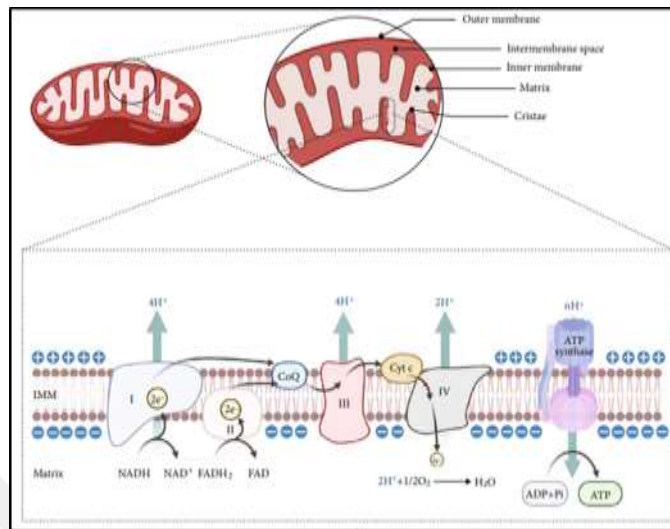


Figure 9. Mitochondrial general structure and ETC components (From Yuan, 65)

OXPHOS is a strictly controlled process, yet some of the electrons flowing through ETC may leak out of the protein complexes. The prematurely released electron reacts with and reduces oxygen to the superoxide anion (O_2^-); the cell most abundant and major ROS. This makes mitochondria the main source of cellular ROS as a byproduct of ATP synthesis and paradoxically very sensitive to ROS-induced pathological changes (67). ROS have been linked to oxidative stress and cellular damage. However, ROS are now known to have beneficial physiological effects through different pathways. ROS at low levels are involved in a signaling process called redox signaling which activates biological events such as regulation of cellular growth, and enzymatic activities (67). For example, mild mitochondrial production of ROS plays a cytoprotective role and assists in preventing the damaging effects of oxidative stress on the cell. This is achieved as ROS themselves activate transcription factors of detoxification enzymes' genes which are redox sensitive. Superoxide dismutase (SOD) and glutathione peroxidase (GPx) are considered to be the most important enzymes in the endogenous antioxidant defense system (68). SOD rapidly dismutates the superoxide ion formed from leaked electrons into hydrogen peroxide (H_2O_2) and oxygen (O_2) in the mitochondria. H_2O_2 produced by this reaction is uncharged thus more stable than any of ROS. H_2O_2 exits to the cytosol and mediates cell signaling by oxidizing metal cofactors or thiols of cytosolic proteins which alters their function.

Glutathione peroxidase in turn scavengers H_2O_2 by converting it into water (H_2O) in the presence of glutathione, an intracellular antioxidant. To keep biological balance intact, the oxidized glutathione (glutathione disulfide) is reduced back to glutathione by the action of glutathione reductase to be used again in another similar reaction (69). In addition to these enzymatic antioxidants, glutathione and other compounds represent the non-enzymatic part of endogenous antioxidant system. These are molecules present in the body or taken from dietary sources that aid in the whole antioxidant defence process. Other examples include vitamin E (α -tocopherol), vitamin C (ascorbic acid), bilirubin, and many others (70). In this way, mitochondria are protected from excessive amounts of ROS which are sequestered and neutralized by an endogenous detoxifying system.

2.4.2. Mitochondrial Dynamics and Oxidative Stress

Mitochondrial production of ROS varies in response to alterations in the availability of oxygen and metabolic substrates in addition to the pathological changes of the cell. Around 2% of electrons normally leak out of ETC during aerobic respiration and get converted into different ROS. Healthy and normally functioning mitochondria are capable of sequestering and detoxifying the formed ROS by the endogenous antioxidant system. However, ROS could be present in the cell in excessive amount that may exceed the antioxidant capacity of the cell and result in a pathological state of oxidative stress. High nutrient availability, inflammation and cytokines accumulation, and hypoxia are some examples of endogenous oxidative stress factors. Exogenous oxidative stress is the result of exposure environmental sources such as radiations, pollutants, heavy metals, cigarette smoke, and many other (71). Regardless of the source, excessive amounts of ROS will overwhelm mitochondrial endogenous capacity whether due to enzymes' saturation or depletion of antioxidant molecules - especially glutathione- which eventually will impair the antioxidant protection mechanisms of the cell. Moreover, when the balance between the amount of superoxide and the level of endogenous detoxifying antioxidant enzymes is disrupted, the production of ROS, and eventually oxidative stress, in mitochondria increases even more. In other words, any disturbance in mitochondria, including ROS-induced damage, can cause changes in the respiratory chain complexes that lead to a decrease in electron transfer efficiency. This leads to more ROS production and a vicious cycle characterized by oxidative stress-induced damage and decreased energy production (70).

The mitochondrial function and integrity is highly compromised in the presence of oxidative stress. ROS accumulation causes direct damage to vital biomolecules such as DNA, lipids, and proteins. This damage results in pathological changes in the cell which could be seen as reduced ATP production, DNA mutation/fragmentation, structural and enzymatic protein degradation, and membrane lipid peroxidation. These changes are thought to be destructive for the cell due to altered signal transduction pathways, inflammation, and increased permeability of mitochondrial and cellular membranes. Subsequently, pro-apoptotic proteins may get activated in the intermembrane space of the mitochondria which announces the beginning of cell cycle arrest and cell death (71). For this reason, mitochondria are now believed to be involved in important physiological processes throughout the body like programmed cell death, autophagy, innate immunity, redox signalling, and cells reprogramming, where damage to the mitochondria directly affects the general health of tissues and organs. Notably, mitochondria are dynamic organelles thus they are able to adapt to oxidative stress and disrupted endogenous antioxidant enzymes by modulating their shape and structure through balanced quality control mechanisms that aid in maintaining their normal functions inside the cell (72).

Mitochondrial dynamics is a term used to describe the coordinated mitochondrial processes that are regulated in response to cellular environment to enable mitochondria to maintain their normal shape, size, number, and consequently cellular homeostasis in normal and pathological situations. These processes include mitochondrial mitophagy, fission, and fusion. Besides preserving normal mitochondrial function in the cell, mitochondrial dynamics also respond to intra and extracellular changes, such as increased ATP demand or oxidative stress, by adapting to nutrients availability and altered cellular metabolic state (73). Mitophagy is a selective self-renewal mechanism through which elimination of dysfunctional or injured mitochondria takes place via autophagosomes to be degraded and recycled later on by cellular lysosomes. It is thought to govern the quality control system of the mitochondria by activating multiple signal transduction pathways especially during differentiation and programming processes of the cells. Mitophagy itself is activated by different biological molecules targeting the OMM receptors that are still not fully understood. Among these molecules are the ROS generated from the oxidation of mitochondrial fatty acid that could also initiate the crosstalk between mitophagy and fission/fusion processes (Figure 10) (74).

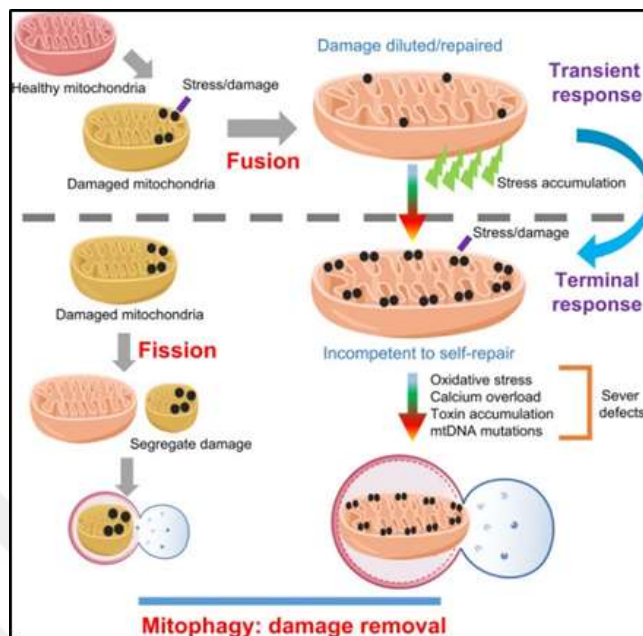


Figure 10. Crosstalk between mitochondrial dynamics and mitophagy (From Xian, 74)

Fission and fusion are two opposite processes that cause mitochondrial membrane division and merging, respectively, and controlled by the dynamin family of Guanosine triphosphatase (GTPase) proteins. During fission, one mitochondrion is divided into two smaller round mitochondria which then face one of two biological fates. Normal functioning daughter mitochondrion leaves the fission cycle and shares its contents with another mitochondrion to dilute any damage for complete recovery; all done through fusion process (75). However, daughter mitochondria with any type of damage, like DNA mutation or protein misfolding, get tagged for degradation and elimination through mitophagy cascade. In this way, mitochondrial fission facilitates mitophagy by providing smaller mitochondria that are easier to be engulfed by autophagosome and eliminated during mitophagy.

Despite being associated with mitochondrial dysfunction or cell death in stress situations, balanced mitochondrial fission is vital to the cell cycle to provide an adequate number of healthy mitochondria are inherited during mitosis (76). Dynamin-related protein 1 (Drp1) is a cytosolic protein that regulates the multi-step fission process of the mitochondria. Fission sites are thought to be marked on the OMM as mitochondria gets attached to the endoplasmic reticulum by the aid of actin and myosin cytoskeleton to mark and form the site of constriction.

Next, Drp1 is recruited to the OMM and binds to the adaptors located on constriction sites and oligomerizes into rings that constrict the mitochondria through the GTP hydrolysis-induced conformational changes. Other fission proteins get activated and complete the scission of mitochondrial OMM at these constrictions. IMM constriction is thought to be a calcium (Ca^{2+})-dependent process where calcium is released from the stimulated endoplasmic reticulum upon contact with mitochondria which in turn uptake Ca^{2+} and several downstream proteins activation ensue to complete the constriction of the inner membrane compartment (77).

Fusion is another multi-step process by which a healthy mitochondrion is merged with another defective one to form a new normal functioning mitochondrion. The new fused mitochondria take an elongated shape where the matrix components are redistributed and OXPHOS activity is retained after any dysfunction as molecules like DNA and ETC proteins become available from the healthy mitochondria. This elongation also impedes the engulfment of mitochondria that is activated due to stress situations like ROS accumulation or cellular starvation which ensures the integrity and survival of the cell to stay intact (76).

Fusion of mitochondria is mediated by three GTPase proteins; mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and Optic Atrophy 1 (OPA1). Mfn proteins oligomerize on the OMM of a mitochondrion to prepare it for fusion, and on two adjacent ones to connect them together while increasing the contact surface. As in fission process, oligomers of Mfn proteins change in conformation by GTP hydrolysis to finally result in the fusion of the inner membranes while controlling the normal architecture of their cristae (74). Notably, mitofusin protein homologues Mfn1 and Mfn2 overlap in their regulatory functions in fusion process, yet Mfn2 started gaining more interest in research due to its higher importance in maintaining normal mitochondrial fusion and integrity in general. Recently, cumulative evidence showed that Mfn2 is the responsible protein of endoplasmic reticulum tethering to mitochondria and its knockout showed significantly impaired close contacts needed for fusion process. In addition, Mfn2-induced tethering enables the exchange of calcium and lipids between the two organelles to maintain metabolic and signaling pathways coordination. For this reason, Mfn2 is now believed to not only be involved in fusion process but it is a key factor and regulator of mitochondrial dynamics and survival (78).

In addition to fission and fusion proteins, OMM contains a unique protein complex that is essential for maintaining mitochondrial function and dynamics. For efficient functioning, mitochondria need around 1500 different proteins though mitochondria can only produce 13 of them by genome encoding which are then incorporated in the respiratory chain as core protein components. Cytosolic ribosomes produce precursors for the remaining mitochondrial proteins that need to be imported to their mitochondrial destination.

Translocase of the outer mitochondrial membrane complex (TOM) is a core protein in the OMM working as the import machinery to facilitate the import of mitochondrial proteins in a selective and synchronized manner (79). The importance of TOM complex is that it is the primary gate for the entry of more than 90% of mitochondrial protein precursors independently from ATP. TOM20 is a major subunit of this complex with special characteristics that make it a key component of the mitochondrial structure. It is anchored in the outer membrane by its N-terminal whereas its C-terminal segment has cytosolic orientation. This arrangement allows TOM20 to function as a receptor that recognizes the polypeptides and selectively uptakes the needed ones by its hydrophobic groove (80). Besides serving as a receptor and initial protein precursors binding site, TOM20 is used as a marker of mitochondria and plays a wide range of roles in the biology of mitochondria. Being associated with the TOM complex only transiently, several studies have shown that it is responsible of many reactions and pathways in the mitochondria and its knockdown caused pathological changes in the cell including increasing the cell's sensitivity to apoptosis. For this reason, dysfunction of any component of TOM complex and TOM20 in particular causes the clogging and accumulation of protein precursor inside the gate causing increased stress in the cell (81).

Mitochondrial dynamics and protein translocases function in the favor of mitochondrial homeostasis. However, during prolonged stress or damage to the cell, these vital processes may get dysregulated with changes in structure and expression levels of their relative proteins that affect the mitochondria negatively. For example, excessive fission and fusion result in a large number of small, fragmented, and round or elongated and over connected mitochondria, respectively. In both cases mitochondria are abnormal and their malfunction will be reflected on the cellular or even systematic level (73). Likewise, defective TOM complex has been linked to mitochondrial dysfunction that is translated into different clinical pictures.

Decreased import system activity is now considered to be both a causative and consequential factor in increased ROS production especially in studying the pathology of neurodegenerative diseases and it is emerging as a new interest in many research fields (82). In this context, the examination of mitochondrial proteins/enzymes and understanding mitochondrial bioenergetics help to evaluate the cell under both physiological and pathological conditions. The evaluation of the expression levels of proteins such as TOM20 and fusion-fission proteins (Drp1, mitofusin) are currently used in research to understand the pathogenesis of many diseases and to develop agents to reduce mitochondrial damage. Furthermore, interventions targeting mitochondrial dynamics and the TOM complex are highly needed in order to impede and counteract stress-induced damage in the whole cell (83).

2.4.3. Role of Mitochondria in SCI-Induced Bladder Dysfunction

Uncontrolled ROS production and oxidative stress have been linked to the incidence of many chronic diseases including cardiovascular, renal, lung, and neurological diseases, and cancer. Oxidative stress has been observed as a direct threat and the primary cause in certain cases like atherosclerosis and lung radiation-induced injury (84). As a second cause, oxidative stress is also engaged in the progression of many diseases where the formation of superoxide ensues after the onset of the primary causative factor. This is widely seen in neurodegenerative disorders such as Parkinson, Alzheimer's, and SCI-induced diseases. It has been recently proposed that Parkinson and Alzheimer's diseases have mitochondrial dysfunction and the subsequent oxidative stress as common features in their pathophysiology even as an early event in their progression (72). Because of its complicated pathophysiology, SCI-induced damage of cellular organelles has lately gained much interest in research. Since ROS production and the resulting oxidative stress are common features of both acute and subacute phases of secondary injury of the spinal cord, mitochondria became the center of interest and have been linked to several SCI-induced dysfunctions. Jia et.al studied the change in the morphology and function of mitochondria in rats with SCI. Their results showed abnormality in the shape, size, cristae, and membranes of mitochondria in rats with SCI. In addition, SCI caused dysregulated mitochondrial dynamics that were thought to be responsible of these changes.

Immediately after injury, fission protein Drp1 expression was diminished while fusion protein (Mfn1) expression was increased. By time, these kinetics were reversed as Drp1 was overexpressed while Mfn1 expression was suppressed. The study demonstrated that early SCI stage is marked with fusion while later stages are dominated by fission dynamics. Moreover, this dysregulation in dynamics was believed to be responsible of further SCI-induced mitochondrial damage as with time morphological changes appeared and eventually led to the rupture of mitochondrial membrane (85). The results of this study and other similar ones highlighted the importance of mitochondrial changes in SCI secondary outcomes in different disease models.

Kidneys are the second organs with the highest mitochondrial content and the most sensitive to mitochondrial damage after the heart. This observation motivated researchers to expand their research and include mitochondrial ROS as a possible cause in urinary system disorders. The research results indicated that the progression of chronic kidney disease has a strong correlation with mitochondrial homeostasis disruptions and increased mitochondrial ROS production (86). Moreover, the mitochondrial role in the progression of acute kidney injury to chronic stage suggested that dysfunctions in mitochondrial bioenergetics and redox balance is a main contributor in the transition from acute to chronic kidney injury, both *in vivo* and *in vitro* studies. This correlation relied on the observation that mitochondrial injuries and disrupted homeostasis were the cause of further renal injury, fibrosis, inflammation, and even renal failure (87). Within the same scope, a study conducted on patients with confirmed mitochondrial diseases showed that 81.5% of these patients had severe overactive bladder symptoms and 34.5% of them suffered from voiding problems. Surprisingly, none of these patients was receiving treatment for the urinary symptoms despite being described as negatively affecting their quality of life (88). These findings were a milestone in understanding the molecular aspects of urinary diseases with high mortality/morbidity rates and resulted in including mitochondrial dysfunctions and their secondary outcomes in multidisciplinary medical care practice guidelines. In clinical practice, it is now recommended that patients of mitochondria-related diseases should receive more attention regarding their urological health.

Despite that these recommendation are consensus-based due to the lack of enough randomized trials, specialists built their decisions depending on previous studies that provided evidence of the mitochondrial importance for urinary bladder muscle contraction during micturition and the urinary system disorders accompanying mitochondrial dysfunction (89). Most of the research previously conducted on SCI-induced secondary effects – including mitochondrial dysfunction- focused on the post injury histological or molecular changes in the affected neurons of the spinal cord but less articles discussed the SCI consequences in the urinary system. On the other hand, studying SCI-induced bladder dysfunction was more interested in monitoring the pathological changes in the LUT after SCI and their correlation with the injury level, or in evaluating the effect of different therapeutic agents rather than understanding the submolecular origin of these changes (90, 91).

Nonetheless, more recent research on the SCI-induced dysfunctions is now more directed towards neurotransmitters, receptors, and molecular changes in spinal neurons and the bladder in an attempt to understand their pathophysiology better. For instance, neurotrophins such as nerve growth factor and brain-derived neurotrophic factor have been studied thoroughly due to the involvement of their level increase and the subsequent neuronal changes in micturition after SCI. It was documented that SCI-induced neurotrophins level increase cause hyperexcitability of bladder afferent neurons resulting in detrusor overactivity, non-voiding contractions, DSD, and eventually impaired micturition and voiding process (92, 93). Although the improvement in bladder urodynamics was significant, the recovery of micturition process was only partial suggesting the involvement of other molecules and pathways in SCI-induced bladder dysfunction thus demanding further research on the submolecular lever (94).

A recent review article provided a comprehensive analysis of the research conducted in the last decade that investigated the molecular mechanism of neurologic lesions-induced bladder and micturition dysfunction. 51 studies were included in the review and demonstrated several changes on the molecular level in the neurons and urinary bladder such as inflammatory and fibrosis markers or receptors expression, and SCI was the most frequently used model to induce bladder dysfunction. Yet, none of these studies mentioned mitochondrial involvement in the pathophysiology of the disease and the authors of the review agreed on the need to explore other key players with possible roles in the disease progression (95).

To fill this gap in research, a study conducted by Kullman et al., discussed SCI-induced mitochondrial changes in LUT using a transection-induced SCI in animal model and showed interesting findings in this area (96). It was shown that SCI caused mitochondrial dysfunction and excessive ROS production that in turn resulted in renal fibrosis and urothelial damage. Notably, SCI caused mitochondrial accumulations in the cytoplasm of bladder umbrella cells and increased levels of Drp1 protein suggesting decreased mitophagy and increased fission processes, respectively. These observations were consistent with SCI-induced mitochondrial damage that resulted in negative outcomes in urinary bladder tissue. Taken together, these research findings and gaps support the rationale for targeting mitochondrial changes post SCI with therapeutic agents and guarantee the need for evaluating their effect on reversing the resulting dysfunctions in the LUT on functional and molecular/histological levels.

2.4.4. Mitocellulars as Pharmacological Agents

Mitocellulars are pharmacological agents developed to target the mitochondria and restore their bioenergetics to preserve neurons from damage after injuries or lesions of different sources such as SCI. The high demand of mitochondrial ATP and limited ability to use glycolysis as an alternative, along with narrow capacity to neutralize oxidative stress make targeting mitochondria after nerve injury reasonable in treating SCI secondary dysfunctions (12).

Mitocellulars have different mechanisms of action at the mitochondrial level, still they all share the main aim of improving neuronal homeostasis. They work by enhancing mitochondrial function and integrity and/or decreasing oxidative stress to eventually stop neuronal degeneration. The most commonly examined mechanisms in preclinical studies are the inhibition of apoptosis or ROS production and mitochondrial dynamics modulation (97). Targeting mitochondrial dynamics is one of the mechanisms used to inhibit cell apoptosis. It has been shown earlier that cell death in the spinal cord is attributed to post-injury apoptosis. The later is related to mitochondrial dynamics and it is inhibited by fusion while promoted with excessive fission that is prominent within 24 hours of SCI. Mitocellulars that restore these dynamics represent a promising option to decrease apoptosis and neuronal loss after SCI (97).

Among all mitoceuticals modes of action, reducing oxidative stress gained the greatest share of recent research where several reviews talked only about mitochondria-targeted antioxidant role of mitoceuticals. This research resulted in many possible therapeutic agents for SCI-induced mitochondrial damage. These agents work either by scavenging produced ROS or inhibition of its formation, improving the activity of antioxidant enzymes, or by inhibiting the lipid peroxidation process itself (98).

In the mid 1980's, vitamin E was found to be the main antioxidant in the human blood with the naturally occurring α -tocopherol is the most effective radical scavenger among all forms of vitamin E. In early SCI treatment studies, α -tocopherol administration showed functional recovery due to scavenging lipid peroxidation products in different animal models. Paradoxically, the free radical neutralization process results in the production of α -tocopheroxyl, which is also a free radical itself. Although the formed radical is stable, it reduces α -tocopherol's antioxidant ability and needs vitamin C as an antioxidant to reduce it back to its original state. Surprisingly, although many antioxidants like vitamin E are mitochondria-targeted, the fraction taken up and penetrating the mitochondria is small depending on their lipophilicity and/or size. This explains why vitamin E and its derivatives should be given at high doses for long duration that increase their risk of toxicity and even mortality, especially if combined with other antioxidants like vitamin C (99, 100). It has been speculated that targeting a single mechanism in mitochondrial dysfunction may be insufficient for SCI and its secondary dysfunctions. To overcome these barriers, it has been suggested to modify mitoceutical agents that are taken up better by the mitochondria and modulate mitochondrial functions through more than one mechanism of action to achieve better outcomes in SCI patients (97).

MitoTEMPO (CAS 1334850-99-5) is a new generation antioxidant agent that targets the mitochondria to scavenge superoxide molecules and other mitochondrial free radicals. The ability of mitoTEMPO to selectively accumulate in the mitochondrial matrix and exert a potent antioxidant action is the result of its unique developed structure (101). It is synthesized by combining TEMPO, a piperidine nitroxide potent antioxidant, covalently with the lipophilic cation triphenylphosphonium (TPP⁺) that is able to pass through mitochondrial phospholipid bilayers to deliver TEMPO to the matrix efficiently (Figure 11) (102).

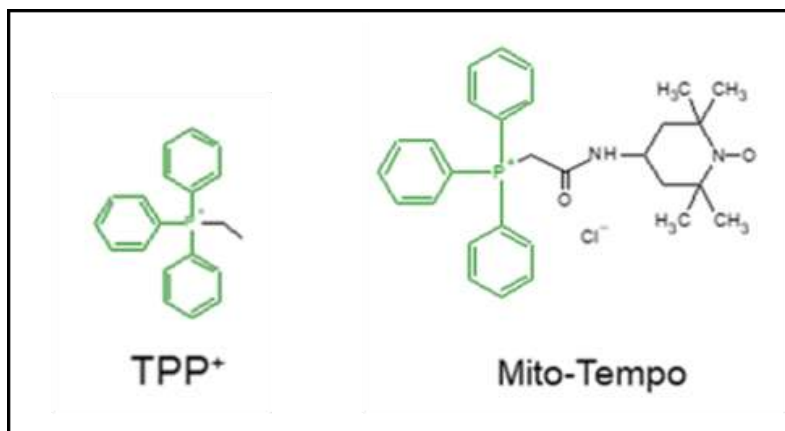


Figure 11. Structure of MitoTEMPO and TPP⁺. TPP⁺; triphenylphosphonium (From Sanchez-Aranguren, 102)

TEMPO is a synthetic molecule from the nitroxide antioxidant family which are stable free radicals with ROS scavenging ability. Previously, nitroxides were considered to be SOD enzyme mimetics as they neutralize ROS and reduce oxidative stress like the endogenous enzyme does. Due to controversy, it is more acceptable to describe TEMPO and its derivatives as ROS scavengers in recent research rather than SOD mimetics. This description fits nitroxides better as they do not have catalytic activity but they interact directly with ROS and scavenge them to restore the oxidative stress-induced mitochondrial dysfunction (103). As a nitroxide, Tempo reacts with different radicals with its nitroxide group in one of two reactions depending on the target radical species. In the presence of a reducing radical, such as hydroxyl radical ($\bullet\text{OH}$), TEMPO receives one electron with its accompanying proton and this reduction reaction and gets reduced to hydroxylamine. If the target radical is of oxidizing nature like superoxide anion ($\text{O}_2^{\bullet-}$), TEMPO loses an electron and exits the reaction in its oxidized state, oxoammonium (Figure 12). Every molecule of TEMPO can scavenge one free radical at a time and the produced redox forms of TEMPO lack the antioxidant properties. To regain its scavenging activity, TEMPO is recycled and reduced back to its nitroxide radical state by the action of endogenous agents like nicotinamide adenine dinucleotide phosphate (NADPH) or glutathione (104, 105).

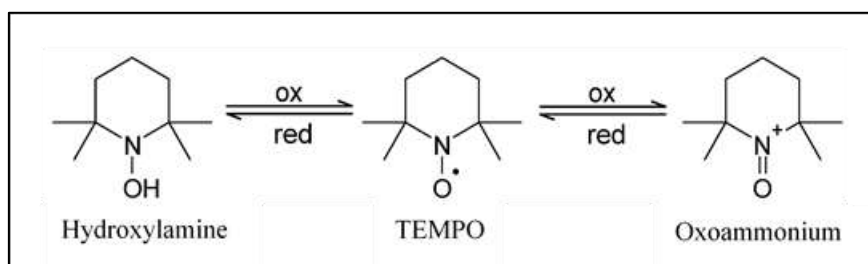


Figure 12. The three radical forms of TEMPO molecule (From Gwozdinski, 105)

The antioxidant activity of TEMPO has been studied in multiple disease models. The ROS scavenging ability of TEMPO showed positive results in vascular dysfunctions as it reduced ischemia-reperfusion and angiotensin-induced endothelial injury by inhibiting lipid peroxidation, TEMPO protected cells from radiation-induced injury and apoptosis and this cytoprotective effect was in a dose-dependent manner. In the urinary system, TEMPO reduced the expression of cyclooxygenase-2 that was increased after osmotic pressure thus providing protection for the cells in the collecting duct (106). When compared to other antioxidants, TEMPO was superior in reducing DNA damage resulting from increased oxidation and nitration of proteins by hydrogen peroxide and nitric oxide (NO) donors which further confirmed its cytoprotective effect (105). In spite of its proven antioxidant effect, TEMPO in its original structure is not well-distributed nor taken up in sufficient amounts by organnells like mitochondria. This drawback limited the use of TEMPO despite its wide-ranging efficacy in oxidative stress-induced conditions. To maximally benefit from its effects, structural modification of TEMPO molecule is one of the suggested stratetgies to deliver the potent antioxidant selectively to the mitochondrial matrix, where its radical scavenging action is needed the most (107).

Since its development by Skulachev and his group, the lipophilic triphenylphosphonium cation (TPP) has been among the most used tools in the investigation of mitochondrial physiology and delivering several molecules to its matrix (108). When coupled to potent antioxidants, TPP results in several-hundred fold increase the mitochondrial accumulation of these agents. MitoQ (TPP + ubiquinone), mitoVitE (TPP+ α -tocopherol), and mitoTEMPO (TPP + TEMPO) are examples of the frequently used antioxidant conjugates in current research (107).

TEMPO derived mitochondrial targeting derivatives showed extensive efficacy in different settings like pulmonary, inflammatory, and neurodegenerative oxidative stress-related conditions. Particularly, mito-TEMPO has shown distinct effectiveness in models of vascular and neuronal dysfunctions among all other TEMPO radical derivatives (106). Preclinically, mitoTEMPO was first studied in hypertension and vascular diseases animal models. Most of the studies used angiotensin 2-induced hypertension as it is related with increased ROS levels in addition to vascular and structural changes. Early results showed that mitoTEMPO decreased the activation of vascular NADPH oxidases, inhibited superoxide production, and restored NO levels thus improved endothelium-dependent vasodilatation and improved blood pressure values (109). ROS were further linked to vascular stiffness due to altered elastin structure in the same hypertension model. MitoTEMPO administration was effective in reducing ROS levels and improved the hallmarks of hypertension as it improved elastin structural changes and vessel stiffness in both mice and rats. The study also stated that the antioxidant action of mitoTEMPO was effective in decreasing oxidative stress by reducing all different sources of ROS. This was concluded depending on the observation that mitoTEMPO restored all vascular changes that are the result of multiple cross-talking ROS sources working in the oxidative stress vicious cycle (110). These findings broadened the area of research to investigate mitoTEMPO in other disease models.

To evaluate its effectiveness in neurodegenerative diseases, mitoTEMPO was tested mainly in Parkinson's and Alzheimer's disease models. H_2O_2 production and the subsequent apoptosis that underlie Parkinson's pathology were remarkably suppressed after treating rat mitochondrial toxin sensitive cell line with mitoTEMPO. After the induction of Alzheimer's disease in mice neuronal cells, tau oligomers aggregation and ROS-induced lipid peroxidation were observed which are typical in the disease settings. MitoTEMPO was able to reduce these changes to levels comparable to control and prevented DNA damage and SOD inhibition (111). The interest in mitoTEMPO application in neuronal cells health grew even more to include other aspect of cellular and subcellular level. Tuncer and colleagues were interested in evaluating mitoTEMPO effect on the alterations of neuronal conduction after ischemia reperfusion-induced sciatic nerve injury (112). The disrupted conduction velocity is thought to be a result of ROS-induced apoptosis of Schwann cells and demyelination.

By reducing ROS, mitoTEMPO preserved the structure and function of nerve fibers and restored the normal conduction velocity. Accordingly, this work introduced mitoTEMPO as a potential agent for the prevention of nerve damage-induced secondary outcomes. Functional evaluation of isolated nerve performed in this work provided novel methodology that was not fully examined in previous literature. Adding functional aspect to the biochemical, molecular, and histopathological analysis in research provided more robust evidence that further supports the the experimental results.

Later on, research started evaluating mitoTEMPO efficacy using more comprehensive methodology that included functional evaluation of the studied target of interest. MitoTEMPO antioxidant effect on the function of corpus cavernosum and vascular smooth muscle was evaluated fir the first time by Yıldız and colleagues. In their work, mitotempo demonstrated protective effect from ROS-induced endothelial dysfunction using rats with hyperglycemia-induced diabetes (113). The functional analysis revealed better relaxation response of the aorta and corpus cavernosaum in diabetic animals after 4 weeks treatment with mitoTEMPO that was comparable to the relaxation of control ones. These findings brought the attention toward mitoTEMPO effect on the function of urogenital organs affected by oxidative stress. MitoTEMPO showed promising results in different models of renal diseases. MitoTEMPO decreased mitochondrial ROS overproduction by modulating the expression of mitophagy inducing proteins. As a result, mitoTEMPO attenuated the deterioration of kidney function in models of acute kidney injury, renal fibrosis, nephrotic syndrome, and podocytes apoptosis in chronic kidney disease (114).

3. MATERIALS and METHODS

3.1. MATERIALS

3.1.1. Chemicals

Acrylamide Bis solution 29:1 40%	BioRad, 1610146
Ammonium persulfate (APS)	Sigma Aldrich, A3678
Ammonia water	Norateks, 100542
Calcium chloride (CaCl ₂)	Tekkim, TK.800202.01002
Carbachol (CCh)	Sigma Aldrich, 4342
Citric acid monohydrate	Sigma Aldrich, 27109
Cyclopiazonic acid (CPA)	Cayman, 11326
Dithiothreitol (DTT)	Sigma Aldrich, 43815
Enrofloxacin	Elanco, Baytril-K®
Entellan	Sigma Aldrich, 107960
Ethyl alcohol (Ethanol)	Merck, 100983
Ethylene glycol tetraacetic acid (EGTA)	Sigma Aldrich, 324628
Eosin	Isolab, 919.003.0050
Formaldehyde	Tekkim, TK.060160.02501
Glucose monohydrate	Isolab, 927.013.1000
Glycerol	Sigma Aldrich, 15524
Glycine	Sigma Aldrich, G8898
Hematoxylin	Moslab, 517-28-2
Hydrochloric acid (HCl)	Tekkim, TK.08023001001
Ketamine hydrochloride	VEM İlaç San ve Tic.A.S., Ketax®
Magnesium sulphate heptahydrate (MgSO ₄ .7H ₂ O)	Tekkim, TK.120310.0100
MitoTEMPO	MedChem Express, 112879
Methyl alcohol (Methanol)	Sigma Aldrich, 1.06007.2500
Nonfat dry milk powder	Aksüt Gıda
Nonidet P 40 (NP-40)	Sigma Aldrich, N-0896
Normal saline (NS)	Polifarma, 200/44
Phenylephrine (Phe)	Sigma Aldrich, P6126

Phenyl methyl sulfonyl fluoride (PMSF)	Sigma Aldrich, 78830
Phosphate buffered saline (PBS)	Oxoid, BR00
Ponceau S staining powder	Sigma Aldrich, 78376
Potassium chloride (KCl)	Isolab, 960.036.1000
Potassium dihydrogen phosphate (KH ₂ PO ₄)	Tekkim, TK.200990
Protease inhibitor cocktail	Cell Signaling, 5871S
Sodium bicarbonate (NaHCO ₃)	Isolab, 969.103.1000
Sodium chloride (NaCl)	Sigma Aldrich, S7920
Sodium citrate (Na ₃ C ₆ H ₅ O ₇)	Santa Cruz , sc203383A
Sodium deoxycholate	Santa Cruz Biotechnology, sc-253579A
Sodium dodecyl sulfate (SDS)	Sigma Aldrich, L3771
Sodium EDTA (Na ₂ EDTA)	AFG Bioscience, 399168
Sodium fluoride (NaF)	Sigma Aldrich, 201154
Sodium hydroxide (NaOH)	Tekkim, TK.1318-79-2
Sodium pyrophosphate (NaPPi)	Sigma Aldrich, 8010
Tetramethylethylenediamine (TEMED)	Applichem, CA1148
Tramadol hydrochloride	Haver Farma İlaç A.Ş., Tramosel®
Tris-Base	Merck, 6791
Tris-hydrochloride (Tris-HCl)	Sigma Aldrich, 3734
Tween 20	AFG Bioscience, 224594
Xylazine	Xylazine® Alvira Animal Health, India
Xylene	Moslab, BKKS00002

3.1.2. Antibodies

Anti- β -Actin mouse monoclonal antibody	Santa Cruz Biotechnology, sc-47778
Anti-DRP1 mouse monoclonal antibody	Santa Cruz Biotechnology, sc-271583
Anti-Mfn2 mouse monoclonal antibody	Santa Cruz Biotechnology, sc-100560
IRDye® 800CW Goat anti-Mouse IgG secondary antibody	LI-COR Biotech, 925-32210
Protein™ Dual Color Standards	BioRad, 1610374
Anti-TOM20 rabbit recombinant monoclonal antibody	Thermo Fisher Scientific, MA5-34964

3.1.3. Kits

Anti-Polyvalent Horseradish Peroxidase (HRP) Diaminobenzidine (DAB) System kit	PatoLab, PL-015-HD, Türkiye
Bicinchoninic Acid (BCA) Protein Assay kit	Thermo Fisher Scientific, 23227, USA

3.1.4. Softwares

Graphpad prism 8	GraphPad, USA
Image Studio™ Software	Li-Cor Bio, USA
Labchart data acquisition software	AD Instruments, New Zealand
ToupView software	Hangzhou ToupTek Photonics, China

3.1.5. Devices and Equipment

Analytical lab balance	ENTRIS 2241-1S, Sartorius, Germany
Autoclave	Hiramaya HV-110, Artisan Technology, Japan
Automatic staining device	Leica Autostainer XL, Germany
Automatic pipettes	Pipetman, Gilson, France
Blot scanner and imaging system	LiCor Odyssey CLx Imager®, Li-Cor Bio, USA
Cellulose acetate (CA) syringe filters	CLS431229, Merck, Germany

Chemicals fume hood	MCO-150, Mikrotest, Türkiye
Cooling centrifuge	Z32HK, Hermle, USA
Data acquisition hardware	Powerlab, AD Instruments, New Zealand
Deep freezer (-20 °C)	Uğur UED, Türkiye
Deionized water device	Sartorius, Germany
Electrophoresis power supply	BioRad, USA
Glass organ bath setup, 20 mL	May, IOBS 99, Türkiye
Gel casting setup	BioRad, USA
Hydrophobic pap pen	PatoLab, Türkiye
Ice machine	Hoshaizaki, Japan
Individually Ventilated Cage (IVC)	Easy Flow, Tecniplast, Italy
Isometric force transducer	MLT0402, AD Instruments, New Zealand
Light microscope	ZEISS Primostar 1, Germany
Liquid nitrogen tank	Thermo Fisher Scientific, USA
Magnetic hotplate stirrer	Dlab Scientific Co.,Ltd,China
Microplate reader	BMG LABTECH, Germany
Microtome	Leica RM 2255, Germany
Mini rocker-shaker	Biosan, Latvia
Oven	Nükleon, Türkiye
pH meter	Mettler Toledo, USA
Pulse stimulator	A310 Accupulser™ Pulse Generator World Precision Instruments, USA
Rat metabolic cage	May, MC01, Türkiye
Refrigerator (+4 °C)	Haier Biomedical, China
Surgical clip	Fine Science Tools, USA
Surgical sutures USP 3/0 S4185	Doğsan Tibbi Malzeme Sanayi A.Ş Türkiye
Ultra deep freezer (-80 °C)	Thermo Scientific, USA
Ultrasonic homogeniser	Bandelin, Germany
Vortex	Heidolph™, Germany
Water heating bath circulator	Julabo/CORIO CD 104101994, Germany

3.1.6. Solutions and Buffers

Unless mentioned otherwise, dH₂O and ddH₂O used in solutions and buffers preparation were autoclaved for sterilization.

Ammonium Persulfate (APS) (10%, 1 mL)

To prepare the working solution, 100 mg APS (MW: 228.18 g/mol) was dissolved in 1 mL ddH₂O. The prepared solution was stored at +4°C for maximum of two weeks.

Calcium Free Krebs-Henseleit Buffer with EGTA (1X, 1 L)

To prepare the working calcium free Krebs-Henseleit physiological solution, the following chemicals were weighed (in grams) and added to 600 mL nonsterile dH₂O to achieve the required molarity (mM) for each: NaCl, 6.9 g (118 mM); KCl, 0.35 g (4.7 mM); KH₂PO₄, 0.16 g (1.2 mM); MgSO₄·7H₂O, 0.14 g (1.6 mM); NaHCO₃, 2.1 g (25 mM); Glucose. H₂O, 1 g (5.5 mM); and EGTA, 3.8 g (mM). During addition, the solution was mixed by placing it over a magnetic stirrer. The final volume was adjusted to 1 L and the solution was oxygenated with carbogen (95% O₂ + 5% CO₂) for 15 minutes. The prepared solution was stored at +4°C for maximum of 2 days.

Carbachol (CCh) (10⁻³ M, 1 mL)

To prepare the stock solution, 0.0294 g of carbachol, (MW 147.2 g/mol) was dissolved in 1 mL of nonsterile dH₂O and vortexed until well dissolved. To prepare working solutions as serial dilutions, 100 µL of the prepared stock solution (10⁻³ M) was added to 900 µL nonsterile dH₂O and vortexed until well dissolved. The same procedure was repeated to prepare solutions in concentrations ranging from 10⁻⁴ M to 10⁻⁸ M of carbachol. Stock and working solutions were prepared in eppendorf tubes and stored in -20 °C for the duration of the study.

Citrate Antigen Retrieval Buffer (10 mM citrate, 0.05% Tween 20, pH 6.0)

To prepare the working buffer solution, 1.92 g of anhydrous citric acid was dissolved in 950 mL of nonsterile dH₂O and the pH was adjusted to 6.0 with 1N NaOH. 0.5 mL of Tween 20 was added to the solution, mixed well and the final volume was brought to 1000 mL with dH₂O. The prepared solution was stored at +4°C for maximum of 2 days.

Krebs-Henseleit Buffer (Krebs solution) (1X, 1 L)

To prepare the working Krebs-Henseleit physiological solution, the following chemicals were weighed (in grams) and added to 600 mL nonsterile dH₂O to achieve the required molarity (mM) for each: NaCl, 6.9 g (118 mM); KCl, 0.35g (4.7 mM); KH₂PO₄, 0.16 g (1.2mM), MgSO₄·7H₂O, 0.14 g (1.6 mM); NaHCO₃, 2.1 g (25 mM), and Glucose. H₂O, 1 g (5.5 mM). During addition, the solution was mixed by placing it over a magnetic stirrer. In a small beaker, 0.56 g of CaCl₂·2H₂O (2.5 mM) was added to 20 mL of nonsterile dH₂O. The solution was then oxygenated with carbogen (95% O₂ + 5% CO₂) for 15 minutes. CaCl₂·2H₂O was added during oxygenation to prevent CaCO₃ formation and the final volume was adjusted up to 1 L. The prepared solution was stored at +4°C for maximum of 2 days.

Milk Powder Solution (5%, 50 mL; 2.5%, 10 mL)

To prepare the stock solution, 2.5 g of nonfat dry milk powder was dissolved in 50 mL of Tris-buffered saline with Tween 20 (TBST) solution and vortexed until dissolved. To prepare 10 mL working solution with concentration of 2.5%, 5 mL of stock solution was added to 5 mL of TBST solution and vortexed until dissolved. The prepared stock and working solutions were stored at +4°C for maximum of 2 days.

MitoTEMPO (1 mg/mL, 1 mL)

To prepare the working solution of mitoTEMPO, 1 mg of mitoTEMPO was dissolved in 1 mL of normal saline (NS) and vortexed until dissolved. The prepared solution was stored at +4°C for maximum of 2 days.

Phenylephrine (Phe) (10⁻³ M, 1 mL)

To prepare the stock solution, 0.0333 g of Phe (MW 167.2 g/mol) was dissolved in 1 mL of nonsterile dH₂O and vortexed until well dissolved. To prepare working solutions as serial dilutions, 100 µL of the prepared stock solution (10⁻³ M) was added to 900 µL nonsterile dH₂O and vortexed until well dissolved. The same procedure was repeated to prepare solutions in concentrations ranging from 10⁻⁴ M to 10⁻⁸ M of Phe. Stock and working solutions were prepared in eppendorf tubes and stored in -20 °C for maximum of 2 days.

Phenyl Methyl Sulphonyl Fluoride (PMSF) (100 mM, 100X, 10 mL)

To prepare the stock solution, 0.174 g of PMSF (MW: 174.19 g/mol) was dissolved in 10 mL of ethyl alcohol. The solution was divided into eppendorf tubes and stored at -20 °C for the duration of the study. The working concentration of the solution used during the study was 1 mM.

Potassium Chloride (KCl) (80 mM, 10 mL)

To prepare the working solution, 2.384 g of KCl was added to 10 mL of nonsterile ddH₂O and vortexed until well dissolved. The prepared solution was stored at room temperature for the duration of the study.

Ponceau S Dye (100 mL)

To prepare Ponceau S dye, 0.1 g of Ponceau S was added to 90 mL of ddH₂O. 5 mL of acetic acid was then added and the final volume was adjusted to 100 mL with ddH₂O. The prepared dye was stored at room temperature for the duration of the study.

RIPA Cell Lysis Buffer (1X, 1 mL)

To prepare the working RIPA cell lysis buffer solution, the following solutions were added to 525 µL of ddH₂O: 50 µL of 50 mM Tris-HCl (pH 8), 30 µL of 150 mM NaCl, 50 µL of 1%, NP-40, 2 µL of 1 mM Na₂EDTA, 50 µL of 0.5% sodium deoxycholate, 10 µL of 0.1% SDS, 10 µL of EDTA-free-1X protease inhibitor cocktail, 100 µL of 5 mM NaPPi, 40 µL of 20 mM NaF, 10 µL of 2 mM NaVan, 10 µL of 1 mM PMSF, and 113 µL of 10% glycerol. The solution was freshly prepared prior to use; any remaining portion was stored at -20 °C until required for a subsequent step in the experiment.

SDS-PAGE Loading Buffer (5X, 20 mL)

To prepare the working solution, 11.62 mL of glycerol (86%), 2 mL of ddH₂O, 2 g of SDS and 0.002 g of bromophenol blue were added to 5 mL of 1 M Tris-HCl (pH 6.8). The solution was slowly stirred at room temperature for 24 hours on a magnetic stirrer to completely dissolve the SDS. The final volume was adjusted to 20 mL with ddH₂O. Solution was divided into aliquots of 1 mL each and stored at -20 °C. Upon use, the aliquot was dissolved by heating

at 60 °C for 10 minutes and 0.077 g of dithiothreitol (DTT) was added to 1 mL of buffer to make the final concentration of 0.5 M. The 1 mL aliquots to which DTT was added were also stored at -20 °C for the duration of the study.

SDS-PAGE Running Buffer (1X, 1L)

To prepare the working solution, 100 mL of 10X Tris-Glycine buffer and 10 mL of 10% SDS solution (final concentration 0.01%) were added to 800 mL of ddH₂O. The final volume was adjusted to 1 L with ddH₂O. Running buffer was freshly prepared prior to use.

SDS-PAGE Transfer Buffer (1X, 1L)

To prepare the working solution, 100 mL of 10X Tris-Glycine buffer and 100 mL of methyl alcohol were added to 800 mL of ddH₂O, respectively. Transfer buffer was freshly prepared and kept at +4°C until use in transfer step of western blot.

Sodium Chloride (NaCl) (5 M, 100 mL)

To prepare the working solution, 29.22 g of NaCl (MW: 58.44 g/mol) was dissolved in 80 mL of ddH₂O. The final volume was adjusted to 100 mL with ddH₂O and stored at room temperature for the duration of the study.

Sodium Dodecyl Sulfate (SDS) (10%, 500 mL)

To prepare the working solution, 50 g SDS (MW: 288.37 g/mol) was dissolved in 400 mL sterile ddH₂O. The final volume was adjusted to 500 mL with ddH₂O and stored at room temperature for the duration of the study.

Sodium EDTA (Na₂EDTA) (0.5 M, pH 8.0, 100 mL)

To prepare the stock solution, 18.612 g Na₂EDTA (MW: 372 g/mol) was added to 60 mL ddH₂O. Solution's pH was adjusted to 8.0 with 10 M NaOH. The final volume was adjusted to 100 mL with ddH₂O and stored at room temperature for the duration of the study. The working concentration of the solution used during the study was 1 mM.

Sodium Fluoride (NaF) (0.5 M, 25X, 100 mL)

To prepare the stock solution, 2.1 g of NaF (MW: 41.99 g/mol) was dissolved in 90 mL of ddH₂O. The final volume was adjusted to 100 mL. The solution was divided into eppendorf

tubes and stored at -20 °C for the duration of the study. The concentration of the working solution used during the study was 20 mM.

Sodium Orthovanadate (NaVan) (200 mM, 100X, 100 mL)

To prepare the stock solution, 3.68 g of NaVan (MW: 183.91 g/mol) was added to 80 mL of ddH₂O, and pH was adjusted to 10.0 with 1 N HCl. The solution, which turned yellow with the use of HCl, was heated at high temperature until it became colorless, approximately for 10 minutes. The solution was left to stand to cool at room temperature. The pH was checked again and adjusted to 10.0 and the final volume was adjusted to 100 mL with ddH₂O. The solution was divided into eppendorf tubes and stored at -20 °C for the duration of the study. The concentration of the working solution used during the study was 2 mM.

Sodium PyroPhosphate (NaPPi) (50 mM, 10X, 100 mL)

To prepare the stock solution, 2.22 g of NaPPi (MW: 444.06 g/mol) was dissolved in ddH₂O. The final volume was adjusted to 100 mL and divided into eppendorf tubes and stored at -20 °C for the duration of the study. The final concentration of the working solution used during the study was 5 mM.

TBS Stock (10X, 1 L, pH 7.6)

To prepare the stock solution, 24 g Tris-HCl (MW: 157.60 g/mol), 5.6 g Tris base (MW: 121.14 g/mol) and 88 g NaCl (MW: 58.44 g/mol) were combined and the final volume adjusted to 800 mL with ddH₂O. The chemicals were dissolved by stirring with a magnetic stirrer, pH was adjusted to 7.6 with 12 N HCl, the final volume was adjusted to 1 L with ddH₂O, and stored at room temperature for the duration of the study.

TBS-Tween 20 (TBST) (1X, 500 mL)

To prepare the working solution of 1X TBS, 50 mL of 10X TBS solution was added to 445 mL ddH₂O. 1250 µL of 20% Tween 20 solution was added to the prepared 1X TBS to make the final concentration 0.05%. The final volume was adjusted to 500 mL with ddH₂O and stored at room temperature for the duration of the study.

Tris Glycine Buffer (10X, 1L)

To prepare the stock solution, 30.3 g Tris base (MW: 121.14 g/mol) and 144 g Glycine (MW: 75.07 g/mol) were dissolved in 800 mL ddH₂O. The final volume was adjusted to 1 L with ddH₂O and stored at room temperature for the duration of the study.

Tris-HCl (1 M, pH 8.0, 100 mL)

To prepare the working solution, 12.114 g of Tris base (MW: 121.14 g/mol) was dissolved in 60 mL of ddH₂O. Solution's pH was adjusted to pH 8.0 and final volume was adjusted to 100 mL with ddH₂O. Solution was filtered through a cellulose acetate (CA 0.2 µm) filter and stored at room temperature for the duration of the study.

Tris-HCl Loading Gel Buffer (0.5 M, pH 6.8, 100 mL)

To prepare the working solution, 6.057 g of Tris base (MW: 121.14 g/mol) was dissolved in 80 mL of ddH₂O. Solution's pH was adjusted to 6.8 and final volume was adjusted to 100 mL with ddH₂O. Solution was filtered through a CA 0.2 µm filter and stored at room temperature for the duration of the study.

Tris-HCl Separation Gel Buffer (1.5 M, pH 8.8, 200 mL)

To prepare the working solution, 36.33 g Tris base (MW: 121.14 g/mol) was dissolved in 150 mL ddH₂O. Solution's pH was adjusted to 8.8 and final volume was adjusted to 200 mL with ddH₂O. Solution was filtered through a CA 0.2 µm filter and stored at room temperature for the duration of the study.

TWEEN 20 (20%, 100 mL)

To prepare the stock solution, 20 mL of TWEEN 20 was added to 80 mL of ddH₂O. Solution was homogenized and stored at +4 °C for the duration of the study.

3.2. Methods

3.2.1. Experimental Animals

In the study, 2-3 months old and 225 – 300 g Sprague Dawley female rats obtained from Karadeniz Technical University (KTU) Surgery Practice and Research Center were used in this study. The rats were housed at KTU İLAFAR in temperature and humidity controlled environment using Individually Ventilated Cage (IVC) system (Easy Flow, Tecniplast, Germany) with a 12-hour light/12-hour dark cycle. During the experiment, the rats were given *ad libitum* tap water and standard feed (Figure 13). The experiments were initiated with the ethics committee approval from the KTU Local Ethics Committee for Animal Experiments (File No: 2022/51, Appendix-1).



Figure 13. IVC housing system of the experimental animals

3.2.2. Experimental Design

According to the treatment groups specified below (Table 2), mitoTEMPO (MT; 1 mg/kg/day) or vehicle (normal saline, NS) (1 mL/kg) was administered intraperitoneally (i.p.) to experimental rats. Administration was performed once a day (at the same time each day) for 3 and 14 days, starting on the day of surgery (day 0) (Figure 14).

Table 2. Experimental groups

Group name	Treatment and duration	Number of animals
Sham	Sham + NS (1 mL/kg/day, i.p., 3 days)	10
	Sham + NS (1 mL/kg/day, i.p., 14 days)	10
SCI	SCI + NS (1 mL/kg/day, i.p., 3 days)	10
	SCI + NS (1 mL/kg/day, i.p., 14 days)	10
SCI + MT	SCI + mitoTEMPO (1 mg/kg/day, i.p., 3 days)	8
	SCI + mitoTEMPO (1 mg/kg/day, i.p., 14 days)	8
Total number of animals		56

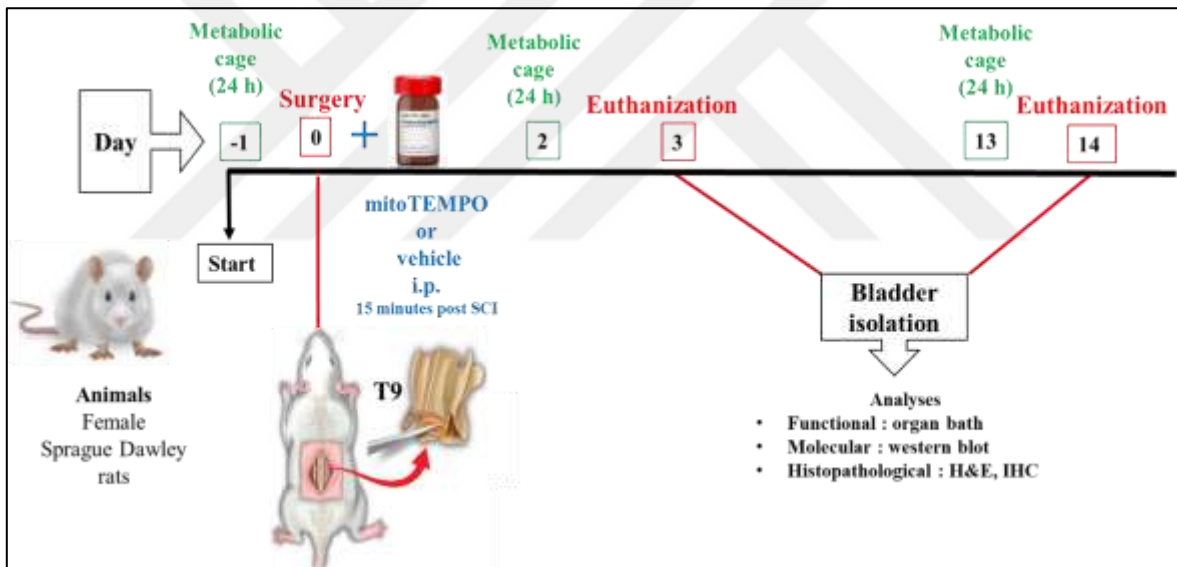


Figure 14. Study design and timeline

3.2.3. Surgical Induction of SCI

In our study, SCI was induced by a suprasacral compression model that was validated in literature (68, 126). The compression was applied on the thoracic vertebra at T9 level to induce bladder dysfunction (127). Before surgical procedures, enrofloxacin (10 mg/kg) was administered subcutaneously (s.c) once a day (starting 3 days before surgery) as prophylaxis to reduce the risk of infection. On the day of the surgical procedure (day 0), rats were anesthetized with ketamine (90 mg/kg i.p)/xylazine (10 mg/kg i.p).

After anesthesia, rats were fixed on the dissection table in prone position and their lower thoracic regions were shaved (Figure 15a). Using surgical instruments, a 5-6 cm incision was performed to identify the thoracic vertebra at T9 level (T9) (Figure 15b). In SCI groups, T9 vertebra was removed (laminectomy) to expose the spinal cord for injury (Figure 15c). Compression was applied to the spinal cord at T9 level for 60 seconds using a surgical clip (Fine Science Tools, USA) (Figure 15d). In the control groups, to perform sham surgery incision site was closed with surgical suture without laminectomy. Successful establishment of SCI was confirmed by the following observations:

- a) Hind limbs and tail spasm and/or twitching during spinal cord compression with the clip
- b) Appearance of tightened spinal cord after removal of the clip (Figure 15e).
- c) The number of the removed vertebra was confirmed by exposing and counting the thoracic ribs after rat euthanization. Correct removal of T9 vertebra was confirmed when the excised vertebra was found to be attached to the ninth rib (Figure 15f).

The muscle layer over the incision was approximated and sutured over injury site without applying pressure or disrupting the spinal cord with surgical sutures in an interrupted manner (Figure 15g). The skin was sutured over incision area with 2/0 sutures (Figure 15h). After surgical procedures, all rats were put in individual cages in recovery room and warmed with blanket, (Figure 15i). After the surgical procedure, animals were injected with 3 mL of s.c 0.9% NS for hydration. An opioid-derived analgesic, tramadol, was administered (10 mg/kg/ day, ip) for 3 days starting from the first day of the procedure (day 0). Due to the continuous risk of infection, enrofloxacin (10 mg/kg, s.c) was administered once a day for 3 days after the surgical procedure (128, 129). The bladders of the rats were manually emptied every day - except days in metabolic cage – until partial spontaneous micturition was observed.

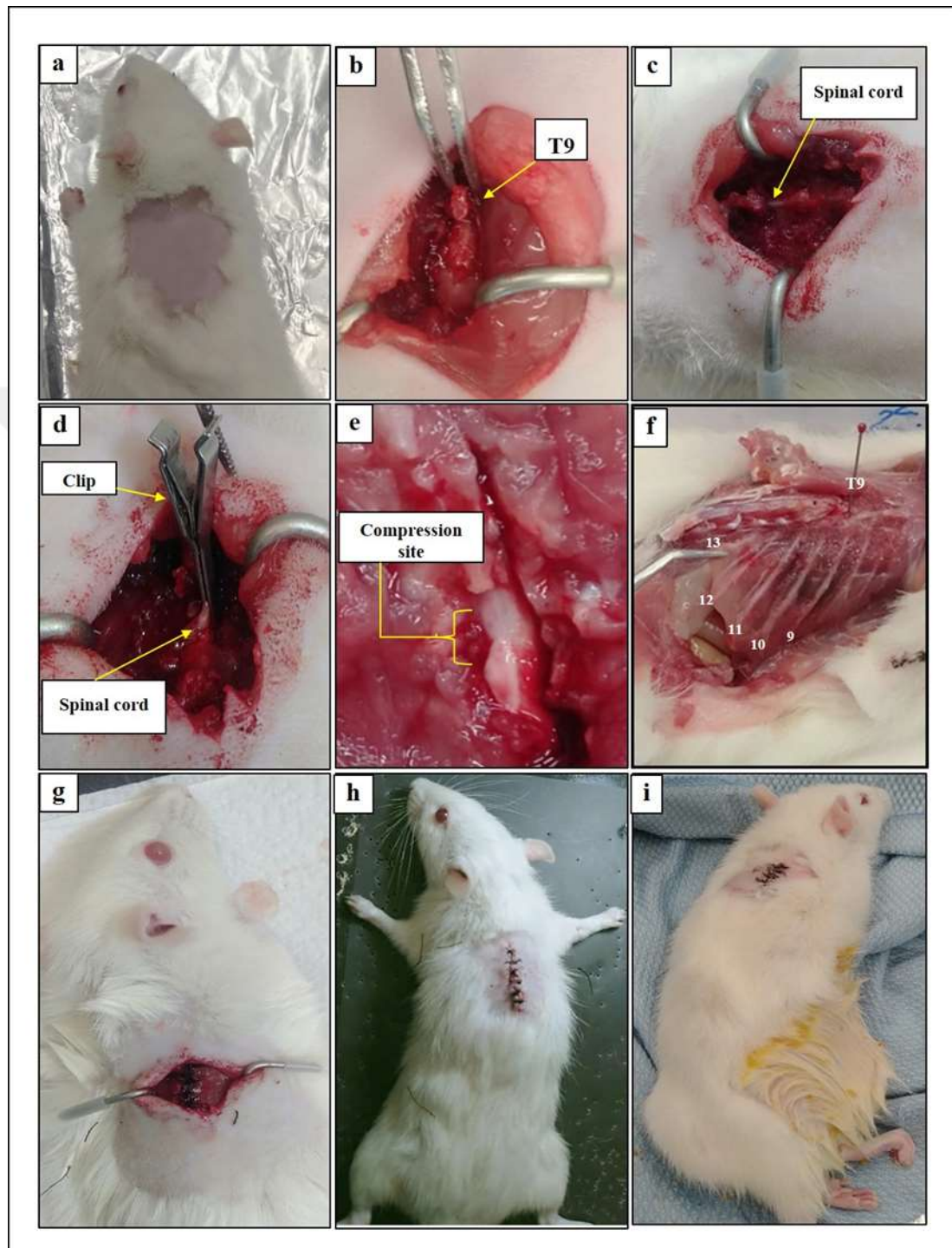


Figure 15. Surgical induction of SCI. **(a)** Anesthetized rat in prone position for surgery. **(b)** Locating T9 level for laminectomy. **(c)** Exposed spinal cord at T9 level. **(d)** Induction of compression with a clip. **(e)** Injured spinal cord. **(f)** Confirmation of injury site. **(g)** Closure of muscle layer with sutures. **(h)** Incision site skin closure with sutures. **(i)** Post injury recovery period.

3.2.4. Water Intake, Urine Outputs, and Fecal Outputs Measurement

Starting one day before surgery (day -1), body weight of each rat was measured and recorded on daily basis until the end of experimental protocol. Rats were placed in single metabolic cages (May, MC01, Türkiye) a day before surgery (day -1) and the last day of drug administration (days 3 and day 14), and 24-hour urine volumes and feces amount in metabolic cages were measured and recorded (Figure 16a and b). Urine was collected in a graduated cylinder to measure the volume (in mL) while feces pellets were collected and weighed on a normal scale (in grams) (Figure 16c). Water was administered in a graduated bottle filled with 120 mL of tap water and the remaining amount was recorded after 24 hours to obtain water intake for each rat (Figure 16d). Placing and removing rats in and out of metabolic cages, surgical procedures, and drug administration were performed between 10.00 am and 12.00 pm by the same investigator to minimize variability and maintain consistency and reproducibility of the experimental outcomes.

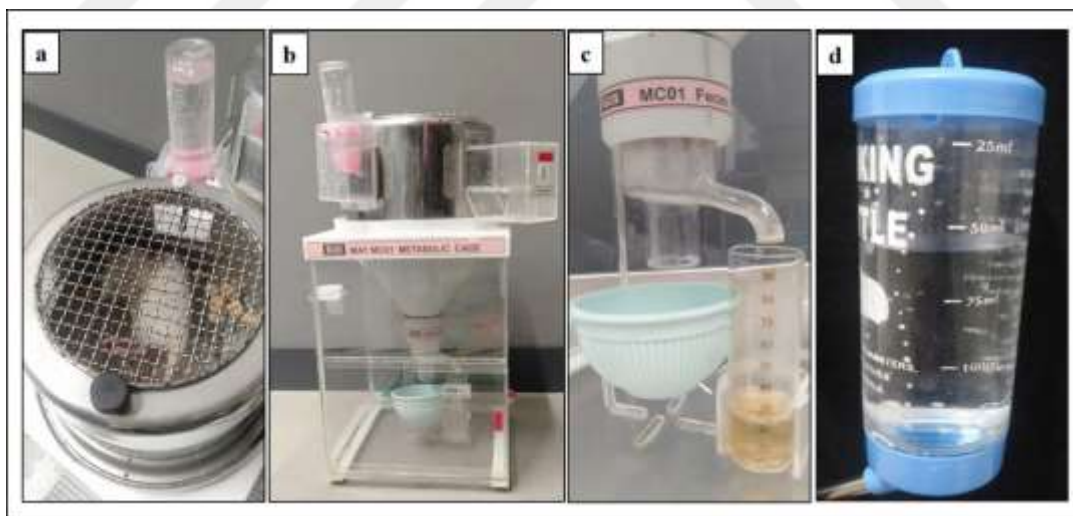


Figure 16. Measurement of metabolic outputs. **(a)** Rat in metabolic cage **(b)** Metabolic cage setup. **(c)** Urine and feces collection units of the metabolic cage. **(d)** Graduated water bottle.

3.2.5. Bladder Collection and Processing

At the end of experimental protocol (day 3 or day 14), rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) i.p. (Figure 17a). After the rat was anesthetized, it was placed in the supine position, a midline incision was made, tissues were retracted and the bladder was exposed (Figure 17b). The bladder was isolated and rinsed with cold Krebs–Henseleit (Krebs solution) solution to remove any residual urine. Surrounding connective tissue was removed and the bladder was placed in a petri dish filled with cold Krebs solution (Figure 17c). Each bladder was processed as described below:

- a) Using fine scissors, the bladder was cut longitudinally along the midline from the apex to the base, flattened, and the dome and trigone were trimmed away (Figure 17d).
- b) The muscle tissue of the bladder (detrusor) was cut into multiple longitudinal strips.
- c) Two strips from each rat were used for functional analysis.
- d) Remaining strips were quickly put in cryotubes and immediately immersed in liquid nitrogen then stored at -80°C for molecular (western blot) analysis.
- e) The whole bladder was isolated from another set of animals and fixed in formaldehyde solution for histopathological analysis.

After the isolation of the tissues, all rats were euthanized by exsanguination (abdominal aorta incision).

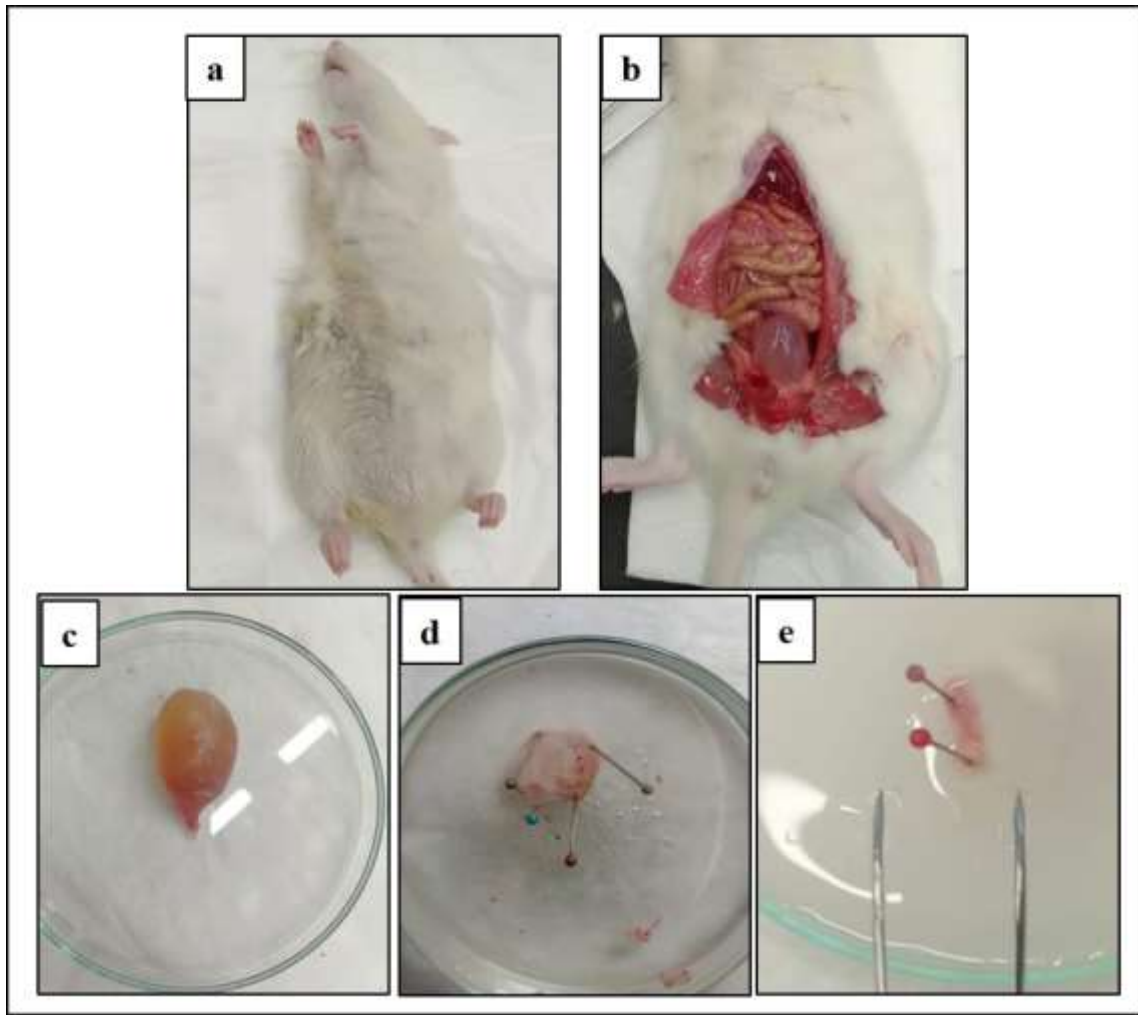


Figure 17. Bladder collection and processing (a) Anesthetized rat in supine position. (b) Bladder exposure in abdominal cavity. (c) Isolated bladder. (d) Flattened and pinned bladder. (e) Bladder detrusor strip.

3.2.6. Functional Analysis

The function of bladder tissue as the contractility response of the detrusor muscle strip to various agents was evaluated using the organ bath system (Figure 18a). The tissue strips were mounted in isolated organ bath (20 mL glass chamber; May, IOBS 99, Türkiye), containing Krebs solution (Figure 18b). The solution in the glass chamber was gassed with carbogen (95% O₂ + 5% CO₂) and kept at 37°C with a water heating circulator system (Julabo/CORIO CD, Germany) (Figure 18c and Figure 18d).

The tissue strip was fixed vertically in the glass chamber with one end fixed to a hook and the opposite end connected to isometric force transducer (MLT0402, AD Instruments, New Zealand) (Figure 18c). The contractile responses of the bladder (detrusor) strips were recorded as isometric tension changes by the force transducer. The mechanical tension was converted into electrical signals that were recorded through data acquisition system (Powerlab, AD Instruments, New Zealand) connected to a software (Labchart data acquisition software, AD Instruments, New Zealand) for subsequent analysis (Figure 18d). Resting tension of 1 g was applied to the suspended strips and the tissues were allowed to equilibrate for 45 minutes. During equilibration period, the bath solution was replaced with fresh Krebs solution every 15 minutes to remove metabolites and maintain optimal physiological conditions for tissue responsiveness.

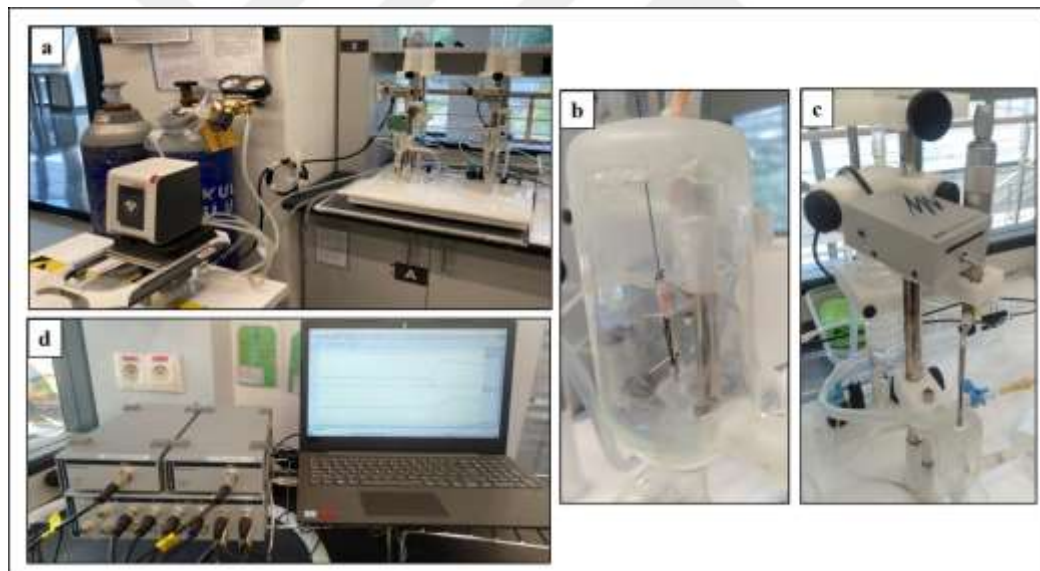


Figure 18. View of the organ bath setup. **(a)** Units for the maintenance of the isolated tissue (carbognrn tanks, glass chambers, heated water bath). **(b)** Placement of bladder strip in the glass chamber. **(c)** Isometric force transducer. **(d)** Units for the data acquisition and recording.

After equilibration, tissues were challenged with 80mM KCl to evoke a depolarization-induced contraction and verify tissue viability (115). After the contraction responses reached a plateau and peak response was recorded, tissues were washed with Krebs solution and allowed to re-equilibrate for 20 minutes with replacing Krebs solution at 5 minutes intervals. Experiments were conducted simultaneously on two bladder (detrusor) strips that were obtained from each rat (Strip I, Strip II). To assess pharmacological responses, each strip received a different treatment protocol as follows:

Strip I: Evaluation of detrusor muscle contractility

Electrical field stimulation (EFS) was applied to induce contraction (Figure 19). Strips (n=4) were placed between two platinum ring electrodes connected to electrical pulse generator (A310 Accupulser™, World Precision Instruments, USA) (Figure 19a and Figure 19b). EFS was applied at 50V, 1 ms duration, increasing frequency (1-64 Hz) with 3 minutes resting intervals. The responses of the detrusor strips were recorded and normalized to the strip weight (Figure 19c). After testing the last frequency, the tissues were left to rest for 45 minutes with replacing Krebs solution at 15 minutes intervals. To evaluate the bladder (detrusor) strips contractile responsiveness to muscarinic receptors stimulation, cumulative concentrations of CCh (muscarinic agonist) ranging from 10^{-9} to 10^{-4} M were added to the organ bath. Contraction responses were recorded and normalized to the strip weight.

Strip II: Evaluation of detrusor muscle relaxation

To evaluate the bladder (detrusor) strips relaxation responsiveness to α 1-adrenergic receptors stimulation, contraction was induced with submaximal concentration of CCh (10^{-5} M) followed by cumulative concentrations of Phe (adrenergic agonist) ranging from 10^{-9} to 10^{-4} M were added to the organ bath. Obtained responses were expressed as percentage of relaxation to contraction induced by submaximal concentration of CCh. After washing, the bath was drained, and a Ca^{2+} -free Krebs-Henseleit solution containing 1 mM EGTA, KCl (80 mM) and CPA (10 μ M) was added to the baths to elucidate the role of the sarcoplasmic reticulum in the process. After a 15 minutes equilibration period, a cumulative response curve was obtained with CaCl_2 (0.01-100 mM) to evaluate the receptor-independent, direct effects of extracellular Ca^{2+} influx on bladder contraction under depolarizing conditions.

The same procedure was used to test the other strip, with the addition of a CPA-free Krebs-Henseleit solution. The response of the drugs was expressed as Emax (maximum response) and pD₂ (negative logarithm of drug concentration at which half of the maximal response is obtained). The values obtained were recorded with the LabChart 8.0.1 software (Labchart data acquisition software, AD Instruments, New Zealand).

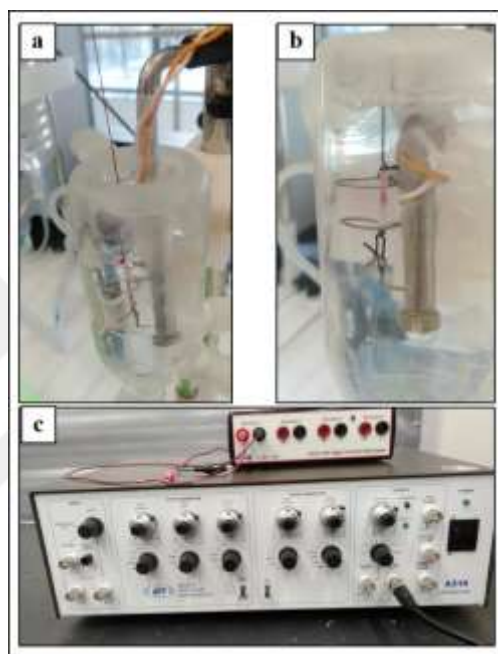


Figure 19. View of the EFS setup. (a) Placement of bladder strip in the glass chamber with the electrode hooks. (b) Close-up view of the EFS electrodes. (c) Electrical pulse stimulator.

3.2.7. Molecular Analysis

Preparation of bladder homogenates

For western blot analysis, bladder tissue was placed in 0.5 mL ice-cold RIPA cell lysis buffer. The tissue was minced into small fragments with surgical scissors followed by homogenization using a mechanical homogenizer (Ultrasonic homogenizer, Bandelin, Germany) at 20,000 rpm for 10 seconds at 5 seconds intervals and incubation on ice for 30 minutes. Homogenates were centrifuged at 10,000 x g for 30 minutes at +4°C. The clear supernatant was collected into new tubes and stored at -80°C until protein quantification and following analysis.

Total protein quantification

The total protein concentration of bladder homogenates was measured using the BSA protein concentration measurement kit as recommended by the company. Based on the stock BSA (2 mg/mL) solution, 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 $\mu\text{g/mL}$ BSA standard solutions were prepared. Solution A and Solution B in the kit were mixed at a ratio of 50:1 to prepare the working solution. 10 μL of the homogenates whose protein concentration was desired to be determined together with the BSA standards were added to the microplate wells in triplicates. 200 μL of working solution was added to the samples.

The microplate was closed, shaken gently and incubated in a 37°C pre-heated oven (NST, Nükleon, Türkiye) for 30 minutes. Meanwhile, the green working solution turns purple over time in the presence of protein (Figure 20). This color change absorbance was measured in a microplate reader (BMG LABTECH, Germany) at a wavelength of 562 nm and the concentration-absorbance graph was drawn using the absorbance of BSA standards and the graphic equation was determined. The absorbance of the samples were placed in the graphic equation and the total protein amount for each sample was calculated in mg/mL.

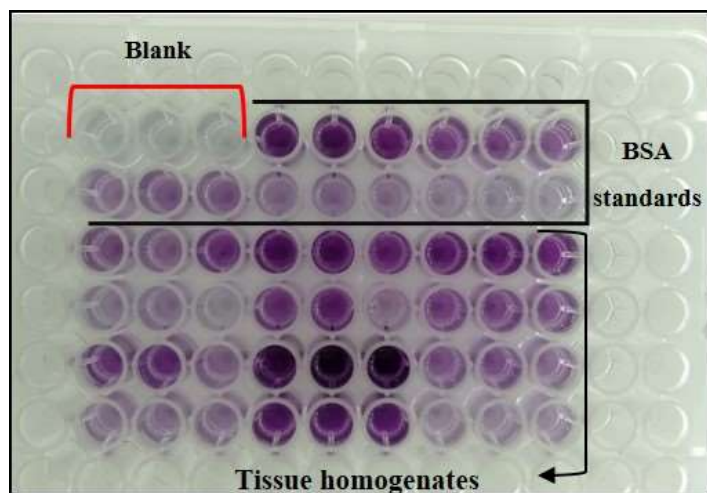


Figure 20. Microplate containing BSA standards and tissue homogenates used for protein quantification

SDS-PAGE gel preparation

Two cassettes containing short and long glasses spaced 1 mm apart were rinsed with ddH₂O and cleaned using 70% ethyl alcohol. Clean cassettes were positioned on the gel casting stand (Bio-Rad, USA), and ddH₂O was introduced just prior to pouring the gel to assess for any leakage from the stand (Figure 21a). The appropriate amount of each component of the gel mixture was chosen by looking at the molecular weight of the protein of interest. 10% SDS-PAGE separation gel was first prepared by mixing the following in order: 2.43 mL ddH₂O, 1.25 mL of acrylamide mixture (40%), 1.25 mL of 1.5 M Tris-HCl (pH 8.8), 50 µL of 10% SDS, 50 µL of freshly prepared 10% APS, and 5 µL of TEMED. The prepared mixture was poured into gel cassettes, overlaid with ddH₂O to create a level interface, and left to polymerize for 45 minutes at room temperature (Figure 21b). After gel polymerization, ddH₂O overlay was discarded and a 5% loading gel mixture was prepared using the following components: 1.57 mL ddH₂O, 0.25 mL of acrylamide mixture (40%), 625 mL of 0.5 M Tris-HCl (pH 6.8), 25 µL of %10 SDS, 25 µL of freshly prepared 10% APS, and 1.5 µL of TEMED. The mixture was carefully poured over the separation gel and a clean comb was inserted into the separation gel mixture between the two glasses to create wells for samples loading. The gel was left to polymerize for 45 minutes at room temperature. After gel polymerization, the gels cassettes were removed from the holder, wrapped with a damp stretch film to prevent air exposure and stored at +4 °C. The prepared SDS-PAGE gels were stored at +4 °C for maximum of 1 week.

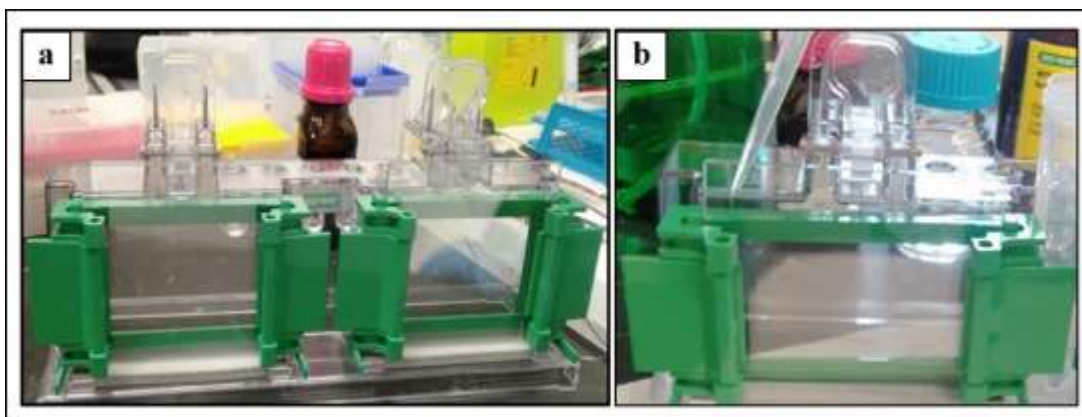


Figure 21. SDS-PAGE gel preparation. (a) Gel casting setup. (b) Gel pouring into cassettes fixed in casting frame.

SDS-PAGE sample preparations

Tissue homogenates were quantified and adjusted to equal concentration of 40 or 60 μg of protein. A volume of tissue homogenates sufficient to attain the desired protein content was transferred to an Eppendorf tube. RIPA lysis buffer and 5X dithiothreitol (DTT) were added to complete the final volume for loading into each well of the gel. Protein denaturation was achieved by vortexing the tubes, incubating them in a water bath at 90°C for 5 minutes, and subsequently centrifuging at 15,000 $\times g$ for 10 seconds. In a separate Eppendorf tube, 2 microlitres of protein standard were added to 23 μL of 1X DDT and vortexed.

SDS-PAGE samples loading and gel electrophoresis

The combs of the prepared gel cassettes were removed, the wells were rinsed with dH_2O , the cassettes were fixed on the gel running module, and positioned the electrophoresis tank. The space between the two cassettes in gel running module was filled with 1X SDS-PAGE running buffer in amount enough to cover the wells. The tank was filled with running buffer to the level required for two gels as indicated on it. The first well of the gel was left empty, 25 μL of protein marker was loaded into the second well, and samples were loaded into the remaining wells in volumes ranging from 25 to 40 μL according to calculations (Figure 22a). For electrophoresis, the tank was closed and the electrodes on the cover were connected to power supply (Figure 22b and 22c). The gel was first run at 75 volts for 15 minutes and then at 100 volts for 90 minutes.

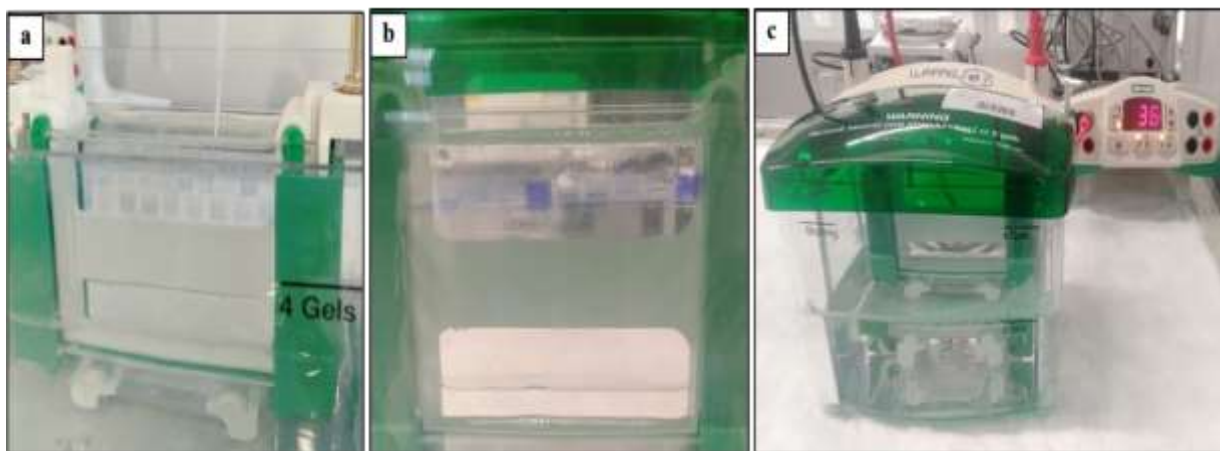


Figure 22. SDS-PAGE samples loading and gel electrophoresis. **(a)** Loading of prepared samples into the gel wells. **(b)** Gel running. **(c)** Gel electrophoresis setup.

Protein wet transfer

Gel running module was removed from the electrophoresis tank after the run and rinsed with dH₂O several times. The small and large glasses were carefully separated from each other with the help of the spatula to keep the gel placed on the small glass. The gel was removed carefully with the spatula (Figure 23a) and equilibrated in cold 1X SDS-PAGE transfer buffer for 5-10 minutes. Depending on the gel size, the pre-cut polyvinylidene difluoride (PVDF) membrane was activated in cold methyl alcohol for 1 minute and then equilibrated in 1X SDS-PAGE transfer buffer for 10 minutes. All layers and membranes were pre-wetted in cold SDS-PAGE transfer buffer and gently rolled with a rod to remove air bubbles. Layers were assembled to form transfer stacks in a blotting cassette (black cathode to transparent anode) in the following order: sponge, 2 Whatman filter papers, gel, PVDF membrane, 2 Whatman filter papers, and sponge (Figure 23b). The cassette was closed (Figure 23c), placed in the gel transfer module, and oriented so that the gel is toward the cathode, marked in black, and the PVDF membrane is toward the anode, marked in red (Figure 23d). The gel transfer module was placed in the tank filled with cold SDS-PAGE transfer buffer. To prevent heat buildup, a stirrer magnet was put in the tank and all apparatus was put in a container full of ice on a magnetic stirrer. The transfer process was carried out at 100 volts for 75 minutes (Figure 23e).

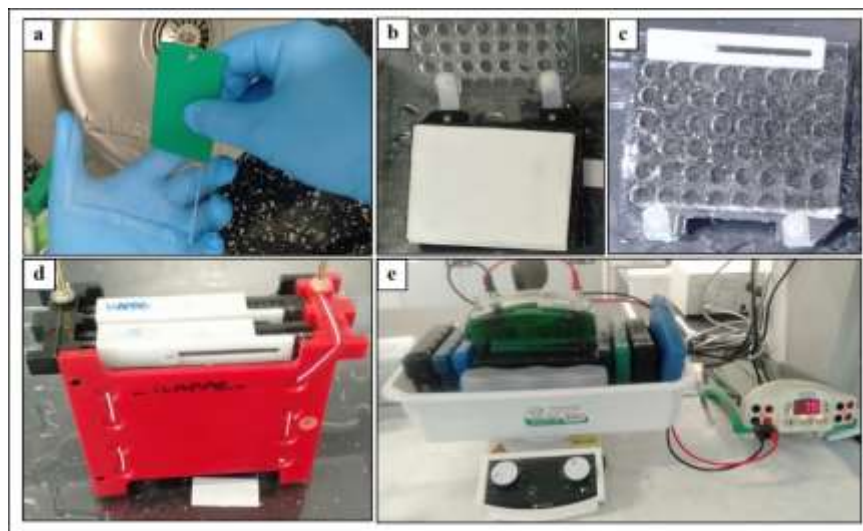


Figure 23. Wet transfer process. **(a)** Gel removal from cassette. **(b)** Layers placed on blotting cassette. **(c)** Closed blotting cassette. **(d)** Gel transfer module with blotting cassettes. **(e)** Transfer process.

After the transfer, the membranes were stained with Ponceau S solution to check the accuracy of the transfer process (Figure 24). After the membranes were washed with distilled water once or twice, Ponceau S dye was added to completely cover the membrane. When protein bands became visible on the membrane, the membranes were washed with deionized water to remove the Ponceau S solution. After the control, the surface of the stained membrane where the samples were located was marked with a pencil and transferred to TBS (1X TBST) buffer containing 0.05% Tween 20 and washed once or twice.

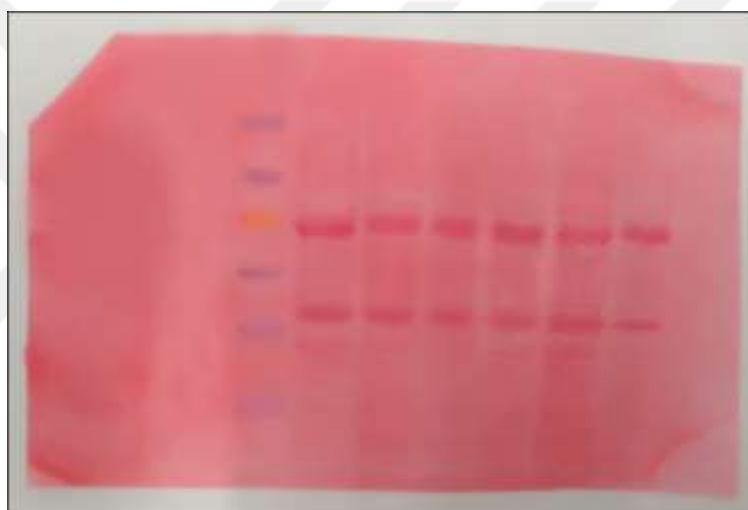


Figure 24. PVDF membrane stained with Ponceau S after transfer process

Immunoblotting and imaging

After washing with TBST, the membranes were subjected to a blocking process for 60 minutes on a shaker, covered in 5% nonfat milk powder prepared in TBS solution containing 0.05% Tween 20 to close nonspecific binding sites. Primary antibodies were prepared at the concentrations recommended in the user manuals (1:200 dilution ratio) in TBS solution containing 0.05% Tween 20. Membranes were incubated with anti-dynamin-related protein 1 (Drp1) or anti-mitofusin 2 (Mfn2) primary antibodies at +4°C at 15 rpm on an orbital shaker overnight (Figure 25a). β -actin was used as a loading control.

After primary antibody incubation (12-16 hours, +4°C), membranes were washed with TBS-T at 30 rpm on an orbital shaker (5 min x5) and incubated with secondary antibody (Goat anti-Mouse IgG, 1:10000 dilution ratio) at room temperature at 15 rpm speed on an orbital shaker for 1 hour. After the completion of secondary antibody incubation, the membranes were washed 5 times for 5 minutes with TBS-T solution. After washing, the membrane was placed in a container with a clean forceps and put in the imaging device. Protein bands on the membranes were made visible with the help of an infrared (IR) imaging device (LICOR Odyssey® CLx Imaging System) (Figure 25b).

The luminescence in the bands was recorded and the relative band intensity was presented as a ratio to the standard protein (β -actin) band intensity on the same membrane using Image studio® 5.5 software (Figure 25c). The average ratio in the sham groups was set to 100% and the data were expressed as % of sham groups.



Figure 25. Immunoblotting and imaging. **(a)** Antibody incubation (primary or secondary). **(b)** LICOR Odyssey® CLx Imaging System. **(c)** Protein bands shown in Image Studio® software.

3.2.8 Histopathological Analyses

Preparation of paraffin blocks from tissues

After euthanization and dissection, bladders were immediately isolated and fixed in 10% formaldehyde solution for 72 hours at room temperature. Bladders were removed from formaldehyde solution, reduced in size and placed in cassettes. After fixation of the tissues in the cassette, the cassette were washed with running tap water to remove fixation residuals and treated with ethyl alcohol series for dehydration (Table 3). After dehydration, samples were cleared in xylene, embedded in molten paraffin wax (Table 3), and allowed to solidify at room temperature to form paraffin blocks (Figure 26a). After sufficient cooling was achieved, the blocks were removed from the base molds and kept at room temperature until sectioning (116).

Table 3. Standard tissue tracking procedure

Application	Duration
Ethyl alcohol (70%)	45 minutes
Ethyl alcohol (80%)	45 minutes
Ethyl alcohol (90%)	45 minutes
Ethyl alcohol (96%)	45 minutes
Ethyl alcohol (96%)	45 minutes
Ethyl alcohol (96%)	45 minutes
Xylene	15 minutes
Xylene	15 minutes
Xylene-Paraffin (1:1)	1 hour
Paraffin	15 minute
Paraffin	2.5 hours

Obtaining tissue sections

Using a microtome device (Leica RM 2255, Germany), serial sections of 5 μm thickness were obtained from tissue paraffin blocks. To flatten the ribbons of paraffin sections, they were floated on the surface of a water bath (bain-marie) at 37°C. Subsequently, 5 μm sections were taken from the blocks, mounted onto charged slides, and dried at 37°C overnight. Slides were stored at room temperature until histological and immunohistochemical processing.

Hematoxylin & Eosin staining

A set of prepared charged slides with paraffin sections were placed on staining racks and incubated at 60 °C for 1 hour to melt excess paraffin and ensure adhesion. For staining, section were treated with Hematoxylin & Eosin staining protocol listed below (Table 4) in an automatic staining device (Leica Autostainer XL, Germany) (117).

Sections were deparaffinized with xylene, rehydrated through a decreasing ethanol series, and rinsed with dH₂O. Hematoxylin dye was applied followed by acid alcohol for differentiation and background reduction. Bluing of the sections was performed by using ammonia water as an alkaline solution. Subsequently, slides were counterstained with eosin, dehydrated through ascending grades of ethyl alcohol, and cleared in xylene. Finally, slides were mounted with drops of entellan and covered with coverslips. Stained slides were kept at room temperature until examination with microscope.

Table 4. Hematoxylin & Eosin staining procedure

Application	Duration
Xylene	5 minutes
Xylene	5 minutes
Xylene	5 minutes
Ethyl alcohol (100%)	5 minutes
Ethyl alcohol (96%)	5 minutes
Ethyl alcohol (70%)	5 minutes
Distilled water (Washing)	1 minute
Hematoxylin	2 minutes
Distilled water (Washing)	1 minute
Acid alcohol (1%)	1 second
Distilled water (Washing)	1 minute
Ammonia water (0.2 %)	10 seconds
Distilled water (Washing)	1 minute
Eosin	1.5 minute
Distilled water (Washing)	1 minute
Ethyl alcohol (96%)	5 seconds
Ethyl alcohol (100%)	5 minutes
Xylene	5 minutes
Xylene	5 minutes

Immunohistochemical staining

To assess the presence of mitochondrial proteins in urinary bladder tissue cells, prepared sections of urinary bladder were stained with primary antibodies using DAB (diaminobenzidine) staining method (118). To deparaffinize the tissues, slides with tissue sections of 5 μm thickness were incubated in an oven at 60°C for 2 hours. After cooling to room temperature, slides were placed vertically in the slide holders and run through 3 xylene series, 10 minutes each. Afterwards, slides were then dipped in decreasing ethyl alcohol series (100%, 96%, 80%, and 70%) and then kept in distilled water. After drying the slides, a pap pen was used to mark the edges of every tissue section as more than one antibody will be added to the same slide in addition of the no primary antibody (NP) section. The antipolyvalent HRP DAB system kit was used according to manufacturer instructions. To prevent non-specific background staining caused by endogenous peroxidase, sections were first incubated with a hydrogen peroxide block for 15 minutes then rinsed twice with PBS. For antigen retrieval, slides were placed in slide holder containing the retrieval solution (citrate buffer) and left in a pre-heated steamer for 20 minutes (Figure 26b). PBS was used again to rinse the slides for 4 times after they cooled to room temperature.

To prevent non-specific background staining, one drop of protein blocker was added to each section in the slide and slides were incubated for 5 minutes at room temperature. For incubation with primary antibody, 20 μL of PBS (NP) or antibody was added on its corresponding marked tissue section on the slide (dilution ratios were 1:150 for TOM20 and 1:100 for Drp1 and Mfn2) (Figure 26c). Slides were incubated with antibodies overnight at +4°C. On the next day, slides were taken out from +4°C and left to warm to room temperature. After that, slides were washed with PBS for 4 times followed by the addition of one drop of biotinylated goat anti-polyvalent secondary antibody on each section. After 10 minutes, slides were rinsed with PBS for 4 times and one drop of streptavidin peroxidase was added on each tissue section to complete the labeled streptavidin-biotin immunoenzymatic antigen reaction, and slides were rinsed again with PBS for 4 times after 10 minutes.

To start the staining process, 20 μ L of DAB substrate and DAB chromogen mixture was added to each section and the slides were monitored for the appearance of brown color (Figure 26d). Once the brown color was seen, the slides were immediately dipped in distilled water. For counterstaining, slides were dipped in hematoxylin for 30 seconds then washed under running tap water for two minutes. Slides were dehydrated through ascending grades of ethyl alcohol, and cleared in xylene. Finally, slides were dried and mounted with a drop of entellan on the slide surface. Coverslip was placed over the slide and left to dry for complete sealing.

Microscopic evaluation

After staining, slides were examined under a light microscope to detect and score histological changes in the bladder tissue. Images of representative areas (10 μ m) were captured at 200x magnification and then analyzed with ToupView software (Hangzhou ToupTek Photonics, China) (Figure 26e). The presence of epithelial irregularity and hemorrhage were assessed. The presence of pathological changes in these parameters was scored as 1, and no change was scored as 0. For immunohistochemical staining, areas that appeared in brown color were considered as positive staining areas containing the protein of interest.



Figure 26. Wet transfer process. **(a)** Samples in paraffin blocks. **(b)** Antigen retrieval. **(c)** Antibody addition. **(d)** Samples staining **(e)** Microscopic evaluation.

3.2.9. Statistical Analyses

All data obtained in this study were analyzed using GraphPad Prism 8.0.1 program (GraphPad Software, USA) and expressed as mean $\pm$ standard error of the mean (SEM). Differences between groups were considered statistically significant when $p < 0.05$. Body weights (g), water intakes (mL), urine outputs (mL), and fecal outputs (g) of animals were analyzed using two-way analysis of variance (ANOVA) with repeated-measures analysis followed by post hoc Tukey multiple comparison test.

Concentration response curves were analyzed using two-way ANOVA analysis followed by post hoc Tukey multiple comparison test. Potency expressed as pD_2 (the negative logarithm of the EC_{50} , $-\log EC_{50}$) that represents the agonist concentration required to produce half of the maximal effect (E_{max}), was determined through nonlinear regression analysis. pD_2 and E_{max} values across more than two groups were analyzed using one-way ANOVA analysis followed by post hoc Tukey multiple comparison test. Western blot densitometrical results were analyzed using one-way ANOVA analysis followed by post hoc Tukey multiple comparison test.

4. RESULTS

4.1. Effects of Mitotempo Treatment on Metabolic Parameters in SCI-Induced Bladder Dysfunction

To evaluate the metabolic parameters, body weight, water intake, urine output, and fecal output of each rat were recorded at designated days and durations during the experimental protocol (Figure 27-30).

Body weights were recorded daily and at day 0 (preoperative day), in both 3-day and 14-day treatment groups of Sham, SCI, and SCI+MT animals had similar body weights (Figure 27a and Figure 27b). In the 3-day treatment groups (Figure 27a), Sham group did not show a significant change in body weight after sham surgery when compared to day 0. However, both the SCI and SCI+MT groups showed a significant decrease in body weight starting on the first day of SCI surgery when compared to day 0, without a significant difference between the SCI and the SCI+MT groups at day 3. This decrease indicates that animals who underwent SCI surgery lost body weight, and this was not prevented with mitoTEMPO treatment.

In the 14-day treatment groups (Figure 27b), starting from day 9, the Sham group showed a significant increase in body weight, which was maintained up to day 14. SCI group had a significantly lower body weight from the first day after SCI surgery when compared to its day 0 that was maintained up to day 14, on which it also became significantly lower than Sham group. However, in the SCI+MT group, body weight showed significant decrease after SCI surgery when compared to its day 0 that persisted only for four days, and was not different from Sham group. After the fourth day and up to the end of the experiment, body weights of the SCI+MT group were not different from day 0 nor from Sham group. These results indicate that loss of body weight after SCI surgery was recovered in animals with mitoTEMPO for 14 days.

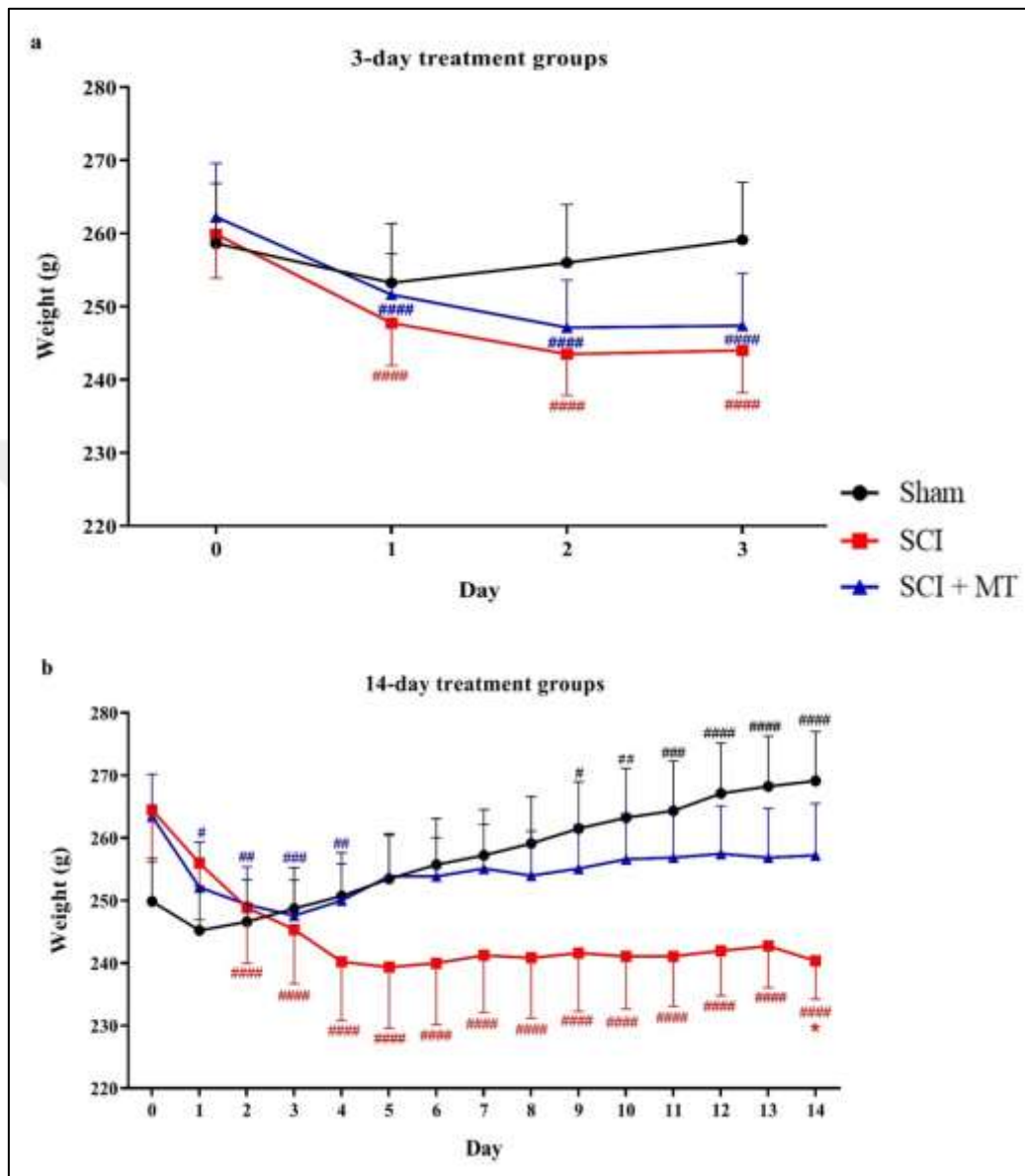


Figure 27. Daily body weights of the animals in different treatment groups. **(a)** 3-day treatment groups. **(b)** 14-day treatment groups. The data were presented as mean $\pm$ SEM (n=8 animals/group). *p<0.05 versus Sham group; #p<0.05, ##p<0.01, ###p<0.001 and ####p<0.0001 versus day 0 in the same group, analyzed with two-way repeated measures of ANOVA followed by Tukey's multiple comparison test. SCI, spinal cord injury; MT, mitoTEMPO.

Water intake, urine output, and fecal output were recorded for twenty-four-hour pre-operatively (pre-op; day 0) and post-operatively (post-op; day 3 or day 14, for 3-day treatment groups and 14-day treatment groups, respectively) in Sham, SCI and SCI+MT groups using the metabolic cage system.

For the water intake; in the 3-day treatment groups and 14-day treatment groups (Figure 28a and Figure 28b), there was no difference between pre-op and post-op water intake in any of the experimental groups, indicating that SCI or mitoTEMPO did not alter the water consumption of the animals.

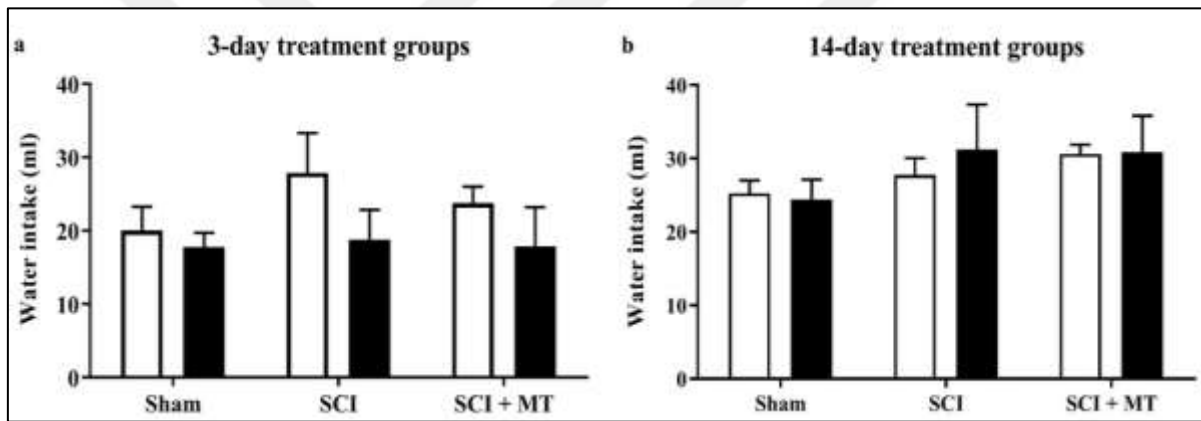


Figure 28. Twenty-four-hour water intake of the animals in different treatment groups. **(a)** 3-day treatment groups (pre-op water intake was measured at day 0 and post-op water intake was measured at day 3). **(b)** 14-day treatment groups (pre-op water intake was measured at day 0 and post-op water intake was measured at day 14). The data were presented as mean $\pm$ SEM; (n=8 animals/group), analyzed with one-way ANOVA followed by Tukey's multiple comparison test, student t-test within the same group were conducted, *SCI*, spinal cord injury; *MT*, mitoTEMPO.

For the urine output; pre-op (at day 0), there was no difference in the twenty-four hour urine output of both 3-day and 14-day treatment groups of the Sham, SCI, and SCI+MT animals (Figure 29a and 29b). In the 3-day treatment groups (Figure 29a), post-op (day 3) urine output was significantly low in the SCI and the SCI+MT groups, compared to both their pre-op urine output and the post-op urine output of the Sham group; suggesting that urination was significantly diminished after SCI and treatment of SCI animals with mitoTEMPO did not effect the urinary impairment.

In the 14-day treatment groups (Figure 29b), post-op (day 14) urine output was significantly low in the SCI group, compared to both its pre-op urine output and the post-op (day14) urine output of the Sham group; suggesting that urination remained significantly diminished after 14 days of SCI. In the SCI+MT group, although urine output was still lower than the pre-op urine output, it was significantly higher than the post-op urine output of the SCI group. Improved urination of SCI animals treated with 14 days of mitoTEMPO (SCI+MT group) indicates time-dependent effect of mitoTEMPO treatment in recovery of urinary bladder function.

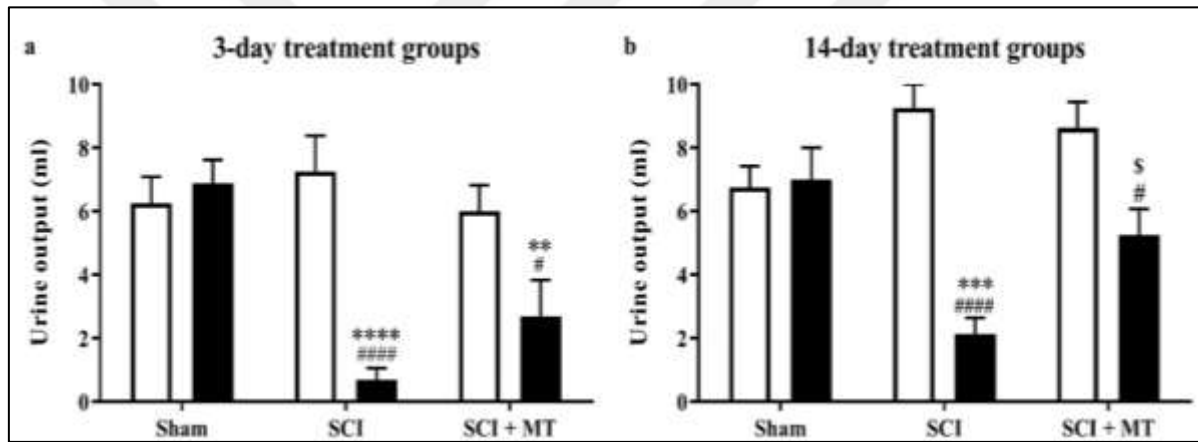


Figure 29. Twenty four-hour urine output of the animals in different treatment groups. **(a)** 3-day treatment groups (pre-op urine output was measured at day 0 and post-op urine output was measured at day 3). **(b)** 14-day treatment groups (pre-op urine output was measured at day 0 and post-op urine output was measured at day 14). The data were presented as mean $\pm$ SEM; (n=8 animals/group). **p<0.01, ***p<0.001 and ****p<0.0001 versus Sham group; #p<0.05 ###p<0.001, and #####p<0.0001 versus day 0 in the same group, \$p<0.05 versus SCI group, analyzed with one-way ANOVA followed by Tukey's multiple comparison test, student t-test within the same group were conducted. *SCI*, spinal cord injury; *MT*, mitoTEMPO.

For the fecal output; pre-op (at day 0), there was no difference in the twenty-four hour fecal output of both 3-day and 14-day treatment groups of the Sham, SCI, and SCI+MT animals (Figure 30a and Figure 30b). In the 3-day treatment groups (Figure 30a), post-op (day 3) fecal output was significantly low in the SCI and SCI+MT groups, compared to both their pre-op fecal output and the post-op fecal output of the Sham group; suggesting that defecation was significantly diminished after SCI and treatment of SCI animals with mitoTEMPO did not effect the intestinal impairment. In the 14-day treatment groups (Figure 30b), post-op (day 14) fecal output was significantly low in the SCI group, compared to both its pre-op fecal output and the post-op (day14) fecal output of the Sham group; suggesting that defecation remained significantly diminished after 14 days of SCI. In the SCI+MT group, fecal output was not different than its pre-op fecal output and the post-op (day14) fecal output of the Sham group. These results indicate the time-dependent effect of mitoTEMPO treatment in recovery of intestinal function.

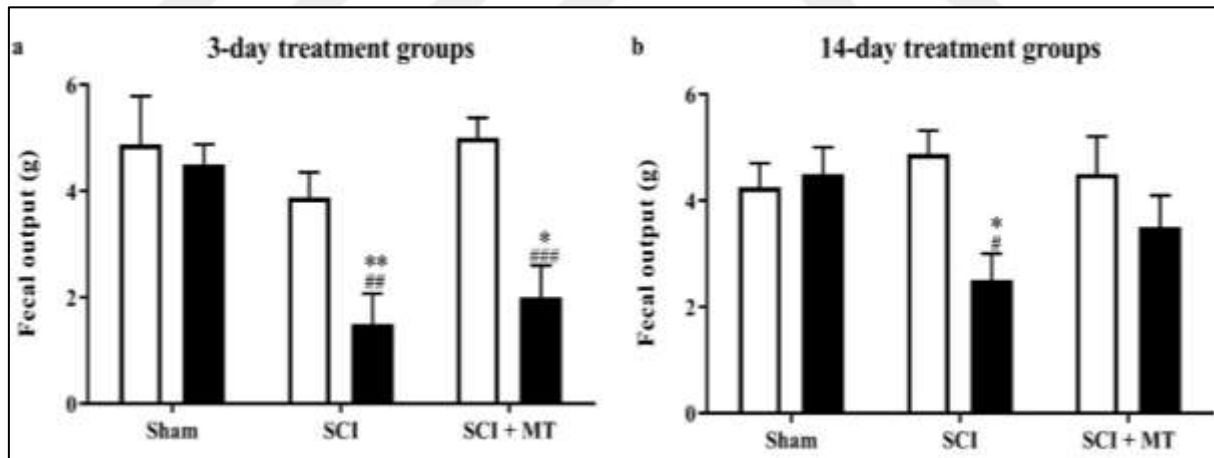


Figure 30. Twenty-four hour fecal output of the animals in different treatment groups. **(a)** 3-day treatment groups (pre-op fecal output was measured at day 0 and post-op fecal output was measured at day 3). **(b)** 14-day treatment groups (pre-op fecal output was measured at day 0 and post-op fecal output was measured at day 14). The data were presented as mean $\pm$ SEM; (n=8 animals/group). * $p < 0.05$ and ** $p < 0.01$ versus Sham group; # $p < 0.05$ and ## $p < 0.001$ versus day 0 in the same group, analyzed with one-way ANOVA followed by Tukey's multiple comparison test. *SCI*, spinal cord injury; *MT*, mitoTEMPO.

4.2. Effects of MitoTEMPO Treatment on Bladder Function in SCI-induced Bladder Dysfunction

Bladder strips (of animals from different experimental groups) were challenged with various pharmacological agents using the ex vivo organ bath and isometric tension recording system to evaluate the effect of mitoTEMPO treatment on bladder contractility.

To investigate receptor-independent, electromechanical coupling-mediated responses, contraction of bladder strips was induced with KCl (80mM) (Figure 31). In the 3-day treatment groups, KCl-induced contractions were significantly lower in the SCI and SCI+MT groups compared with contraction responses of the Sham group (Figure 31c). In the 14-day treatment groups, KCl-induced contraction remained decreased in the SCI group compared to the Sham group, whereas, contraction response in the SCI+MT group was not different than the Sham group and was significantly higher than the SCI group (Figure 31b and Figure 31d). These results indicate receptor-independent contractile mechanisms of bladder smooth muscle was impaired with SCI, and, this impairment was recovered with time-dependent treatment with mitoTEMPO, since 14-day but not 3-day treatment was effective.

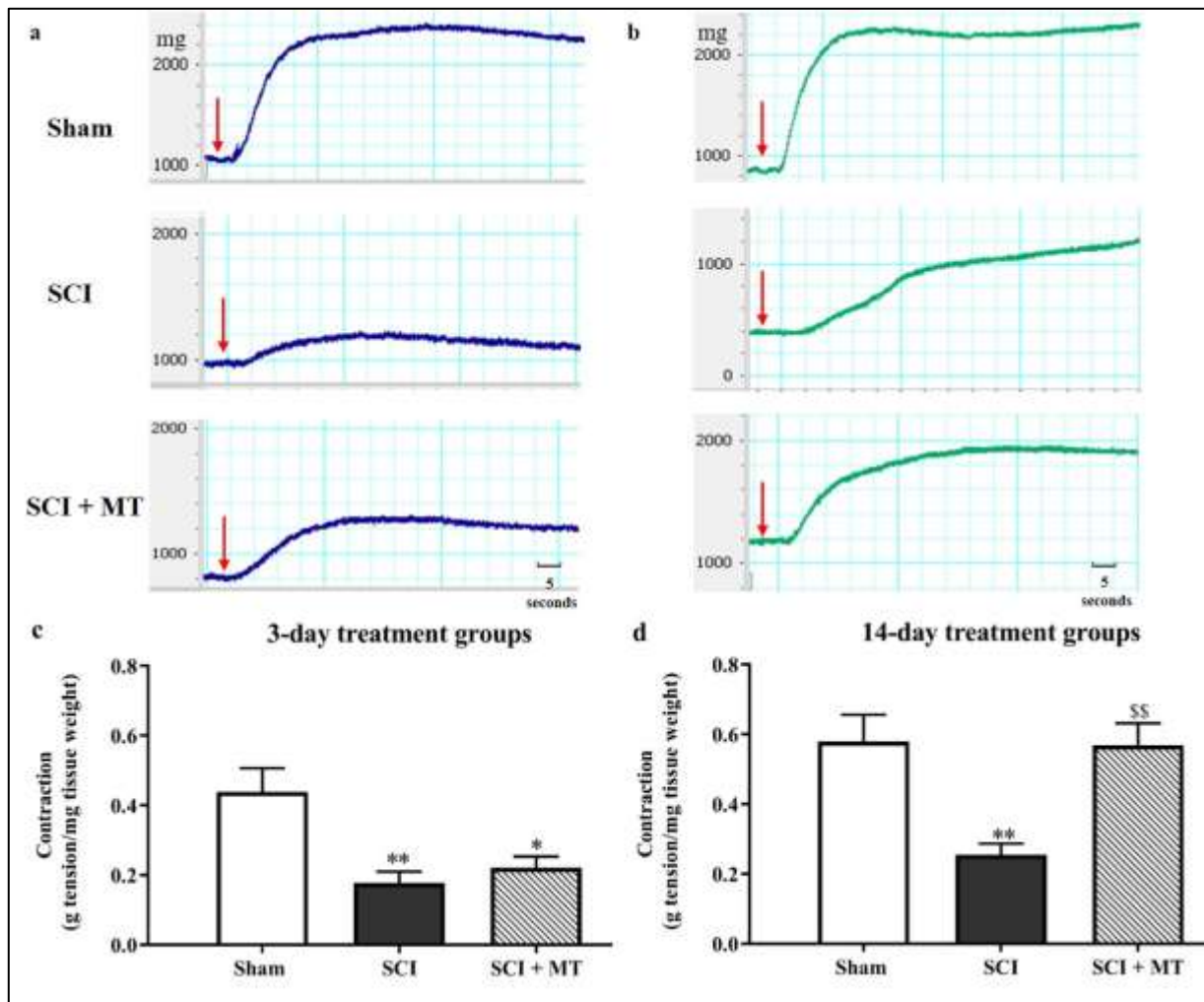


Figure 31. KCl-induced contraction of bladder strips from different treatment groups. Upper panel (**a, b**); representative tracings from 3-day and 14-day treatment groups. Lower panel (**c, d**); KCl-induced contraction response of bladder strips from 3-day and 14-day treatment groups. KCl (80 mM) addition was indicated by ($\downarrow$) symbol. The response was normalized to the wet weight of the respective bladder strip. Data were presented as mean $\pm$ SEM (n=8 strips/group; two strips per animal). * p <0.05 and ** p < 0.01 versus Sham group; \$\$ p <0.01 versus SCI group, analyzed with one-way ANOVA followed by Tukey's multiple comparison test. *SCI*, spinal cord injury; *MT*, mitoTEMPO.

To evaluate neurogenic responses, contraction of bladder strips was induced with EFS (Figure 32). In the 3-day treatment groups, EFS caused a frequency-dependent (1-64 Hz) contraction of bladder strips of the Sham group, whereas EFS-induced contractions were significantly low both in the SCI and SCI+MT groups (Figure 32a and Figure 32c). In the 14-day treatment groups, EFS-induced contraction remained low in the SCI group compared to the Sham group. Frequency-dependent contraction responses in the SCI+MT group were not different than Sham group and were significantly higher than SCI group (Figure 32b and Figure 32d). These results indicate neurogenic mechanisms mediated contraction of bladder smooth muscle was impaired with SCI, and, this impairment was recovered with time-dependent treatment with mitoTEMPO, since 14-day but not 3-day treatment was effective.

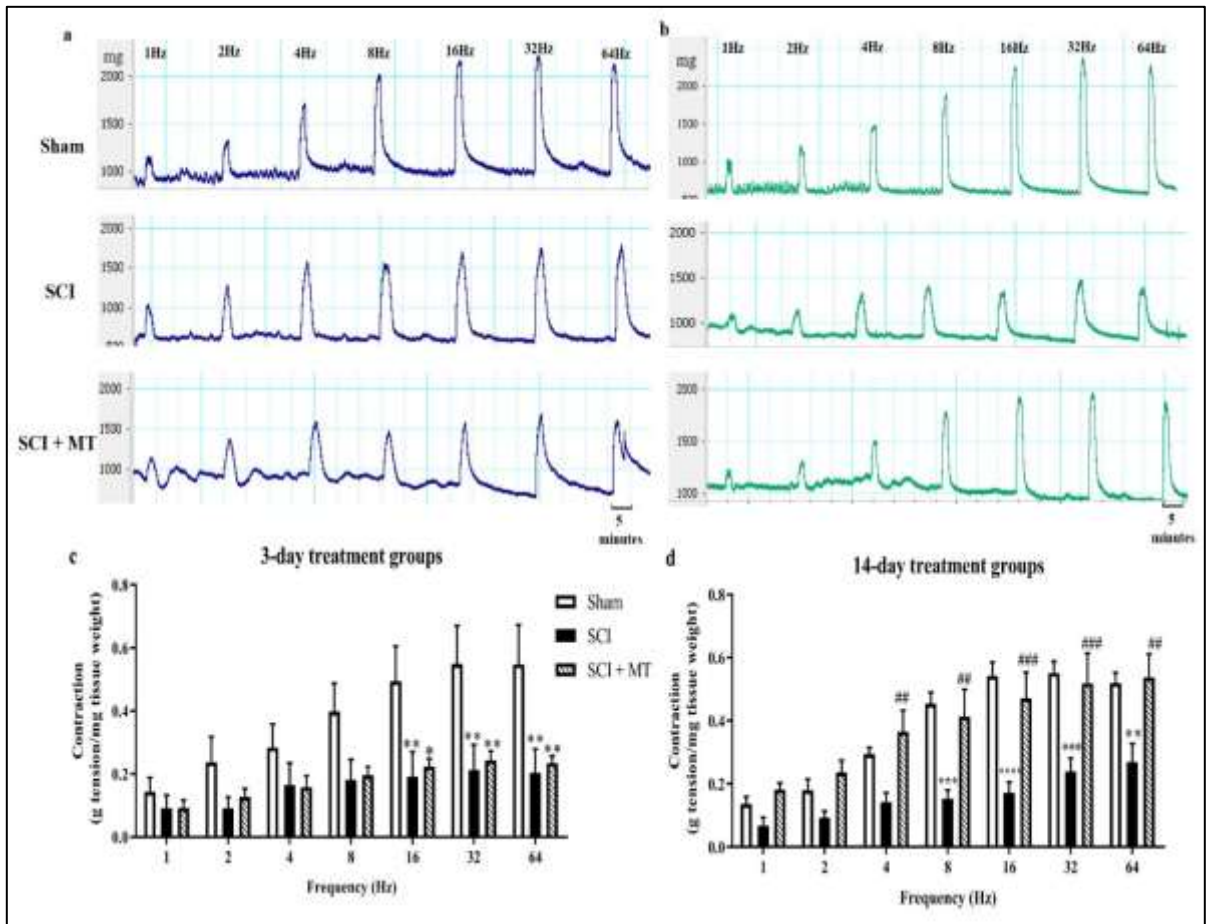


Figure 32. EFS-induced contraction bladder strips from different treatment groups. Upper panel (a, b); representative tracings from 3-day and 14-day treatment groups. Lower panel (c, d); EFS-induced contraction response data of bladder strips from 3-day and 14-day treatment groups. Response was normalized to the wet weight of the respective bladder strip. Data were presented as mean $\pm$ SEM, (n=4 strips/group; one strip per animal). * p <0.05, ** p <0.0, *** p <0.001, and **** p <0.0001 versus Sham group; ## p <0.01 and ### p <0.001 versus SCI group analyzed with two-way ANOVA test. SCI, spinal cord injury; MT, mitoTEMPO, Hz, Hertz.

To assess the muscarinic receptor-mediated (cholinergic) responses, contraction of bladder strips was induced with a muscarinic agonist, carbachol (CCh) (Figure 33). In the 3-day treatment groups, CCh caused concentration-dependent (10^{-9} to 10^{-4} M) contractions of bladder strips in the Sham group, whereas CCh-induced contractions were significantly lower in both the SCI and SCI+MT groups (Figure 33a and Figure 33c). Additionally, E_{max} was markedly lower in the SCI and SCI+MT groups compared to the Sham group, but pD_2 was not different among the experimental groups (Table 5). In the 14-day treatment groups, similarly, CCh caused a concentration-dependent contraction of bladder strips in the Sham group. The contraction of both the SCI group and the SCI+MT group was not different from that of the Sham group (Figure 33b and Figure 33d). Moreover, E_{max} and pD_2 did not change in the experimental groups (Table 5). These results indicate that muscarinic receptor-mediated detrusor contraction was diminished at 3 days after SCI, and mitoTEMPO treatment did not affect this response. However, at 14 days after SCI, muscarinic contractions were not different in the experimental groups, suggesting the time-dependent recovery of muscarinic contractile mechanisms, and without an effect of mitoTEMPO.

Table 5. E_{max} and pD_2 values of carbachol-induced contraction of bladder detrusor strips

Group	E_{max} (%)	pD_2
3-day Sham	0.723 ± 0.068	6.006 ± 0.133
3-day SCI	$0.341 \pm 0.074^{**}$	5.6 ± 0.539
3-day SCI + MT	$0.439 \pm 0.035^*$	6.428 ± 0.233
14-day Sham	0.749 ± 0.123	5.821 ± 0.4
14-day SCI	0.543 ± 0.357	5.179 ± 2.351
14-day SCI + MT	0.996 ± 0.612	5.554 ± 1.455

Data were presented as mean $\pm$ SEM; $p < 0.05$ was considered statistically significant. $^*p < 0.05$ and $^{**}p < 0.01$ indicate significant versus Sham group. Two-way ANOVA test was applied for comparisons ($n = 4$ strips/group; one strip per animal). E_{max} , maximum contraction induced by carbachol; pD_2 , negative logarithm of EC_{50} .

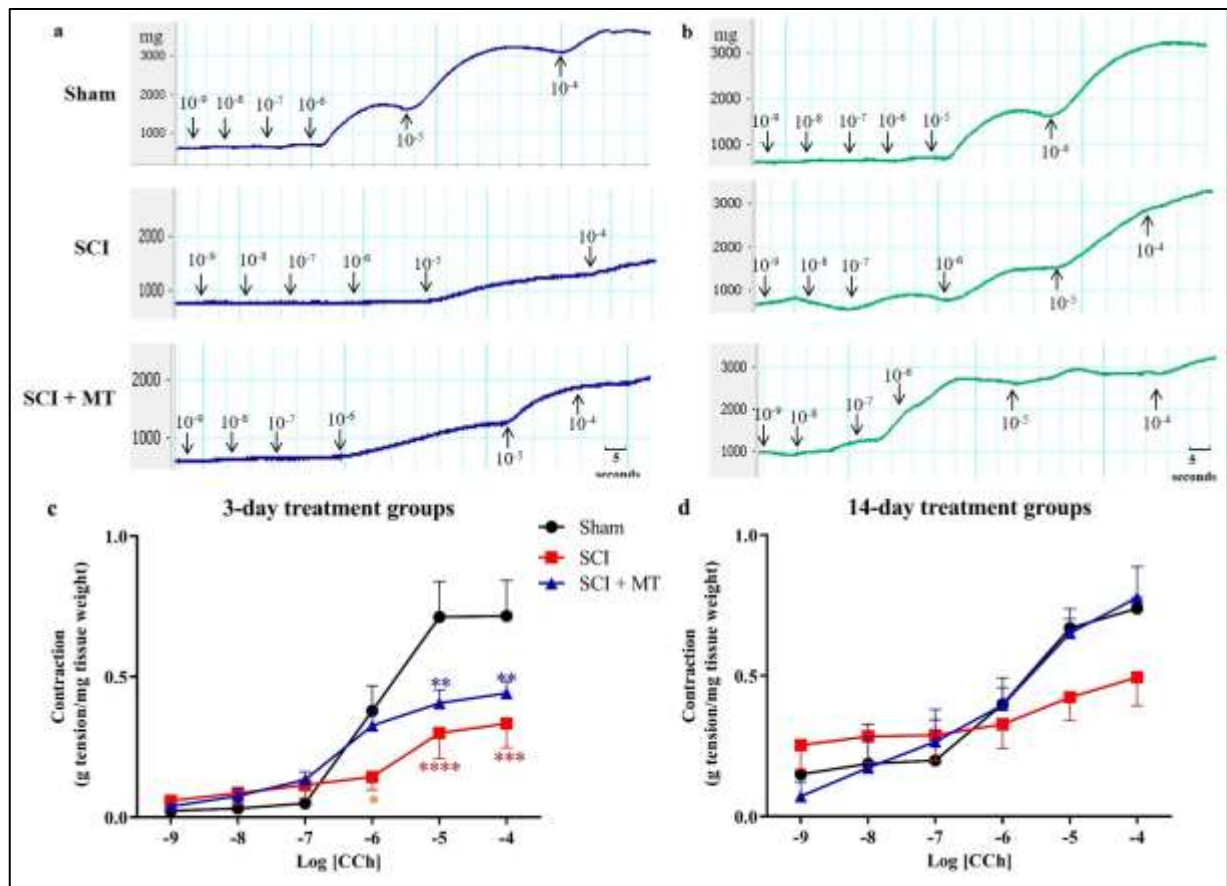


Figure 33. CCh-induced contraction of bladder strips from different treatment groups. Upper panel (**a, b**) representative tracings from 3-day and 14-day treatment groups. Lower panel (**c, d**) CCh-induced contraction response of bladder strips from 3-day and 14-day treatment groups. Cumulative CCh addition was indicated by (↓) and (↑) symbols. The response was normalized to the wet weight of the respective bladder strip. Data were presented as mean ± SEM (n=4 strips/group; one strip per animal). *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 versus Sham group, analyzed with two-way ANOVA test. SCI, spinal cord injury; MT, mitoTEMPO; CCh, carbachol.

To evaluate the receptor-independent, extracellular Ca^{2+} influx-mediated responses, bladder strips were incubated in calcium-free medium and increasing concentrations of CaCl_2 (0.01-100 mM) were added. In a limited number of preliminary experiments, CaCl_2 -induced contraction was not achieved in any of the experimental groups, suggesting the presence of technical challenges that require modifications. The results were not conclusive and were not included in the results section.

To investigate relaxation responses, bladder strips were precontracted with the submaximal concentration of CCh (10^{-5} M), and cumulative concentration-dependent Phe (10^{-8} – 10^{-4} M) responses were recorded (Figure 34). In the 3-day treatment groups, Phe caused concentration-dependent (10^{-8} to 10^{-4} M) relaxation of bladder strips in the Sham group, whereas Phe-induced relaxations were significantly lower in both the SCI and SCI+MT groups (Figure 34a and Figure 34c). Additionally, E_{max} was markedly lower in the SCI and SCI+MT groups compared to the Sham group, but pD_2 was not different among the experimental groups (Table 6).

In the 14-day treatment groups, similarly, Phe caused relaxation of bladder strips in the Sham group (Figure 34b and Figure 34d). The relaxation of the SCI group was significantly lower than that of the Sham group whereas in the SCI+MT group, Phe induced a concentration dependent relaxation that was not different from that of the Sham group. As for the E_{max} , the SCI group but not the SCI+MT group had a significantly low value compared to that of the Sham group (Table 6). Further more, E_{max} for SCI+MT was significantly higher than the SCI group. pD_2 was not different among the experimental groups (Table 6). These results suggest that the diminished Phe-mediated relaxation of bladder after the early and late phases of SCI were recovered with long term (14 days) treatment of mitoTEMPO.

Table 6. E_{max} and pD₂ values of phenylephrine-induced relaxation of carbachol-precontracted detrusor strips

Group	E _{max} (%)	pD ₂
3-day Sham	20.205 ± 1.438	5.978 ± 0.372
3-day SCI	11.600 ± 1.613 **	5.250 ± 0.655
3-day SCI + MT	13.554 ± 1.565*	5.892 ± 0.692
14-day Sham	22.734 ± 3.606	7.303 ± 4.323
14-day SCI	7.187 ± 1.092*	6.66 ± 1.23
14-day SCI + MT	15.383 ± 4.095	5.196 ± 1.076

Data were presented as mean ± SEM; p<0.05 was considered statistically significant. *p<0.05 and **p<0.01 indicate significant differences versus Sham group. Two-way ANOVA test was applied for comparisons (n=4 strips/group; one strip per animal). *E_{max}*, maximum relaxation evoked by phenylephrine; *pD₂*, negative logarithm of EC₅₀.

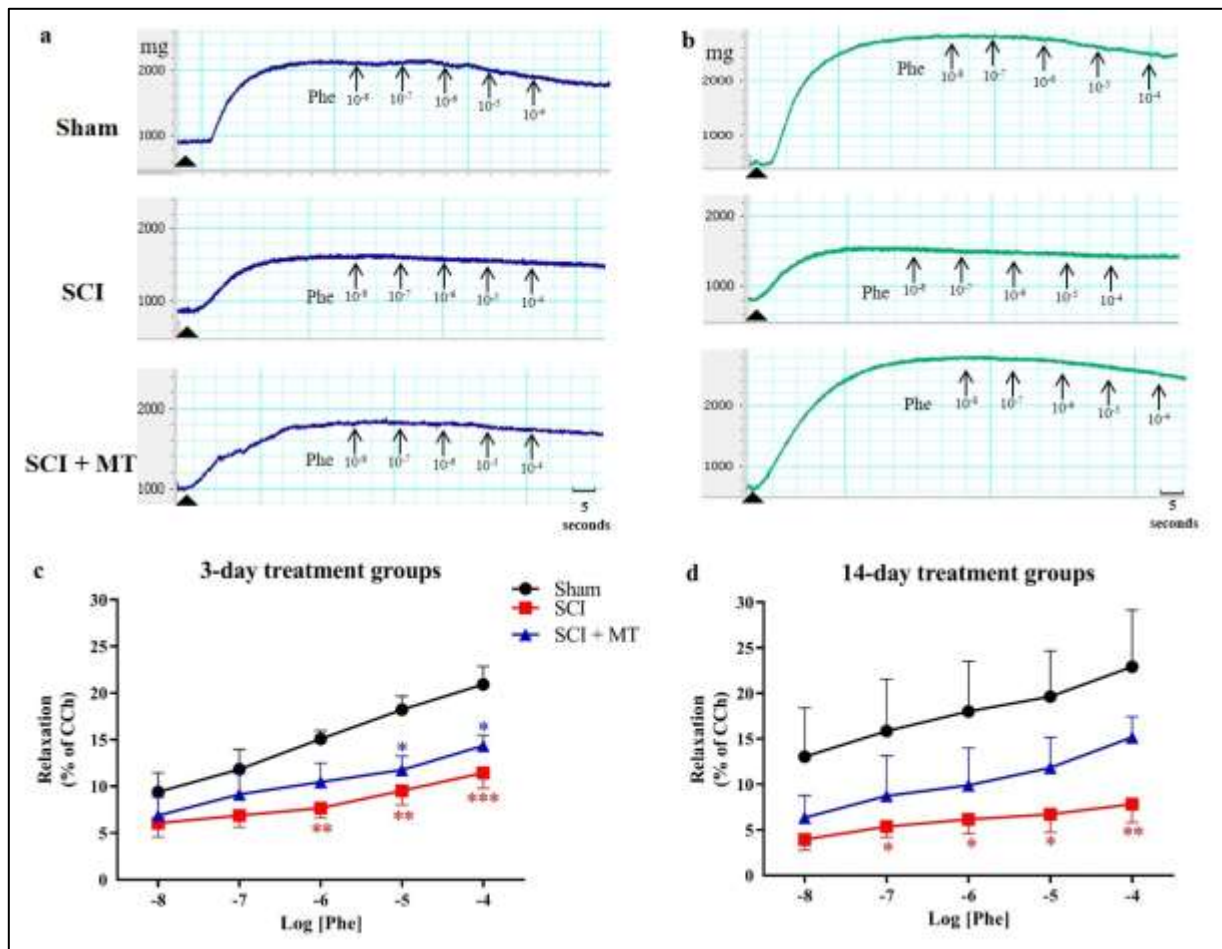


Figure 34. Phenylephrine-induced relaxation of urinary bladder strips from different treatment groups. Upper panel (**a, b**); representative tracings from 3-day and 14-day treatment groups. Time of CCh addition was indicated by ($\blacktriangle$); time of Phe addition was indicated by ($\uparrow$) symbols. Lower panel (**c, d**); Phe-induced relaxation response data of urinary bladder strips from 3-day and 14-day treatment groups. Response was obtained by cumulative addition of phenylephrine to urinary bladder strips from different treatment groups pre-contracted by submaximal dose of CCh. Relaxation was expressed as percentage of submaximal CCh contraction. Data were presented as mean $\pm$ SEM (n=4 strips/group; one strip per animal). *p<0.05, **p<0.01, and ***p<0.001 versus Sham group, analyzed with two-way ANOVA test. SCI, spinal cord injury; MT, mitoTEMPO, CCh, charbachol; Phe, phenylephrine.

4.3. Effects of Mitotempo Treatment on Bladder Mitochondrial Dynamics in SCI-induced Bladder Dysfunction

To evaluate bladder mitochondrial dynamics, the bladder tissue expressions of Drp1 and Mfn2, mitochondrial fission and fusion proteins, respectively, were analyzed by western blotting (Figure 35 and Figure 36). In the 3-day treatment groups, Drp1 protein expression did not change after SCI or with mitoTEMPO treatment (Figure 35b). On the other hand, in the 14-day treatment groups, a significant increase of approximately 3-fold was observed in the SCI group compared to the Sham group. A significant increase of approximately 2.5-fold was detected in the SCI+ MT group compared to the Sham group, whereas Drp1 protein expression was not different in the SCI+ MT group compared to the SCI group (Figure 35c). These results indicate that mitochondrial fission was activated in the later phases of SCI and was not altered by mitoTEMPO treatment.

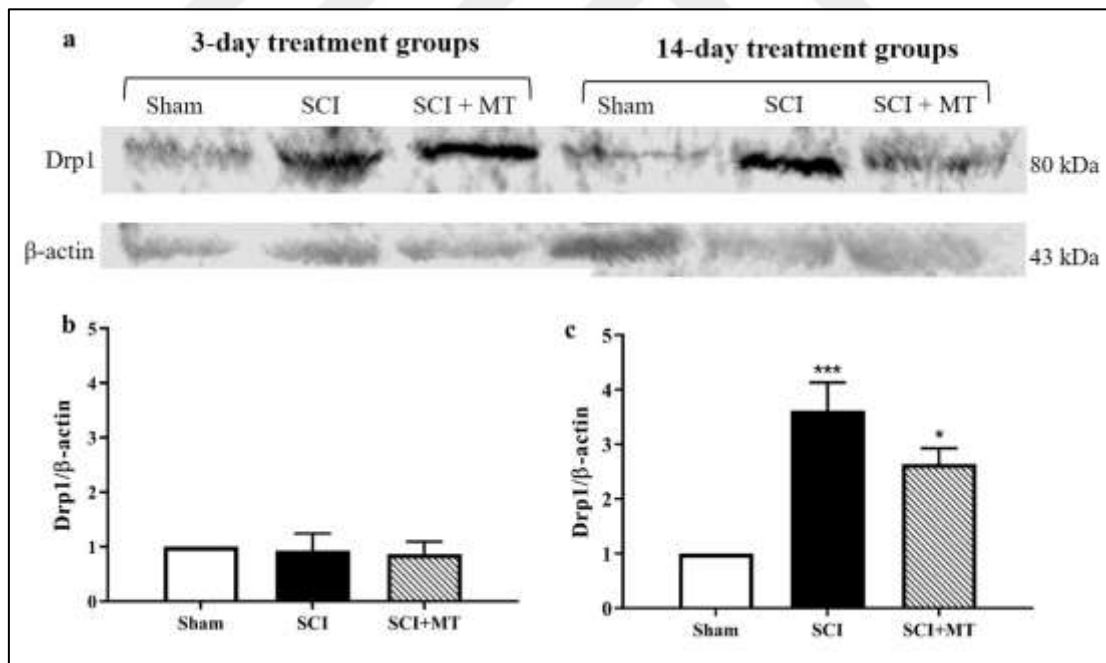


Figure 35. Drp1 protein expression in bladder tissues of different treatment groups. Upper panel (a) representative western blot image of 3- and 14-day treatment groups. Semi-quantitative analysis of Drp1 protein expression level in bladder tissues (b) 3-day treatment groups, (c) 14-day treatment groups. Drp1 protein expression was normalized to β -actin. Data were presented as mean $\pm$ SEM (n = 4 animals/group). *p < 0.05 and ***p < 0.001 versus Sham group, analyzed with one-way ANOVA followed by Tukey's multiple comparison test. SCI, spinal cord injury; MT, mitoTEMPO.

Both in the 3-day and 14-day treatment groups, Mfn2 protein expression, although it showed a trend to increase, was not significantly different in SCI or SCI+MT groups compared to that of the Sham group (Figure 36). These results suggest that mitochondrial fusion was not altered after SCI in the bladder tissue

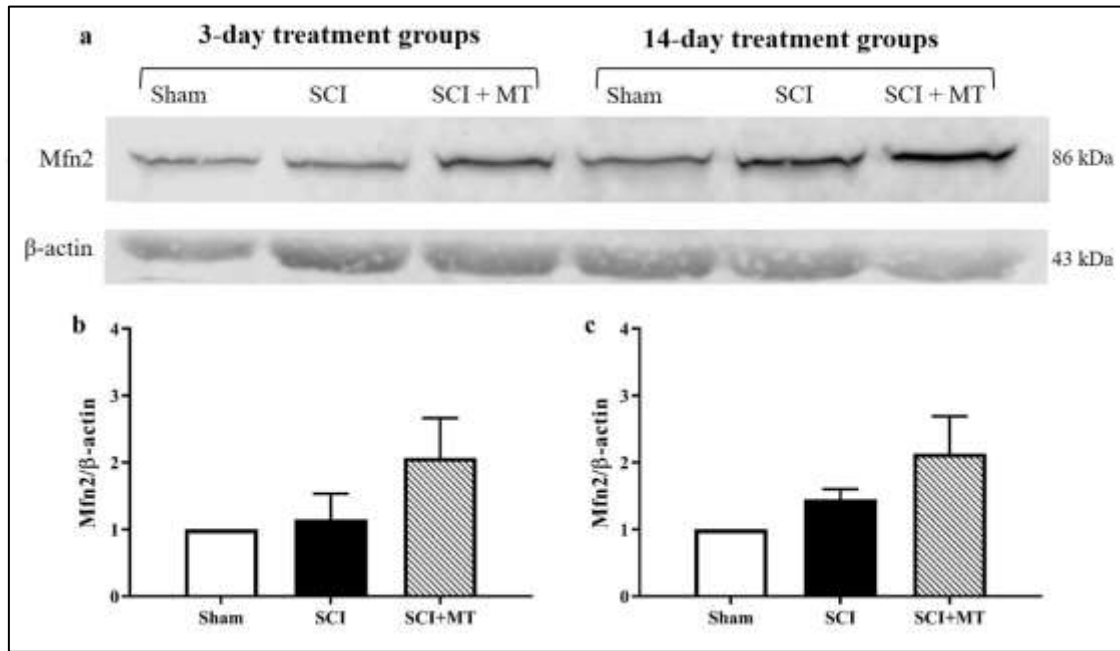


Figure 36. Mfn2 protein expression in bladder tissues of different treatment groups. Upper panel (a) representative western blot image of 3- and 14-day treatment groups. Semi-quantitative analysis of Mfn2 protein expression level in bladder tissues (b) 3-day treatment groups, (c) 14-day treatment groups. Mfn2 protein expression was normalized to β -actin. Data were presented as mean $\pm$ SEM (n = 4 animals/group), analyzed with one-way ANOVA followed by Tukey's multiple comparison test. SCI, spinal cord injury; MT, mitoTEMPO.

4.4. Effects of MitoTEMPO Treatment on Bladder Protein Expression and Structural Alterations in SCI-induced Bladder Dysfunction

4.4.1. Hematoxylin&Eosin Staining

To evaluate structural alterations in the bladder, H&E-stained bladder tissue of different treatment groups was analyzed for the presence of epithelial irregularity, edema, and hemorrhage (Figure 37, Table 7). The bladders of sham animals from both 3- and 14-day treatment groups had a normal histological structure (Figure 37a and Figure 37d).

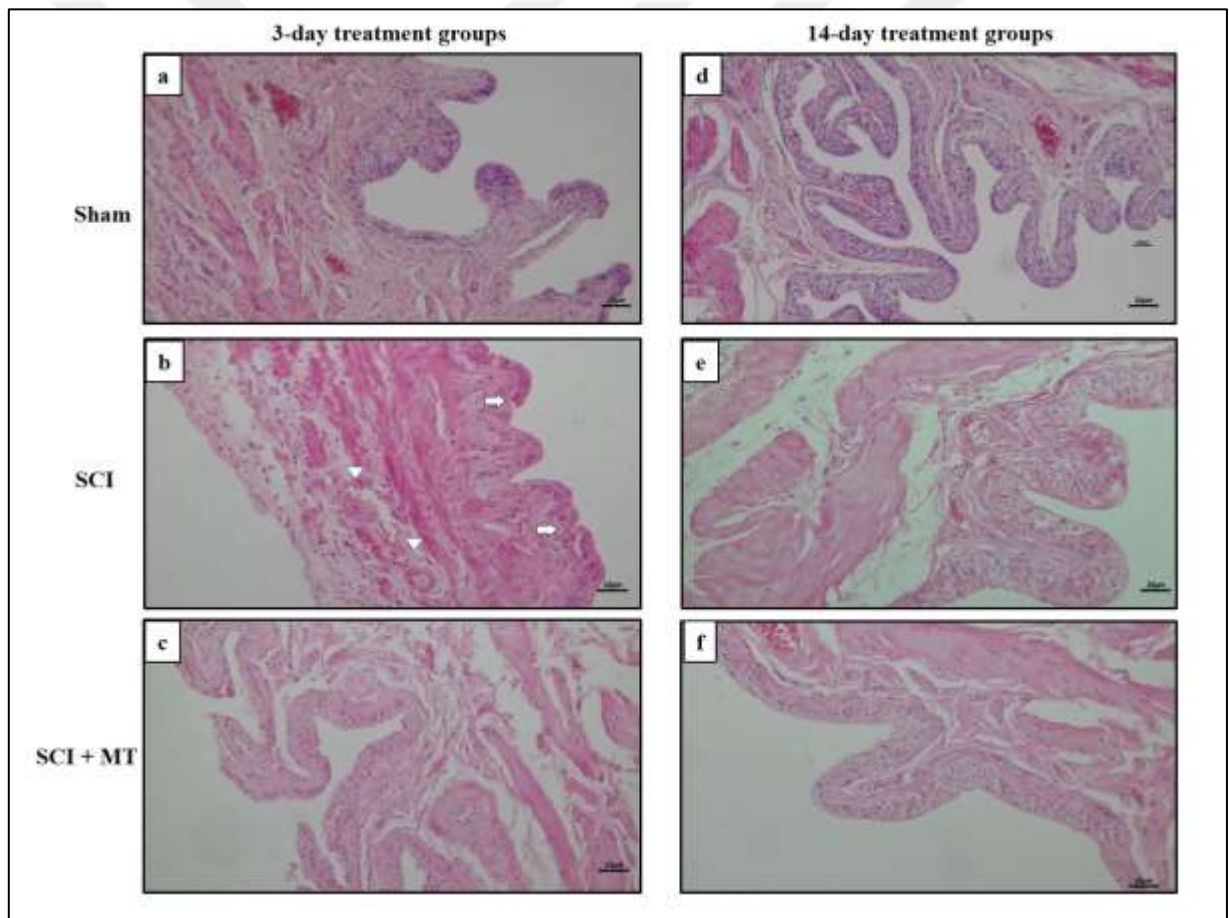


Figure 37. H&E staining of bladder tissues sections from different treatment groups. **(a-c)** 3-day treatment groups, **(d-f)** 14-day treatment groups. ⇨ indicates epithelial irregularity, ∇ indicates hemorrhage. Scale bars: 10 μ m. Original magnifications: $\times 100$. SCI, spinal cord injury; MT, mitoTEMPO.

In the 3-day treatment groups, urothelial irregularity and hemorrhage were observed in SCI bladder tissue, while urothelium was normal, with no signs of hemorrhage in the SCI+ MT bladder tissue (Figure 37b and Figure 36c). In the 14-day treatment groups, normal epithelium and no hemorrhage were detected in SCI or SCI+MT groups (Figure 37e and Figure 37f). Although these results represent a limited number of tissues, histopathological images and scoring data support the fact that mitoTEMPO treatment prevented SCI-induced bladder tissue hemorrhage and infiltration at early/acute stages of SCI. On the other hand, the lack of pathological changes in the bladder tissue at 14 days after SCI, indicated the recovery of damage in the bladder tissue at late/chronic stages of SCI.

Table 7. Scoring of H&E-stained bladder tissue sections from different treatment groups

3-day treatment groups	Edema	Hemorrhage	Infiltration
Sham	0	0	0
(n=2)	0	0	0
SCI	0	1	1
(n=2)	0	1	1
SCI + MT	0	0	0
(n=1)			
14-day treatment groups	Edema	Hemorrhage	Infiltration
Sham	0	0	0
(n=2)	0	0	0
SCI	0	0	0
(n=2)	0	0	0
SCI + MT	0	0	0
(n=1)			

4.4.2. Immunohistochemical Staining

To investigate localization and change in expression of mitochondrial proteins TOM20, Drp1, and Mfn2 in SCI-induced bladder dysfunction, bladder tissues of different treatment groups were evaluated using immunohistochemical analysis (Figure 38, Figure 39, and Figure 40).

TOM20, a mitochondrial outer membrane protein, was localized to the urothelium and vascular endothelial cells in the bladder (Figure 38). In the 3-day treatment groups, TOM20 localization and expression were similar among the experimental groups (Sham, SCI, SCI+MT) (Figure 38a and Figure 38c). In the 14-day treatment groups, the SCI group staining of TOM20 appeared reduced compared to the SCI+ MT group, which also showed a similar staining pattern to the Sham group (Figure 38e - Figure 38g).

Drp1, a mitochondrial fission protein, was localized to the urothelium (Figure 39). In the 3-day treatment groups, SCI and SCI+ MT groups showed lower positive staining in comparison to the Sham group (Figure 39a - Figure 39 c). The 14-day treatment groups, Sham and the SCI+ MT had little to no staining, although SCI group showed strong focal brown deposits of chromogen in the urinary bladder tissue (Figure 39e- Figure 39 g).

Mfn2, a mitochondrial fusion protein, was localized to the urothelium and vascular endothelial cells in the bladder (Figure 40). In the 3-day treatment groups, both the SCI group and SCI+ MT group displayed a higher amount of staining in comparison to the Sham group (Figure 40a- Figure 40c). However, the staining was lower in the 14-day treatment SCI group but remained higher in the Sham and SCI+ MT groups (Figure 40e- Figure 40 g).

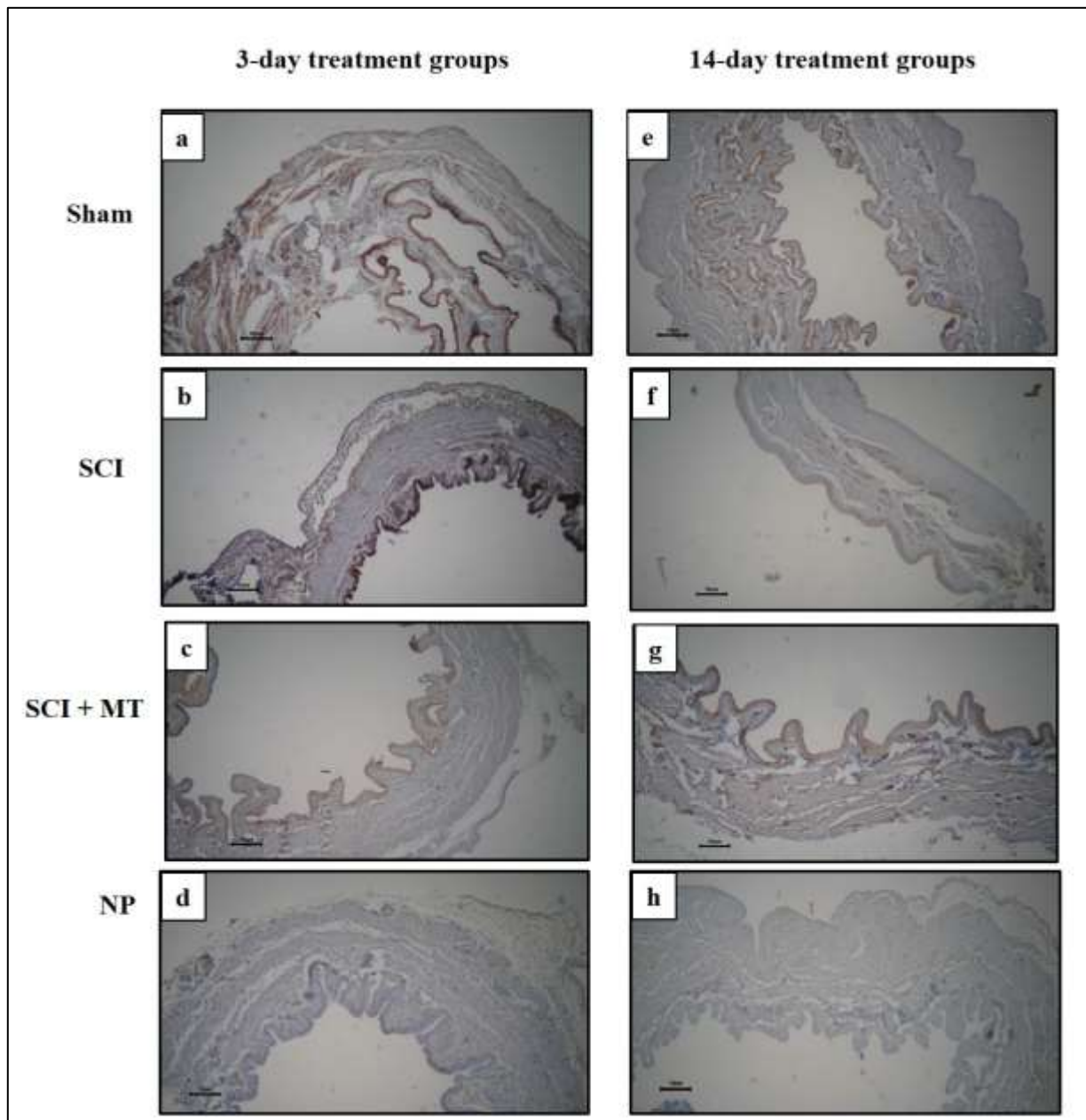


Figure 38. Immunohistochemical analysis of TOM20 in bladder tissue sections from different treatment groups. **(a-d)** 3-day treatment groups, **(e-h)** 14-day treatment groups. Brown spots of diaminobenzidine staining indicate positive immunoreactivity. Primary antibody was omitted in the NP control. Slides were counterstained with hematoxylin. Scale bars: 10 μm. Original magnifications: × 100. NP, no primary; SCI, spinal cord injury; MT, mitoTEMPO.

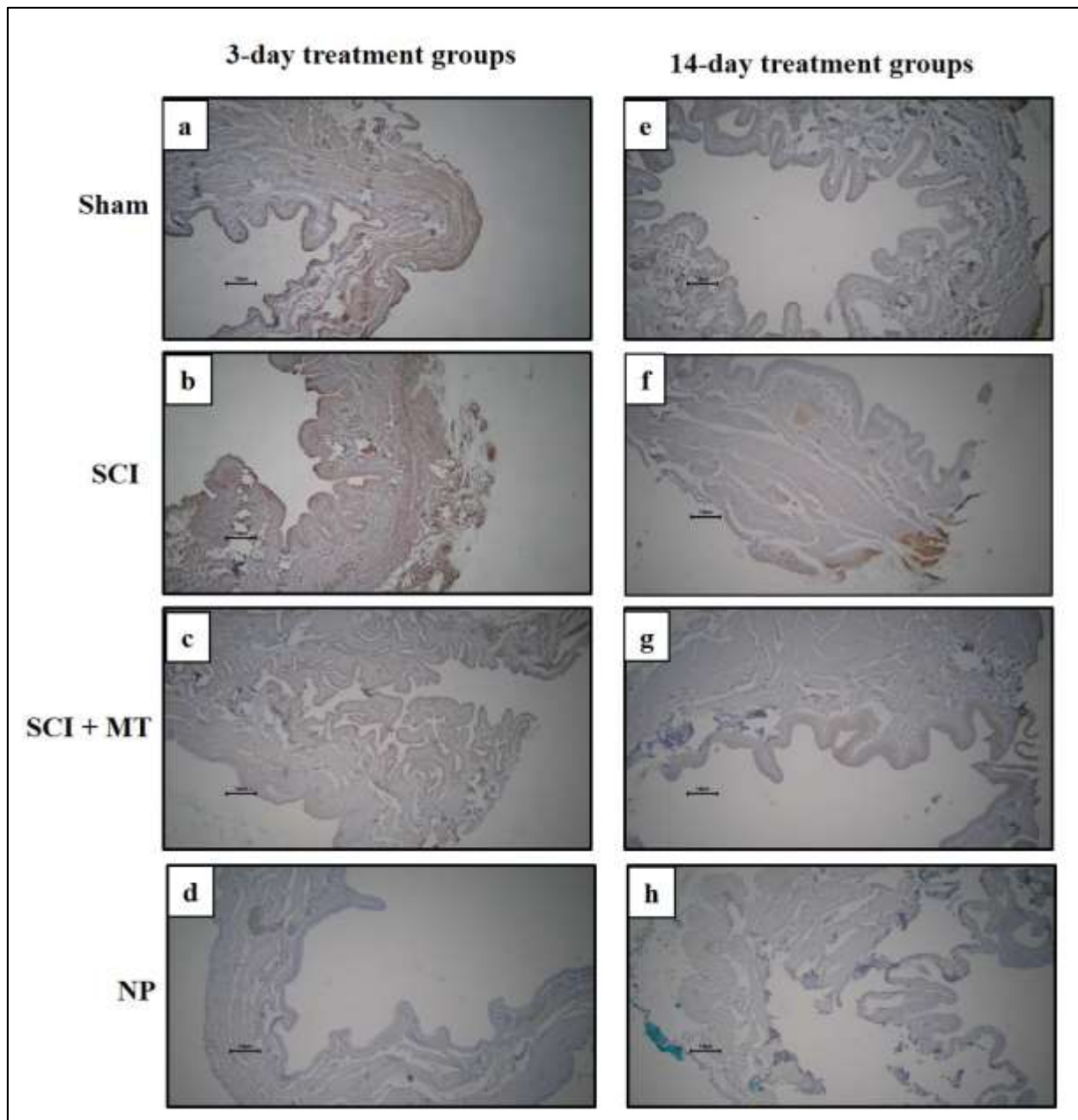


Figure 39. Immunohistochemical analysis of Drp1 in bladder tissue sections from different treatment groups. **(a-d)** 3-day treatment groups, **(e-h)** 14-day treatment groups. Brown spots of diaminobenzidine staining indicate positive immunoreactivity. Primary antibody was omitted in the NP control. Slides were counterstained with hematoxylin. Scale bars: 10 μ m. Original magnifications: $\times 100$. NP, no primary; SCI, spinal cord injury; MT, mitoTEMPO.

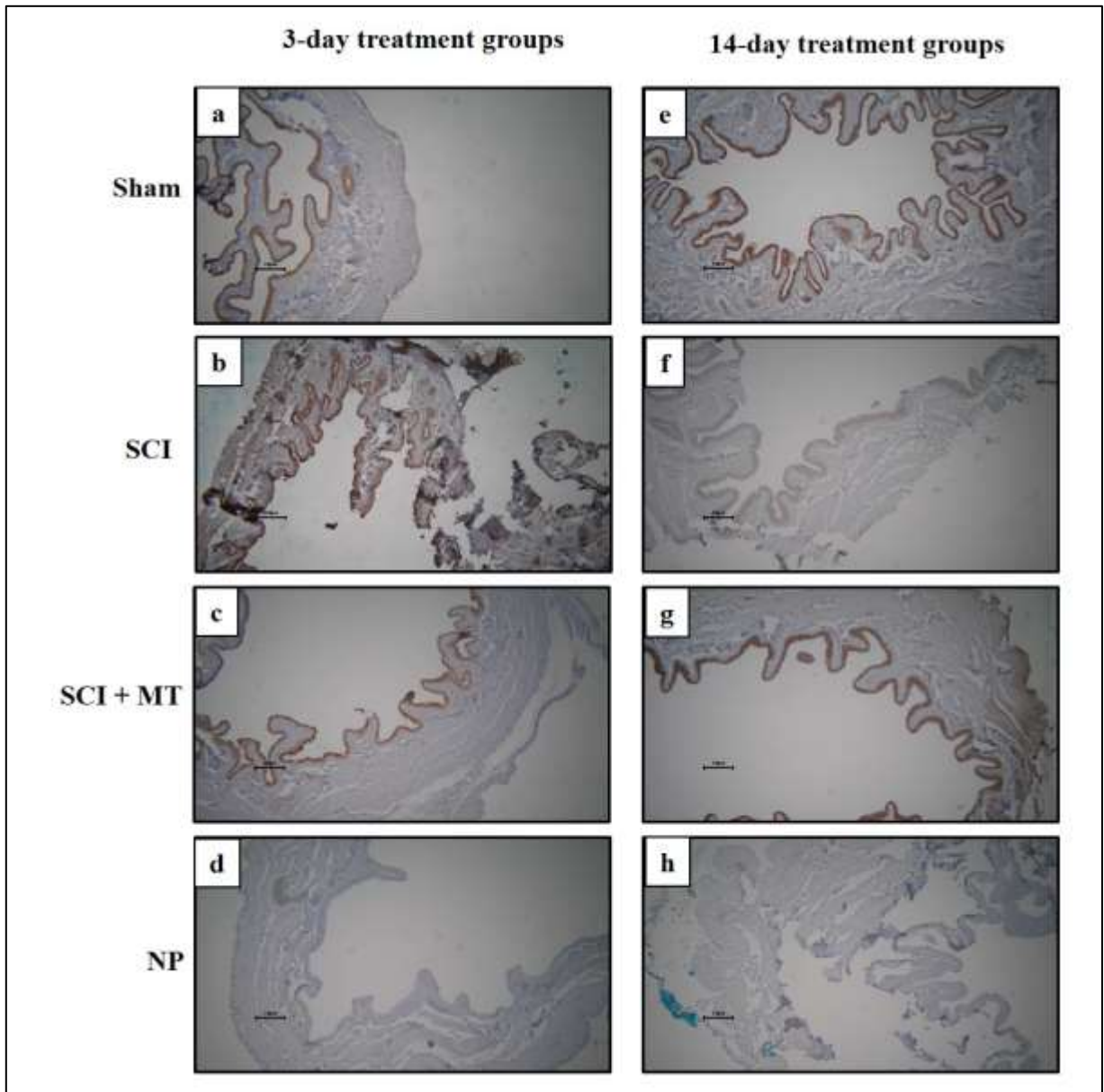


Figure 40. Immunohistochemical analysis of Mfn2 in bladder tissue sections from different treatment groups. **(a-d)** 3-day treatment groups, **(e-h)** 14-day treatment groups. Brown spots of diaminobenzidine staining indicate positive immunoreactivity. Primary antibody was omitted in the NP control. Slides were counterstained with hematoxylin. Scale bars: 10 μ m. Original magnifications: $\times 100$. NP, no primary; SCI, spinal cord injury; MT, mitoTEMPO.

5. DISCUSSION AND CONCLUSION

Bladder dysfunction is an understudied but common comorbidity of SCI that causes impaired storage and/or micturition processes, resulting in serious complications of the urinary system (4). Since there has been no effective treatment, recently the focus of research has shifted toward studying the subcellular level involvement in SCI and SCI-induced dysfunctions with the aim of developing new treatments with better therapeutic outcomes (12). In this study, we evaluated the therapeutic effects of mitoTEMPO, a mitochondria-targeting emerging antioxidant, in a rat model of compression injury model of SCI-induced bladder dysfunction and assessed the effect using metabolic, functional, molecular, and histopathological analysis. We found that mitoTEMPO in a dose of 1mg/kg/day for 3 and 14 days, particularly the 14-day treatment, improved metabolic parameters (water intake, urine output, fecal output) and detrusor muscle contractility in SCI-induced urinary bladder dysfunction. Furthermore, at the molecular level, changes in expression of mitochondrial proteins (Drp1 and Mfn2) and histopathological changes in the bladder tissue caused by SCI were improved with mitoTEMPO treatment. These findings indicate the potential therapeutic benefit of mitoTEMPO in reversing SCI-induced secondary complications. To our knowledge, our study is the first work conducted to examine the effect of mitoTEMPO on SCI-induced bladder dysfunction by combining metabolic, molecular, and histopathological analyses, along with functional analysis of bladder contractility.

Animal models, specifically rat models, of SCI have been established via several different approaches. In this study, SCI was induced using a suprasacral compression model by applying compression to the spinal cord at the level of thoracic vertebra T9 with a surgical clip for 60 seconds. Clip compression model has been favored in SCI research over other models as it allows for controlling closing force and duration, which provides better reproducibility with less variation between animals (63). Female Sprague-Dawley rats were selected for this study based on the comments of studies regarding the effect of gender on experimental outcomes. These studies demonstrated that compared to males, age-matched female Sprague-Dawley rats showed similar changes in functional and histological outcomes in a thoracic contusion SCI model, and the use of a single sex animal model is suitable for similar SCI studies (119). In addition, female rats are preferred for the induction of neurogenic bladder because of their less

complex LUT thus more convenient for manual bladder emptying with lower risk of complications after surgery in this experimental model (95). In our model, the lesion was performed at the T9 level, which is consistent with the standard protocols applied in studies of bladder dysfunction. The thoracic region is considered the most common location for SCI induction due to the reliability, reproducibility, and lower risk of respiratory depression in comparison with injury at the cervical level (56). Specifically, the lower thoracic level of T8 and T9 is favored to disrupt bladder innervation for the induction of SCI-induced bladder dysfunction. It has been shown that contusion of the spinal cord at the T9 level disrupts bladder function of animals, whereas lesions at T1 caused minimal urinary deficits with spared bladder function (95, 120).

Surgical interventions, depending on the extent and duration, commonly result in metabolic stress response due to direct tissue injury or indirect metabolic changes that may affect body weight and composition (121). In our study, the body weights of all the animals were measured daily before any intervention until the last day of the experiment. Although no difference in body weights was observed before the surgical procedure between different experimental groups, animals with SCI experienced a significant decrease in body weight immediately after the procedure. Animals with SCI receiving mitoTEMPO treatment began to regain their body weights one week after the procedure, and reached similar body weights to the animals in the sham group. Nevertheless, the disparity in body weight between the groups with or without mitoTEMPO was apparent at the end of the experiment, with increased weights in the sham and mitoTEMPO treatment groups, while the weights of the SCI group without mitoTEMPO remained significantly low. These data are consistent with previous studies demonstrating chronic body weight reductions after SCI in both humans and animals. In their first year of injury, SCI patients experience notable weight loss from increased metabolic demand, accompanied by lower calorie intake due to psychological reasons, all resulting from their severe trauma. If not reversed, prolonged body weight and fat mass loss will result in lifelong energy and metabolism imbalance due to nutritional deficits and development of pressure ulcers, respectively, which further expand the comorbidities profile of SCI. The improvement in the metabolic state reflects the patient's recovery and the effectiveness of the applied intervention or medication (122, 123).

Our results demonstrated that mitoTEMPO administration ameliorated chronic body weight loss after SCI. As early indicators of increased levels of postoperative stress after surgery, increased inflammatory response leads to elevated ROS levels resulting in lipids and proteins peroxidation (121). In experimental SCI studies, the prolonged body weight and fat loss in animals are thought to be a result of increased stress and muscle atrophy after SCI surgery and not from hypophagia (123). This was further confirmed as the same studies showed no change and even an increase in food intake in experimental animals. Therefore, the positive effects of mitoTEMPO in SCI animal models could also be attributed to its role in improving stress levels, body weight, and composition, and overall body homeodynamics to a level comparable to animals with no injury.

The water intake, urine output, and fecal output are critical metabolic parameters that reflect the urinary and bowel functions. In this study, when water intake was assessed, no change was observed before and after the procedure in any of the experimental groups. This result was in agreement with previous studies that demonstrated unchanged water intake during the study period in SCI and sham groups, with no significant differences between the groups or over the duration of the study (124). Urine output of experimental animals was assessed to monitor functional changes in the SCI-induced bladder dysfunction model. SCI resulted in a significant decrease in urine output compared to sham treatment. The decrease in urine output was less severe in the SCI rats group that received mitoTEMPO for three days. Importantly, prolonged treatment with mitoTEMPO (14 days) eliminated this decrease, and the measured urine output was similar to the sham group. Although urine output was partially improved in the untreated SCI groups after 14 days, it remained lower than the urine output of sham and mitoTEMPO groups throughout the experiment. The partial recovery of urination in the SCI group was likely due to spontaneous recovery of bladder function and recovery from spinal shock. In addition, the bladder after SCI may respond to sensory information in an independent manner even in the presence of small urine volumes. Sensory nerve fibers carrying sensory information travel through and synapse on an interneuron in the spinal cord. The secondary reflex then takes place and sends a signal to the motor neuron, which in turn stimulates the bladder muscle contraction and urine excretion (125). Although urine output is expected to improve due to these secondary changes, coordination between the bladder and the external sphincter is still lost after SCI.

In animals with thoracic spinal cord injuries, decreased urine output is attributed to damage to the ascending and descending urinary tract, leading to loss of control over both the bladder and external urethral sphincter. This results in urinary retention and DSD. If left untreated, these pathological changes can lead to increased bladder pressure, which can damage the upper urinary tract, a life-threatening condition (126). Therefore, the bladder function recovery observed in the SCI is thought to be partial, as urine output remained significantly lower than that of normal rats up to two weeks after surgery (120). In our study, mitoTEMPO treatment caused urine outputs similar to that of the sham group and higher than the SCI group. Moreover, manual bladder emptying was needed for animals with SCI throughout the experiment, whereas mitoTEMPO-receiving animals started voluntary urination around the fifth day after surgery. These observations suggest that mitoTEMPO may have an effect on areas outside the bladder, such as high-level neurons that control urination by coordinating both the bladder and the external urethral sphincter. It is worth noting that urine output change was not the result of decreased water intake as our results showed the later to be stable even after the surgery and among all groups. As in urine outputs, the same patterns of change were seen in the evaluation of fecal output in different experimental groups. Despite moderate improvement, the SCI group showed reduced fecal excretion, which remained significant until 14 days after surgery. Conversely, after SCI, fecal output in rats receiving mitoTEMPO returned to levels similar to those in the sham group after being decreased in the early stages of SCI. This is of great importance because SCI not only causes neurogenic bladder but also results in serious bowel problems that are also called neurogenic bowel. Intestinal problems arise because SCI reduces intestinal contractions, prolongs bowel transit time, and causes evacuation difficulties (4). Based on our results, administration of mitoTEMPO was able to reverse the neurogenic bowel and bladder caused by SCI that were reflected as improvements in the general homeodynamics of experimental animals with SCI.

The bladder is the organ responsible for storing and emptying urine by contracting or relaxing the detrusor muscle. This coordinated function is accomplished by the central and peripheral nervous systems through different signals, which can be electrical or chemical, operating through receptors or ion channels, respectively. Understanding the mechanisms of detrusor muscle contraction in normal and pathological physiological states is important for developing interventions needed to control bladder dysfunction (8).

In our study, we examined the contraction and relaxation of the isolated bladder detrusor muscle in response to different pharmacological agents to assess changes in bladder function associated with SCI and evaluated the effect of mitoTEMPO on these changes. To evaluate contractility responses, KCl was applied to detrusor muscle strips to increase the extracellular potassium concentration, which will cause a reduction in the transmembrane potassium gradient. As a result, the driving force for potassium efflux will be diminished, leading to depolarization of the membrane and the consequent opening of voltage-dependent L-type Ca^{2+} channels in the plasma membrane, allowing for the rapid influx of extracellular Ca^{2+} . In addition to direct Ca^{2+} entry, this depolarization can also enhance the release of sarcoplasmic reticulum Ca^{2+} through Ca^{2+} -induced Ca^{2+} release mechanisms. Along with increased Ca^{2+} sensitization pathways, these events will cause intracellular calcium to bind to the contractile apparatus, and the resultant cross-bridge cycling will eventually cause the contraction of the bladder detrusor muscle (127). In our study, the application of KCl to the bladder detrusor strips induced contraction in all experimental groups. However, SCI resulted in a significant reduction in KCl-induced contraction compared to the sham groups. This reduction persisted throughout the 14-day experimental period in the SCI groups. While the decreased contraction was moderately reversed with three days of mitoTEMPO administration, the 14-day mitoTEMPO group showed KCl contractile responses similar to those in the sham group. Since KCl-induced contraction does not depend on receptor-mediated mechanisms, the blunt contraction in KCl-activated muscle strips suggests an impairment in depolarization-contraction coupling, which could be attributed to several factors. Downregulation of voltage-dependent L-type Ca^{2+} channels, impaired storage capacity or release of Ca^{2+} from sarcoplasmic reticulum, and contractile apparatus reduced sensitivity to Ca^{2+} are possible explanations of the decreased KCl-induced contraction seen with neurogenic bladder (128). In addition, smooth muscle structural and metabolic changes, such as fibrosis, atrophy, or oxidative stress, may also decrease the bladder contractile capacity in response to KCl (129). Mitochondrial dysfunctions leading to increased ROS levels and diminished ATP production are responsible for reduced KCl-induced detrusor contractility in many disease models. In an ischemia-reperfusion-induced partial bladder outlet obstruction rat model, increased oxidative stress in bladder tissue caused a significant decline in KCl-induced contractile response of bladder smooth muscle, which could be reversed by the use of antioxidants (130). These results suggest that mitoTEMPO administration improved KCl-

mediated contraction by modulating one or more depolarization-contraction coupling mechanisms impaired by SCI, including the increased oxidative stress. To further elucidate the effect of mitoTEMPO on other possible mechanisms involved in this bladder dysfunction model, additional functional analyses were employed using different agents in our study.

Another mechanism that causes contraction of detrusor smooth muscle cells is the initiation of excitation-contraction coupling via activation of muscarinic receptors by the neurotransmitter acetylcholine. This contraction can be induced experimentally by stimulating smooth muscles with electrical current via EFS or by applying CCh, an acetylcholine analog, directly to the tissue in the organ bath. Application of EFS to the detrusor strips depolarizes the neuronal terminals that release neurotransmitters, resulting in neurogenic contractions (131). Our results showed a significant reduction in frequency-dependent contractions induced by EFS in the SCI group. The reduction observed in the SCI groups became more pronounced over time, and 14 days after the procedure, a reduction in contractile responses was also noted at lower frequencies. This reduction was reversed in the SCI groups treated with mitoTEMPO. These results align with clinical SCI studies' findings, where a significant reduction in bladder contractions was demonstrated in SCI patients (132). The decrease in contraction amplitude in bladder tissue may be due to SCI-induced bladder denervation and so a lower amount of neurotransmitter release that induces contraction, or to a lower amount of muscle in each bladder strip, which is seen as reduced muscle contraction response. EFS reveals denervation-induced changes in neurotransmitter release, reflecting the effect of SCI on bladder innervation. KCl induces contraction in a receptor-independent manner via depolarization, which provides insight into the intrinsic properties of the bladder detrusor muscle during contraction (133). In light of these findings, the improvement seen after mitoTEMPO administration suggests that it acts on both neurotransmitter release and bladder muscle integrity.

To initiate the process of urine excretion (micturition), the bladder detrusor muscle contracts in response to the activation of muscarinic receptors. Acetylcholine released from parasympathetic nerves to receptors in the detrusor muscle is the neurotransmitter responsible for this contraction. After binding to its muscarinic receptor, mainly the M3 subtype receptor, acetylcholine causes various changes within the cell by coupling with Gq protein- phospholipase C (PLC) pathway. The activated pathway will cause an increase in intracellular Ca^{2+} levels by

opening voltage-gated Ca^{2+} channels and increasing its release from intracellular stores. In addition, the sensitivity of the cell's contractile system to Ca^{2+} will be increased. All of these events will activate the contractile apparatus in smooth muscle, causing bladder contraction and urine excretion. Any disruption of this process, as seen in patients with SCI, can reduce bladder contractility and lead to dysfunction, leading to urinary retention (134). In our study, SCI caused a significant decrease in bladder contractility in response to the cumulative addition of CCh. This decreased contractility was considered consistent with the acute phase of injury because it was present only in the 3-day treatment groups and resolved within two weeks after surgery. The attenuated contractile response of bladder muscle strips to CCh in the SCI group suggests an impairment of muscarinic receptor-mediated signaling. This decrease may result from muscarinic receptors desensitization or downregulation, or impaired coupling of receptors to the Gq/PLC pathway and its downstream transducers. The decrease observed in the pD_2 value in our results indicates that higher concentrations of CCh are necessary to cause a certain contraction. This observation supports the idea that muscarinic receptors were affected after the SCI. On the other hand, the reduced E_{max} seen after SCI suggests the involvement of alterations in calcium mobilization or sensitivity, and structural changes and remodeling of the detrusor muscle that may also contribute to the attenuated CCh-induced response. However, the 14-day groups showed an improvement in CCh-induced contractility that could be attributed to re-sensitization of muscarinic receptors and/or recovery of G-protein/PLC downstream signaling pathways that have been confirmed in literature as a compensatory response of bladder smooth muscle (135).

Urinary continence is the ability to store urine in the bladder through the relaxation of the detrusor muscle. The relaxation is achieved through the indirect function of adrenoreceptors in the bladder. Activation of α -adrenoceptors is responsible for pre-junctional reduction of acetylcholine release, thus reducing muscarinic receptor-induced contraction. On the other hand, β_3 -adrenoceptors activation will cause bladder smooth muscle relaxation by the activation of adenylyl cyclase and cyclic adenosine monophosphate (cAMP) through G_s protein-coupling pathway which will eventually cause muscle relaxation (128). Therefore, impaired bladder relaxation reflects a decreased response to sympathetic stimulation.

This has also been seen in SCI, where disruption of preganglionic sympathetic neurons resulted in less relaxation of the bladder detrusor muscle, preventing proper bladder filling (120). A similar trend was observed with our findings. Our induction of SCI resulted in significantly lower relaxation response of the detrusor muscle, which persisted for 14 days. MitoTEMPO administration increased bladder relaxation, given that the decreased relaxation was less pronounced in the early phase when compared to SCI groups and completely resolved after 14 days. The reduced Phe-induced relaxation of bladder strips precontracted with CCh observed in our study could be attributed to a diminished adrenergic relaxant mechanism such as downregulation of β 3-adrenergic receptors (136). In addition, urothelial NO release and cGMP signaling have been linked to bladder muscle relaxation. Recently, oxidative stress has been shown to reduce NO bioavailability, thus blunting adrenergic-mediated relaxation. For this reason, Increased SCI-induced oxidative stress could also be responsible of the reduction in bladder muscle relaxation seen in our results (137).

Pathological changes in bladder function in SCI vary widely across clinical practice and research. This is attributed to the complex mechanisms of these changes, which differ depending on the level of injury (sacral or suprasacral), degree of injury (complete or incomplete), and species(49). In this study, bladder muscle strips were challenged with various contractile agents to examine the complex pathophysiological changes in the SCI model. This approach facilitated a thorough evaluation of the effect of mitoTEMPO on bladder changes induced by SCI. The decrease in contractions in response to various agents, such as KCl, EFS, and carbachol, and the decrease in relaxation after phenylephrine application to the detrusor strips reflect changes in bladder contractility, i.e., contraction or relaxation. In animal models of SCI, injury to the lower thoracic level of the spinal cord produces initial bladder areflexia with reduced voiding rates that could be translated into increased residual urine volume or decreased urine output (5, 138).

The results of our study are in line with these findings, where metabolic parameters in experimental animals, such as urine output, showed a significant decrease in the first days after SCI induction, which was supported by the early decrease observed in bladder contractility in functional analysis. Over time, the changes in urinary function in our experimental animals tended to increase after injury, whereas metabolic outcomes remained lower than those in the sham experimental group even 14 days after SCI.

A similar pattern has been experienced in earlier research. With time, the SCI-induced areflexic bladder detrusor muscle shows a gradual increase in contraction response after the emergence of spinal cord reflex pathways. Despite bladder hyperreflexia, the bladder may not be emptied efficiently, and voiding may not return to normal levels due to the loss of bladder and urethral sphincter coordination (139). These SCI-induced changes in micturition reported in previous literature are a probable explanation for the improvement in bladder contractility accompanied by a persistent decrease in our metabolic results. Previous studies have found that mitoTEMPO treatment restored the decreased urine output caused by various injuries to the urinary tract to levels similar to those in normal animals (139). In our study, daily administration of mitoTEMPO to rats with SCI resulted in a significant increase in urine output, and within 14 days, these animals exhibited metabolic outcomes comparable to those of normal animals and better than those of animals not receiving mitoTEMPO. Furthermore, the contraction and relaxation response of the bladder detrusor muscle to various experimental agents returned to normal levels after SCI-induced dysfunction. These improvements in bladder function began earlier and were more pronounced when compared to animals not treated with mitoTEMPO. These results suggest that mitoTEMPO treatment resulted in functional improvement that could be attributed to neural and muscular recovery. The beneficial effects of mitoTEMPO were further confirmed by metabolic and histopathological analyses.

Recent research has been interested in exploring the effect of pathological changes on cellular health and how it would be reflected in disease progression to be targeted later for therapeutic interventions. As a vital source of energy for the cell, mitochondria have been the focus of several studies in this area of research. Mitochondrial morphology and function are interconnected, where any pathological alteration in one of them invariably disturbs the other (140). For that, mitochondrial structure or function is greatly affected by SCI-induced secondary changes, and the same applies in reverse. Mitochondrial proteins play a crucial role in cell structure and health, and their expression level provides a good insight into the general state of mitochondria and the cell as a whole. Mitofusin proteins (Mfn1 and Mfn2) are fusion proteins located in the OMM that work by merging two small mitochondria into one large healthy one. Their expression levels have been reported to be reduced in various ROS-induced pathologies,

including SCI models, and conversely, their upregulation has been shown to ameliorate apoptosis and improve tissue and mitochondrial structure and function in these models. Conversely, excessive mitochondrial fragmentation promotes apoptosis, with fission proteins gradually increasing after cellular damage. Drp1 is another mitochondrial protein, located in the MIM, and it is responsible for mitochondrial fragmentation (fission) to prepare it for apoptosis. Inhibition of the mitochondrial fission protein, Drp1, has been shown to block ROS production and restore mitochondrial and cellular health in SCI and other neurotraumatic and neurodegenerative disease models (141) .

In our study, mitochondrial protein expression in the bladder was dysregulated in SCI animals. SCI caused a significant increase in Drp1 expression and a minor increase in Mfn2 expression. These findings align with what has been reported in previous studies (96). After induction of SCI at the T9 level, mouse urothelium also showed a slight increase in Mfn2 expression but a significant one in Drp1 levels, evidenced by accumulation of a large number of dysfunctional mitochondria in the cytoplasm (96). Although the change in Mfn2 was an increase (not a decrease as expected) and not significantly different from the sham group, the pattern of mitochondrial protein expression followed a similar pattern that was previously mentioned in the literature. In their SCI model, the work of Jia et.al and Cao et al. showed that in the first 8 hours of SCI, Drp1 levels showed an initial decrease while Mfn1 levels increased. At later stage, these changes showed the opposite 105 with a dominant mitochondrial fission dynamics, the same trend of dynamics in our work (85, 142). The use of the mitochondria-targeting antioxidants such as mitoQ and mitoTEMPO can increase mitofusin proteins expression and decrease Drp1 expression, thereby improving mitochondrial biodynamic function after SCI (96, 143). In our results, animals receiving mitoTEMPO showed a slight increase in Mfn2 expression and a lesser increase in Drp1 expression compared to SCI groups with prolonged administration. Mitochondrial protein expression is a dynamic process requiring a healthy balance between fission and fusion. Although mitochondrial protein expression levels did not return to normal, mitoTEMPO restored mitochondrial dynamics closer to the balance necessary for mitochondrial functional integrity. In other words, mitoTEMPO treatment restored the balance between mitochondrial fission and fusion mainly by reducing Drp1 fission protein, which resulted in generating a healthy mitochondrial population.

The functional and molecular results of our study were also supported by our histopathological findings. SCI caused bladder tissue damage, reflected by epithelial disorganization and hemorrhage. This damage was only seen in the acute phase (3 days) after injury in the SCI group and healed after 14 days. On the other hand, mitoTEMPO demonstrated a protective effect from tissue injury, with tissues maintaining normal morphology even in the early stages of injury. Protein import into mitochondria is a dynamic process dependent on transport complexes of the mitochondrial outer membrane, such as the TOM complex. The integrity of this complex can be altered in response to various conditions, including oxidative stress, which negatively impacts the overall health of mitochondria and the cell (81). Long-term enhancement of the mitochondrial protein transfer complex has been linked to better mitochondrial function and may be a potential target for protection against neurodegenerative diseases (144). However, the increase in TOM20 may not always indicate a better mitochondrial state. For instance, TOM20 expression had the tendency to increase in the urothelial cells after SCI. This was explained by the demonstrated increase in mitochondrial fission that produced a large number of dysfunctional mitochondria, thus more expression of mitochondrial proteins (96). Likewise, the SCI group in 106 our study showed an initial increase in TOM20 levels in the bladder, which then increased significantly after 14 days where the fission process was increased as shown in our molecular results. MitoTEMPO treatment maintained normal TOM20 levels in both the early and late stages of SCI. This was demonstrated as the bladder tissue of the mitoTEMPO receiving groups exhibited more similarity to the sham group tissue under the microscope which further supports its overall protective effects on mitochondria. Staining of bladder tissue with anti-Drp1 and anti-Mfn2 antibodies and immunohistochemical analysis results further supported our molecular findings. For tissues treated with Drp1 antibody, SCI groups showed low positive staining, thus lower Drp1 levels, in the early phase. The strong focal brown deposits were obvious in the SCI group after 14 days, but were not present in the group that received mitoTEMPO. The early SCI period also showed the same increase in Mfn2 localization in the tissue, but the brown color became low and closer to the sham group in the late phase with mitoTEMPO treatment. The trend in protein expression change between groups in both the early and late phases of SCI was aligned with the results we obtained through western blot analysis. This further confirmed the positive role of mitoTEMPO in the protection of

mitochondria on both the structural and functional level, contrary to SCI which showed negative impact on the mitochondria in general.

In conclusion, this study demonstrated the protective effect of treatment with mitoTEMPO, a mitochondrial targeting antioxidant, administered at a dose of 1 mg/kg i.p, in the rat model of SCI-induced bladder dysfunction. To our knowledge, this is the first study to evaluate the mitoTEMPO effect on the SCI-induced functional, molecular, and histopathological changes in the urinary bladder in the rat model. According to our study, the SCI induced by compression at the thoracic vertebrae T9 caused decreased contractility of the urinary bladder detrusor muscle. Molecular analysis of bladder tissue revealed an imbalance in the oxidative state of the mitochondria, indicated by an increase in fission proteins, which are elevated under oxidative stress and lead eventually to apoptotic cell death. mitoTEMPO administration restored the dysfunction observed in the urinary bladder, including functional, molecular, and histopathological changes. In light of these data, it is thought that mitoTEMPO antioxidant action was effective in modifying mitochondrial dynamics, which in turn resulted in functional improvement in SCI-induced bladder dysfunction. MitoTEMPO is a promising therapy with high potential for treating health problems associated with oxidative stress including diseases resulting from neurotrauma or neurodegeneration. Future research is warranted to elucidate the mechanisms and effects of mitoTEMPO to apply it to other SCI-related dysfunctions. Furthermore, similar future research will allow us to understand the dysfunctions resulting from SCI better and tailor mitoTEMPO use to become suitable for similar health problems.

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ÖZET

Mitokondri Hedefleme Ajanı Olan Mitotempo'nun Sıçan Omurilik Hasarına Bağlı Mesane Disfonksiyonunda Terapötik Etkileri

Omurilik hasarı (OİH; spinal cord injury-SCI), santral merkezler ve periferik sistem arasında sinyal alışverişini sağlayan nöronlarda meydana gelen travmatik bir yaralanmadır. OİH, omurilik sinirlerinde oluşan hasar sonucu omuriliğin işlevlerinde geçici veya kalıcı değişikliklere neden olur. Son epidemiyolojik çalışmalar, trafik kazalarının omurilik yaralanmalarının başlıca nedeni olarak kabul edildiğini ve erkeklerde daha sık görüldüğünü göstermektedir. OİH 'nin diğer nedenleri arasında düşmeler, şiddetli yaralanmalar ve omurilik felci, sıkışması veya omurilik iskemisiyle sonlanan tümörler yer almaktadır. Her yıl rapor edilen 130 bine yakın yeni vakanın büyük bir bölümünü ise çalışma hayatının en verimli döneminde olan sağlıklı genç bireyler oluşturmaktadır. Dolayısıyla hasar sonrası gelişen somatik, otonomik ve psikososyal sorunlar birçok açıdan önemli iş gücü kayıplarına sebep olmaktadır. OİH hastaları, tıbbi tedavi veya bireyin çalışma yeteneğini kaybetmesi yoluyla, hastanın ailesi ve tüm toplum için büyük bir ekonomik yüke yol açan fiziksel, psikolojik ve bilişsel sonuçlarla maruz kalabilmektedir. OİH, anatomik bölgeye ve yaralanmanın şiddetine bağlı olarak birçok sistemde uzun dönemli komplikasyonlara neden olarak yüksek morbidite ve düşük yaşam kalitesine yol açabilmektedir. Çizgili kas/motor sistem fonksiyon kaybı ve nöropatik ağrı başta olmak üzere gelişen üriner, gastrointestinal, kardiyovasküler ve respiratuvar sistem komplikasyonlarının ana kaynağı omurilik kaynaklı efferent nöronlarda veya santrale gelen afferent nöronlarda meydana gelen dejenerasyondur. Bunun sonucunda, hedef dokularda metabolik disfonksiyon ve histopatolojik değişikliklerle gelişen fonksiyon kayıplarına sebep olmaktadır. Mesane disfonksiyonu, OİH sonrası karşılaşılan ve tedavisine yönelik klinik yaklaşımların kısıtlı olduğu önemli bir morbiditedir. OİH'lı bireylerde mesane disfonksiyon prevalansı yaklaşık %60 civarındadır. Klinik tablo meydana gelen travmanın şiddeti, omurga üzerindeki konumu ve etkilenen nöronların tipine bağlı olarak az aktif mesane, mesane aşırı aktivitesi veya inkontinansı şeklinde kendini gösterebilmektedir. Klinikte mesane disfonksiyonun tipine göre farklı farmakolojik seçenekler uygulanabilmektedir.

Yan etki profilleri (bulanık görme, gastrointestinal sistem bozuklukları ve kognitif bozukluklar) ve etkilerinin zamanla azalması sebebiyle bu ajanların kullanımı hastanın yaşam kalitesini etkileyebilmekte; etkin terapötik doza ulaşamamasına, hasta uyuncunun azalmasına ve hatta ilacın kesilmesine neden olabilmektedir. Ayrıca mevcut tedavilerin etkinliğinin sınırlı ve pahalı olması ise hastaların iş gücünü, yaşam kalitesini ve süresini azaltarak sosyoekonomik anlamda önemli bir yük oluşturmaktadır. Klinik gözlemler, OİH kaynaklı mesane disfonksiyonunun diğer OİH kaynaklı problemler göre çok daha az araştırıldığı ve restorasyonu için mevcut tedavi protokollerinden daha etkili ve güvenilirliği daha yüksek yeni terapötik yaklaşımlara ihtiyaç duyulduğunu işaret etmektedir. OİH kaynaklı mesane disfonksiyonu patogenezinde nöronal hasar sonrası bozulan mesane inervasyonu nedeniyle, mesane dokusunda meydana gelen mitokondriyal hasar ve oksidatif stres kaynaklı anomalilerin önemli rolü olduğu bilinmektedir. Bu bilgiler ışığında, nöronal hasar kaynaklı patolojik değişikliklerin yavaşlatılabilmesi veya durdurulabilmesi adına mesane dokusundaki mitokondriyal oksidatif hasarının farmakolojik olarak hedeflenmesinin önemli bir tedavi stratejisi olduğu düşünülmektedir. Mitokondri, elektron taşıma zinciri (ETC) ve ilgili reaksiyonlar yoluyla hücrenin ana enerji kaynağıdır; hücre yaşam siklusunda önemli bir rol oynar. ATP sentezi sırasında, mitokondriyal elektron taşınması elektronları oksijene sızdırarak reaktif oksijen türleri (ROS), özellikle de süperoksit oluşturabilir. Aşırı ROS, oksidatif strese yol açarak mitokondriyal DNA, lipid ve proteinlere zarar verir, işlevi bozar ve hücre ölümüne neden olur. DRP1 tarafından fisyon ve MFN1/2 tarafından füzyon dâhil mitokondriyal dinamikler, stres altında mitokondri bütünlüğünün korunmasına yardımcı olur. Füzyon, sağlıklı ve hasarlı mitokondrilerin içeriklerini karıştırarak hasarı azaltırken, fisyon hasarlı kısımları mitofaji yoluyla bozunmaya karşı izole ederek hücrel homeostazı korur. Mitokondriyal protein sentezi için gereken tüm öncüller, başlangıçta bu öncüller için ortak bir reseptör görevi gören dış zar alt birimi TOM20 ile bir TOM kompleksinden geçerek mitokondriye girer. Mitokondriyal dinamikler ve protein translokazları, hem fizyolojik hem de patolojik koşullar altında mitokondriyal homeostaz için önemlidir. Ancak, uzun süreli stres veya hücre hasarı sırasında, bu süreçler düzensizleşebilir ve mitokondriyi olumsuz etkileyen ilgili proteinlerin yapısında ve seviyelerinde değişiklikler meydana gelebilir. OİH'nin mitokondriyal disfonksiyona ve aşırı ROS üretimine neden olduğu, bunun da ürotelyal hasara ve mesanede birçok olumsuz sonuca yol açtığı gösterilmiştir. MitoTEMPO, mitokondriyal süperoksit ve diğer radikaller için spesifik

olan, mitokondriyi hedefleyen bir antioksidandır. Omurilik yaralanmaları sonrasında mitokondriyal oksidatif stres artışı nöronal dejenerasyon ile birlikte mesane dokusundaki hücresel ve yapısal değişiklikleri indükleyerek mesane disfonksiyonuna sebep olduğundan, mitokondriyal oksidan mekanizmalar üzerinde inhibitör etkili bir mitokondri hedefleme ajanı olan mitoTEMPO'nun sitoprotektif etkisi ile OİH kaynaklı mesane disfonksiyon gelişimini engelleyebileceği düşünülmektedir. Çalışmamızın amacı "OİH sonrasında mitokondriyal oksidatif stresin artması ile nörodejenerasyon ve mesanedeki hücresel/yapısal değişikliklere bağlı olarak gelişen mesane disfonksiyonunu, mitokondriyal oksidan mekanizmalar üzerinde inhibitör etkili sitoprotektif bir mitokondri hedefleme ajanı olan mitoTEMPO engeller" hipotezini test etmek üzere, mitoTEMPO'nun OİH modeli ile indüklenen mesane disfonksiyonu tedavisindeki zamana bağlı etkilerinin metabolik, fonksiyonel, biyokimyasal, histopatolojik ve immunohistokimyasal düzeyde araştırılmasıdır. Çalışmanın ana hedefleri, mitoTEMPO tedavisinin metabolik sonuçlar (vücut ağırlığı, idrar çıkışı vb.) üzerindeki etkisinin araştırılması, OİH modeli uygulanan sıçanlarda zamana bağlı mitoTEMPO tedavisinin mesane detrusor fonksiyonuna etkisinin araştırılması; OİH modeli uygulanan sıçanlarda zamana bağlı mitoTEMPO tedavisinin mesane dokusundaki mitokondri ile ilişkili moleküllere (Mrf2, Drp1, TOM20) etkisinin araştırılması; OİH modeli uygulanan sıçanlarda zamana bağlı mitoTEMPO tedavisinin mesane dokusundaki hücre içi yapılardaki değişiklikler, mitokondri bütünlüğü ve doku morfolojisi üzerindeki etkisinin araştırılması. Çalışmamız hipotezinin prelinik çalışmalarda yeri olan mitokondriyal hedefli bir antioksidan ajan üzerine kurgulanmış olması literatüre yeni bilgiler sunacak olması açısından önemlidir. Çalışma amacına yönelik planlanan ana hedeflerden elde edilen bulgularla günümüzde yeni tedavi yaklaşımları açısından yoğun bir şekilde araştırmaların devam ettiği OİH kaynaklı mesane disfonksiyonu patofizyolojisinin mitokondriyal düzeyde kapsamlı anlaşılması ve mitoTEMPO'nun klinik araştırmalar seviyesine gelebilmesi için hem ön veriler elde edilmesi hem de literatüre önemli katkı sağlaması hedeflenmektedir.

Çalışmamızda 8-12 haftalık (275 ± 25 g) Sprague-Dawley dişi sıçanlar kullanıldı ve altı gruba ayrıldı: 3 günlük sham, SCI (OİH), ve SCI + mitoTEMPO grubu; 14 günlük sham, SCI (OİH), ve SCI + mitoTEMPO grubu. Tedavi gruplarına göre sıçanlara mitoTEMPO (1 mg/kg) veya taşıyıcı (serum fizyolojik, SF), cerrahi girişim günü (1. gün) başlamak üzere 3 ve 14 gün boyunca günde bir kez intraperitoneal (i.p.) olarak uygulandı. OİH kaynaklı mesane disfonksiyonu için suprasakral kompresyon modeli kullanıldı. Model gruplarında laminektomi ardından omuriliğe torasik vertebra T9 seviyesinde 60 saniye boyunca forseps ile kompresyon uygulandı. Lezyon, araştırmalarda uygulanan standart protokolle uyumlu olarak T9 seviyesinde gerçekleştirildi. Literatüre göre, güvenilirlik, tekrarlanabilirlik ve servikal seviyedeki yaralanmaya kıyasla daha düşük solunum depresyonu riski nedeniyle torasik bölge, OİH indüksiyonu için en yaygın lokasyondur. Özellikle, T8 ve T9'un alt torasik seviyeleri, OİH kaynaklı mesane disfonksiyonunun indüksiyonu için mesane innervasyonunu bozmak için tercih edilir. T9 seviyesindeki omurilik kontüzyonunun hayvanların mesane fonksiyonunu bozduğu, T1 seviyesindeki lezyonların ise mesane fonksiyonunu koruyarak minimal idrar retansiyonuna neden olduğu gösterilmiştir. Sham gruplarında, sham cerrahi işlemler yapıldı fakat laminektomi yapılmadan insizyon bölgesi cerrahi sütur ile kapatıldı. Cerrahi işlemler bir gün önce başlanarak, her sıçanın ağırlığı ötanazi gününe kadar günlük olarak ölçüldü ve kaydedildi. Tüm sıçanlar cerrahi işlemlerden önceki gün (0. Gün) ve ilacın uygulanmasının son gününde (3. ve 14. Günler) tek metabolik kafeslere yerleştirildi ve metabolik kafeslerdeki 24 saatlik idrar hacimleri ve dışkı miktarı ölçüldü ve kaydedildi. Metabolik veriler gruplar arasında zamana bağlı olarak değerlendirildi. Son ilaç dozu uygulamasından 24 saat sonra sıçanlara anestezi uygulaması yapıldı ve mesane izole edildi. Mesanenin kas dokusu şeride kesildi. İki şerit fonksiyonel analiz için kullanıldı, kalan şeritleri ise sıvı azotta hızlıca donduruldu ve moleküler (western blot) analizleri için -80°C 'de saklandı. Histopatolojik analiz için mesane çıkarıldı ve parafinle muamele edildi ve analiz için doku kesitleri hazırlandı. Çalışmamızda tüm gruplardaki deney hayvanlarının vücut ağırlıkları, deneyin son gününe kadar herhangi bir müdahaleden önce günlük olarak ölçüldü. Farklı deney grupları arasında cerrahi işlem (OİH/SCI grubu) öncesi vücut ağırlıklarında bir fark görülmemesine rağmen, OİH oluşturulan hayvanlarda işlem sonrası vücut ağırlıklarında önemli bir azalma meydana geldi. Bununla birlikte, OİH ve mitoTEMPO alan hayvanlar (SCI+ mitoTEMPO), işleminden bir hafta sonra başlayarak sham grubuyla benzer değerlere ulaşana kadar vücut ağırlıklarını geri kazanmaya

başladı. Yine de, OİH gruplarındaki hayvanlar, deneyin sonunda sham grubundan önemli ölçüde farklı hale gelen vücut ağırlığındaki azalmayı korudu. OİH cerrahisi kaynaklı stres ve kas atrofisi, OİH modelinde vücut ağırlıklarını kaybından sorumlu olabilir. Bu nedenle mitoTEMPO'nun olumlu etkileri, hayvan çalışmalarında stres seviyelerinde ve genel sağlıkta sham gruplarıyla karşılaştırılabilir iyileşme olduğunu göstermektedir. Deney hayvanları tarafından yapılan su alımları değerlendirildiğinde, işlem öncesi ve sonrası ve tüm gruplar arasında herhangi bir değişiklik görülmedi. Bu durum, stres veya ameliyat nedeniyle görülen değişikliklerin hayvanların susuzluğunu ve su alımını vücut ağırlıklarında yaptığı gibi etkilemediğini göstermektedir. Deney hayvanlarının idrar atım miktarları, OİH kaynaklı mesane disfonksiyonu modelinde fonksiyonel değişiklikleri izlemek için tüm deney gruplarında değerlendirildi. OİH işlemi, sham işlemine kıyasla atılan idrar miktarında ciddi düşüşe neden oldu. İdrar çıkışındaki azalma, 3 gün boyunca mitoTEMPO alan OİH grubu sıçanlarda ise daha az şiddetliydi. Önemli olarak, mitoTEMPO ile uzun tedavi (14 gün) bu düşüşü ortadan kaldırdı ve idrar çıkışı sham grubuna benzer ölçüldü. Tedavi uygulanmayan OİH gruplarında 14 gün sonunda idrar çıkışında kısmen iyileşme olmasına rağmen, yine de 14 gün sonundaki sham ve mitoTEMPO alan gruplardan farklı kaldı. MitoTEMPO uygulaması ile idrar çıkışının sham grubuna benzer ve OİH'li gruptan ise yüksek olması yani idrar fonksiyonunda belirgin bir iyileşmeye neden olması, mitoTEMPO'nun mesane dışındaki alanlarda, örneğin hem mesane hem de dış üretra sfinkteri aracılığıyla idrar yapma sürecini kontrol eden yüksek seviyeli nöronlar üzerinde bir etkiye sahip olabileceğini düşündürmektedir. Aynı değişim farklı deney gruplarında dışkı çıktılarının değerlendirilmesinde de görüldü. OİH grubu, orta düzeyde iyileşmeye rağmen ameliyattan 14 gün sonrasına kadar önemli olmaya devam eden azalmış dışkı atılımı gösterdi. Öte yandan, OİH işleminden sonra mitoTEMPO alan sıçanların dışkı çıktısı, OİH'nin erken evresinde azalma göstermesine rağmen, sham grubuna yakın seviyelere döndü. Sonuçlarımıza göre, mitoTEMPO uygulaması OİH yapılan deney hayvanlarının genel fizyolojisinde iyileşme şeklinde yansıyarak OİH kaynaklı nörojenik bağırsak ve mesaneyi tersine çevirebilmiştir. MitoTEMPO'nun olumlu etkileri, fonksiyonel, metabolik ve histopatolojik analizlerle daha da doğrulandı. Detrusor kasının fonksiyonu organ banyosu sistemi kullanılarak değerlendirildi. Şeritlerinin izometrik gerim değişiklikleri iki kanaldaki güç değiştirici transdüser ile kaydedildi. Çalışmamızda, OİH'ye bağlı mesane fonksiyonundaki değişiklikleri değerlendirmek amacıyla izole mesane detrusor kasının farklı ajanlara yanıt olarak

kasılması ve gevşemesini inceledik ve bu değişiklikler üzerine mitoTEMPO'nun etkisini değerlendirdik. Mesane kontraktilitesinde, voltaj kapılı kalsiyum (Ca^{2+}) kanallarının aktivasyonu düz kasların kasılmasını aktive eder. Detrusordan hazırlanan şerit şeklindeki kesitlere, düz kas hücrelerinin depolarizasyonu ile L-tipi Ca^{2+} kanallarının açılmasına ve hücre içi kalsiyum girişine neden olarak detrusor kasının kasılmasına yol açan potasyum klorür (KCl) uyguladık. Bu çalışmada, KCl'nin mesane detrusor şeritlerine uygulanması tüm deney gruplarında kasılmaya neden oldu. Ancak OİH, sham gruplarıyla karşılaştırıldığında KCl kaynaklı kasılmada önemli bir azalmaya neden oldu. Bu azalma OİH uygulanan gruplarda 14 günlük deney süresi boyunca devam etti. MitoTEMPO'nun 3 günlük uygulaması ile azalmış kasılma orta düzeyde tersine dönerken, 14 gün mitoTEMPO alan grupta, KCl kasılma yanıtları sham grubuna yakın değerler gösterdi. Bu sonuçlar, OİH ile bozulan KCl aracılı kasılma mekanizmalarının mitoTEMPO uygulaması ile düzeldiğini göstermektedir. Detrusor düz kas hücrelerinde kasılmaya neden olan diğer mekanizma, nörotransmitter asetilkolin aracılı muskarinik reseptörlerin aktivasyonu ile uyarılma-kasılma eşleşmesinin başlamasıdır. Bu kasılma uyarısı, deneysel olarak düz kasın elektrik akımı ile uyarılması (elektiriksel alan stimülasyonu-EFS) veya dokunun bulunduğu organ banyosu ortamına doğrudan asetilkolin analogu olan karbakol uygulamasıyla yapılabilir. Detrusor şeritlerine EFS uygulanması, nörotransmitterleri serbest bırakan nöron terminallerini depolarize ederek nörojenik kasılmalara neden olur. Çalışmamızda, OİH uygulanan grupta EFS kaynaklı frekans bağımlı kasılmalarda önemli azalma oldu. OİH gruplarında görülen azalma, zamana bağlı olarak daha belirgin hale geldi ve işlemden 14 gün sonra, daha düşük frekanslarda da kasılma yanıtlarında azalma kayıt edildi. MitoTEMPO uygulanan OİH grubunda bu azalma tersine çevrildi. İdrar atılımı sürecini (miktürisyon) başlatmak için, mesane detrusor kası muskarinik reseptörlerin aktivasyonu etkisiyle kasılır. Parasempatik sinirlerden detrusor kasındaki reseptörlere salınan asetilkolin, bu kasılmadan sorumlu nörotransmitterdir. Reseptörüne bağlandıktan sonra, hücrede voltaj kapılı kalsiyum kanallarının açılması, hücre içi depolardan kalsiyum salınımı ve hücrenin kasılma sisteminin kalsiyuma karşı duyarlılığının artması gibi çeşitli değişikliklere neden olur. Tüm bu olaylar mesanenin kasılması ve idrara çıkma ile sonuçlanır. Bu sürecin herhangi bir nedenle bozulması, OİH hastalarında görüldüğü gibi mesane kontraktilitesini ve idrar retansiyonuna neden olan disfonksiyonu azaltabilir. Çalışmamızda, mitoTEMPO'nun OİH ile indüklenen kolinerjik kasılmalarındaki değişikliklere olan etkisini değerlendirmek için, mesane detrusor

şeritleri artan konsantrasyonlarda karbakol (10^{-9} - 10^{-4} M) eklenerek kasıldı ve kümülatif konsantrasyon-cevap eğrileri elde edildi. Karbakol, tüm tedavi gruplarında mesane detrusor şeritlerinde konsantrasyona bağlı kasılmaları indükledi. Ancak, kasılma genliği SCI ve SCI+mitoTEMPO 3 günlük gruplarında önemli ölçüde azaldı ve bu daha düşük genlik ve Emax% olarak görüldü. Azalmış olmasına rağmen, SCI grubunda karbakol ile indüklenen kasılma parametrelerinde diğer 14 günlük tedavi gruplarıyla karşılaştırıldığında hiçbir fark görülmedi. Hücre dışı Ca^{2+} akışının depolarize edici koşullar altında mesane kasılması üzerindeki reseptör-bağımsız, doğrudan etkilerini değerlendirmek amacıyla $CaCl_2$ kullanılarak kümülatif yanıt eğrisi oluşturulması planlanmıştır. Ancak gerçekleştirilen organ banyosu deneyinden elde edilen veriler bilimsel açıdan yetersiz bulunmuş ve deneysel protokolün modifikasyonu gerekliliği ortaya çıkmıştır. Bu nedenle, söz konusu bulgular bu raporda sunulmamış ve tartışılmamıştır. Mesanede idrarın dolup depolanabilmesi için detrusor kasının gevşemesi gerekir ve bu, sempatik sistemin etkisiyle gerçekleşir. Mesane gevşemesinin azalması, sempatik uyarıya verilen yanıtın azalması anlamına gelir. Bu durum, preganglionik sempatik nöronların bozulmasının mesane detrusor kasının daha az gevşemesine ve mesanenin düzgün dolmasını engellemesine neden olduğu SCI'de de görülür. MitoTEMPO'nun OİH kaynaklı gevşeme değişiklikleri üzerindeki etkisini değerlendirmek için, mesane detrusor şeritleri submaksimal dozda karbakol (10^{-5} M) ile önceden kasıldı ve ardından kümülatif konsantrasyon-tepki eğrileri elde etmek için artan konsantrasyonlarda fenilefrin (10^{-8} – 10^{-4} M) eklendi. Fenilefrinin kümülatif eklenmesi, tüm deney gruplarında konsantrasyona bağlı olarak detrusor kasının gevşemesine neden oldu. Üç günlük gruplarda, SCI, sham grubuyla karşılaştırıldığında fenilefrine karşı önemli ölçüde azalmış gevşeme yanıtına neden oldu. Anlamlı olmasına rağmen, SCI + mitoTEMPO'daki gevşeme yanıtındaki azalma, SCI grubunda görülen azalmadan daha azdı. On dört günlük SCI grubu da sham grubuyla karşılaştırıldığında gevşeme yanıtında önemli bir azalma gösterirken, mitoTEMPO azalmayı tersine çevirdi ve sham grubuyla karşılaştırılabilir gevşeme yanıtı gösterdi. Mesane detrusor kasının farklı deneysel ajanlara karşı kasılma ve gevşeme tepkisi, OİH kaynaklı işlev bozukluğundan sonra normal seviyelere döndü. Mesane fonksiyonundaki bu iyileştirmele daha erken başladı ve mitoTEMPO uygulanmayan hayvanlara kıyasla daha iyi sonuçlandı. Bu sonuçlar mitoTEMPO tedavisinin fonksiyonel iyileşmeye neden olduğunu göstermektedir. Omurilik yaralanmasından sonra mitokondriyal dinamiklerdeki değişiklikleri değerlendirmek amacıyla, mesanedeki Drp1

ve Mfn2 proteinlerinin ekspresyonu western blot yöntemiyle belirlendi. Üç günlük tedavi uygulandıktan sonra mitokondriyal fisyon proteini Drp1 ekspresyonunda, sham grubuna kıyasla SCI ve SCI+ MT gruplarının hiçbirinde anlamlı bir değişiklik görülmedi. 14 günlük tedavi grubundaki Drp1 ekspresyonuna bakıldığında, sham grubuna kıyasla SCI grubunda yaklaşık 3 katlık anlamlı bir artış görüldü. SCI+ MT grubunda ise yaklaşık 2,5 katlık anlamlı bir artış tespit edildi. Ancak SCI grubuna kıyasla SCI+ MT grubundaki Drp1 ekspresyonundaki azalma anlamlı değildi. Omurilik yaralanmasından sonra 3 ve 14 günlük tedavi gruplarındaki mitokondriyal füzyon proteini Mfn2 ekspresyonunda, tedavi gruplarının kendi içerisindeki sham gruplarına kıyasla hem SCI hem de SCI+ MT gruplarında artış görüldü. Ancak bu artışlar anlamlı değildi. Sonuçlarımızda, mitoTEMPO alan hayvanlar OİH gruplarıyla karşılaştırıldığında Mfn2 ekspresyonunda hafif bir artış ve Drp1 ekspresyonunda daha düşük bir artış gösterdi. Mitokondriyal protein ekspresyonu, fisyon ve füzyon süreçleri arasında sağlıklı bir dengenin gerekli olduğu dinamik bir süreçtir. Mitokondriyal proteinlerin ekspresyon düzeyleri normale dönmese de mitoTEMPO, mitokondriyal dinamiklerin, mitokondriyal fonksiyon bütünlüğünü sağlayan gerekli dengeye daha yakın olmasını sağladı. Başka bir deyişle, mitoTEMPO tedavisi mitokondriyal fisyon ve füzyon arasındaki dengeyi yeniden sağlayarak sağlıklı bir mitokondri popülasyonu oluşturdu. Epitel düzensizliği ve hemoraji varlığı, H&E boyası ile boyanmış mesane dokularının slaytlarında değerlendirildi. Tüm kontrol gruplarının mesaneleri normal histolojik yapı gösterdi. Üç günlük gruplarda, SCI grubu epitel düzensizliği ve hemoraji gösterirken, SCI+ MT grubunda epitelyum normal ve düzenli olup hemoraji bulgularına rastlanmamıştır. On dört günlük tüm tedavi gruplarında normal epitelyum gösterildi ve hemoraji bulunamadı. Sonuç olarak mitoTEMPO, dokuların yaralanmanın erken evrelerinde bile normal morfolojisini koruyarak doku yaralanmasına karşı koruyucu bir etki göstermiştir. Dokular öncelikle non-primer ile immunohistokimyasal boyama yöntemiyle boyandı. Dokularda boyanma görülmemesi yöntemimizin doğruluğunu gösterdi. Daha sonra her gruba TOM20, Drp1, Mfn2 antikoruyla muamele edilerek immunohistokimyasal analizi gerçekleştirildi. Elde edilen sonuçlara göre, 3 günlük SCI grubunda daha koyu kahverengi boya gösterdiği için TOM20 miktarında bir artış gözlemlendi. MitoTEMPO benzer miktarda kahverengi boya gösterdi, dolayısıyla TOM20 miktarı sham grubuna benzerdi. Ondört günlük SCI grubunda, TOM20 ile boyama oranları, sham grubu boyanma oranları da benzer görünen SCI+ MT grubuna kıyasla belirgin şekilde azaldı. MitoTEMPO tedavisi, OİH 'nin hem erken hem de

geç evrelerinde normal TOM20 seviyelerini korumuştur. Bu durum, mitoTEMPO alan grupların mesane dokusunun mikroskop altında sham grubu dokusuna daha fazla benzemesiyle gösterilmiştir; bu da mitokondri üzerindeki genel koruyucu etkilerini daha da desteklemektedir. Drp1 antikoru ile tedavi edilen dokularda, 3 günlük gruplar, SCI ve SCI+ MT grupları, sham grubuna kıyasla daha düşük pozitif boyanma gösterdi. 14 günlük sham ve SCI+ mitoTEMPO, çok az veya hiç boyanma göstermezken, SCI grubu idrar kesesi dokusunda güçlü fokal kahverengi kromojen birikintileri gösterdi. Mfn2 antikoruyla bağlı kahverengi boyanma seviyesi, hem 3 günlük SCI hem de SCI+ mitoTEMPO'da sham grubuna kıyasla artmıştı. Ancak, boyanma 14 günlük SCI grubunda çok daha düşüktü, ancak birbirine benzer olan 14 günlük sham ve SCI+ mitoTEMPO'da belirgin kaldı. OİH'nin hem erken hem de geç evrelerinde gruplar arasındaki protein ekspresyonu değişimi, western blot analiziyle elde ettiğimiz sonuçlarla uyumludur. Bu durum, genel olarak mitokondriler üzerinde olumsuz etki gösteren OİH'nin aksine, mitoTEMPO'nun mitokondrilerin hem yapısal hem de işlevsel düzeyde korunmasındaki olumlu rolünü daha da doğrulamaktadır. Çalışmamızda, OİH 'li sıçanlara günlük mitoTEMPO uygulanması idrar çıkışında önemli bir artışa neden olmuş ve 14 gün içinde bu hayvanlar normal hayvanlarla karşılaştırılabilir ve mitoTEMPO almayan hayvanlardan daha iyi metabolik sonuçlar sergilemiştir. Ayrıca, mesane detrusor kasının çeşitli deneysel ajanlara verdiği kasılma ve gevşeme tepkisi, OİH kaynaklı işlev bozukluğundan sonra normal seviyelere dönmüştür. Mesane fonksiyonundaki bu iyileşmeler daha erken başlamış ve mitoTEMPO ile tedavi edilmeyen hayvanlara kıyasla daha belirgin olmuştur. Bu sonuçlar, mitoTEMPO tedavisinin sinir ve kas hücrelerinin iyileşmesine bağlı olabilecek fonksiyonel iyileşmeye yol açtığını göstermektedir. Ek olarak, mitokondriyal protein ekspresyonu OİH hayvanlarında düzensizlik gösterirken, mitoTEMPO bu olumsuz değişiklikleri önledi veya azalttı. Histopatolojik sonuçlarımız da kullandığımız OİH modelinde mitoTEMPO'nun metabolik ve işlevsel yararlı etkisini destekledi. OİH'nin hücresel hasar ve çoklu organ disfonksiyonuna yol açan oksidatif stresle bağlantılı olduğu göz önüne alındığında, OİH kaynaklı mesane disfonksiyonunda görülen iyileşmeler, daha düşük mitokondriyal oksidatif stres seviyelerine bağlanabilir. Sonuç olarak, bu çalışma, sıçan modelinde OİH ile indüklenen mesane disfonksiyonunda 1 mg/kg/gün dozunda uygulanan mitokondriyal hedefleyen bir antioksidan olan mitoTEMPO tedavisinin koruyucu etkisini göstermektedir. Bilgilerimize göre, bu, sıçan modelinde OİH ile indüklenen mesanedeki fonksiyonel, moleküler ve histopatolojik değişiklikler üzerine mitoTEMPO etkisini

değerlendiren ilk çalışmadır. Çalışmamıza göre, torasik vertebra T9'da kompresyonla indüklenen OİH, mesane detrusor kasının kasılabilirliğini azaltmıştır. Mesane dokusunun moleküler analizi, fisyon proteinlerindeki artışla gösterilen mitokondrinin oksidatif durumundaki dengesizliği ortaya koymuştur; bu proteinler oksidatif stres altında yükselir ve sonunda apoptotik hücre ölümüne yol açar. mitoTEMPO uygulaması, fonksiyonel, moleküler ve histopatolojik değişiklikler dâhil olmak üzere mesanede gözlenen disfonksiyonu düzeltmiştir. Bu veriler ışığında, mitoTEMPO antioksidan etkisinin mitokondriyal dinamikleri değiştirmede etkili olduğu ve bunun da OİH kaynaklı mesane disfonksiyonunda fonksiyonel iyileşmeye yol açtığı düşünülmektedir. MitoTEMPO, nörotravma veya nörodejenerasyondan kaynaklanan hastalıklar da dâhil olmak üzere oksidatif stresle ilişkili sağlık sorunlarının tedavisinde yüksek potansiyele sahip, ümit verici bir tedavidir. MitoTEMPO'nun mekanizmalarını ve etkilerini açıklığa kavuşturmak ve diğer OİH ile ilişkili işlev bozukluklarına uygulamak için gelecekte yapılacak araştırmalara ihtiyaç vardır. Dahası, gelecekte yapılacak benzer araştırmalar, OİH kaynaklı işlev bozukluklarını daha iyi anlamamızı ve mitoTEMPO kullanımını benzer sağlık sorunlarına uygun hale getirmemizi sağlayacaktır.

Anahtar Kelimeler: Antioksidan, Mesane, Drp1, Mfn2, Mitokondri, Organ Banyosu, Sıçan, TOM



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PUBLICATIONS (written according to thesis writing rules)

Altarifi AA, Kalha Z, Kana'An SF, Alfaqih MA, Alsalem MI (2019). Effects of combined swimming exercise and non-steroidal anti-inflammatory drugs on inflammatory nociception in rats. *Experimental and Therapeutic Medicine* 17(5): p. 4303-4311.

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