UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE MEDICINA
BIOMEDICINA

(PROYECTO)

EFECTO DE LA TIBOLONA SOBRE LA MUERTE NEURONAL Y LA RECUPERACIÓN

MOTORA EN UN MODELO DE LESIÓN TRAUMÁTICA DE MÉDULA ESPINAL EN RATA

TESIS

(**POR ARTÍCULO CIENTÍFICO**)

TIBOLONE IMPROVES LOCOMOTOR FUNCTION IN A RAT SPINAL CORD INJURY MODEL

BY MODULATING APOPTOSIS AND AUTOPHAGY

QUE PARA OPTAR POR EL GRADO DE:

MAESTRO EN CIENCIAS BIOLÓGICAS

PRESENTA:

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CIUDAD UNIVERSITARIA, CD. MX., FEBRERO, 2024

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M. en C Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **11 de septiembre de 2023** se aprobó el siguiente jurado para el examen de grado de **MAESTRO EN CIENCIAS BIOLÓGICAS** en el campo de conocimiento de **Biomedicina** del alumno **HEREDIA NIETO ALEJANDRO** con número de cuenta **521014371** por la modalidad de graduación de **tesis por artículo científico** titulado: **"TIBOLONE IMPROVES LOCOMOTOR FUNCTION IN A RAT SPINAL CORD INJURY MODEL BY MODULATING APOPTOSIS AND AUTOPHAGY",** que es producto del proyecto realizado en la maestría que lleva por título: **"EFECTO DE LA TIBOLONA SOBRE LA MUERTE NEURONAL Y LA RECUPERACIÓN MOTORA EN UN MODELO DE LESIÓN TRAUMÁTICA DE MÉDULA ESPINAL EN RATA,** ambos realizados bajo la dirección de la **DRA. ANGÉLICA BERENICE COYOY SALGADO,** quedando integrado de la siguiente manera:

Presidente:	DRA. MARÍA DE LOS ÁNGELES ARACELI DÍAZ RUIZ
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Secretario:	DR. JAIME IVÁN VELASCO VELÁZQUEZ

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Ciudad Universitaria, Cd. Mx., a 08 de enero de 2024

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

c. c. p. Expediente del alumno

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Abreviaturas

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Abreviatura	Significado
Akt o PKB	Protein kinase B; proteína cinasa B
AMPK	Adenosine monophosphate activated kinase; proteína cinasa activada por monofosfato de adenosina
Apaf-1	Apoptosis protease-activating factor 1; factor apoptótico 1 activador de proteasas
BAK	Bcl-2 homologous antagonist killer
BAX	Bcl-2 associated X protein; proteína X asociada a Bcl-2
Bcl-2	B-cell lymphoma 2; linfoma de células B2
CA3	Cuerno de Amón 3
Cyt-C	Citocromo C
DAMPs	Danger associated molecular patterns; patrones moleculares asociados a daño
DISC	Death-inducing signaling complex; complejo de señalización inductor de muerte
dpl/dpi	Días post lesión; days post injury
ER	Estrogen receptor; receptor a estrógenos
ERK	Extracellular signal-regulated kinase; cinasa regulada por señal extracelular
Escala BBB	Escala de evaluación locomotora Basso, Beattie y Bresnahan
GPER	G protein-coupled estrogen receptor 1; receptor a estrógenos acoplado a proteína G 1
H&E	Hematoxylin and eosin; hematoxilina y eosina
HS	Hormonas sexuales
IGF-1	Insulin-like growth factor 1; factor de crecimiento 1 similar a la insulina
LAM	Laminectomía
LC3	Microtubule-associated protein 1 light chain 3; proteína asociada a microtúbulos de cadena ligera 3

LTME	Lesión traumática de médula espinal
mTOR	Mammalian target of rapamycin; Diana de rapamicina en mamíferos
PE	Phosphatidylethanolamine; fosfatidiletanolamina
PI3K	Phosphatidylinositol 3-kinase; fosfatidilinositol 3-cinasa
PI3P	Phosphatidylinositol 3-phosphate, fosfatidilinositol 3-fosfato
PTPM	Poros transitorios de permeabilidad mitocondrial
SCI	Spinal cord injury
SERMs	Selective estrogen receptor modulators; moduladores selectivos de los receptores a estrógenos
STEAR	Selective tissue estrogen activity regulator; regulador selectivo de la actividad estrogénica tisular
T9	Novena vértebra torácica
TFE3	Transcription factor E3; factor de transcripción E3
TIB	Tibolona
TIB 2.5	Tibolona 2.5 mg/kg de peso
TNF	Tumor necrosis factor; factor de necrosis tumoral
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VPS15	Vacuolar protein sorting 15; proteína 15 de clasificación vacuolar
VPS34	Vacuolar protein sorting 34; proteína 34 de clasificación vacuolar

1. Resumen en español e inglés

La lesión traumática de médula espinal (LTME) es una condición discapacitante que afecta a millones de personas en el mundo, que puede generar repercusiones negativas en la salud, la vida social y la economía del paciente. Desafortunadamente, a la fecha no existe ningún tratamiento que permita la recuperación completa de las funciones perdidas después de una LTME, lo que hace necesario el desarrollo de propuestas terapéuticas. La apoptosis y la autofagia son mecanismos de muerte celular críticos que ocurren después de una LTME, por lo que constituyen blancos importantes para intervenciones terapéuticas que buscan favorecer la recuperación funcional. La tibolona (TIB) es un regulador selectivo de la actividad estrogénica tisular (STEAR) que tiene propiedades neuroprotectoras que podrían incidir sobre los mecanismos de muerte celular. Por lo anterior, el objetivo del presente trabajo fue evaluar el efecto de la TIB sobre la muerte celular por vía apoptótica y autofágica después de una LTME y demostrar si esto favorece la recuperación funcional motora. Se utilizaron ratas macho de la cepa Sprague Dawley, a las que se les produjo una LTME por contusión de intensidad moderada a nivel de la novena vértebra torácica (T9). Las ratas seleccionadas se administraron diariamente con TIB (2.5 mg/kg) y se sacrificaron 1, 3, 14 y 30 días post lesión (dpl). Se evaluaron algunos marcadores autofágicos y apoptóticos mediante la técnica western blot y ensayos TUNEL, además evaluamos la preservación del tejido y la recuperación funcional motora utilizando la escala BBB (Basso, Beattie y Bresnahan). Los resultados muestran que la TIB regula marcadores autofágicos de manera dependiente al tiempo, inhibiendo su expresión durante los primeros 3 dpl y promoviendo su expresión a partir de 14 dpl. Además, la TIB inhibe la apoptosis de forma consistente, incrementa la cantidad de tejido preservado y mejora la recuperación funcional después de la LTME. Por lo que la TIB puede ser una alternativa terapéutica para promover la recuperación funcional después de la LTME.

1.1 Abstract

Spinal cord injury (SCI) is an incapacitating condition that affects millions of people worldwide which can cause paralysis and has negative consequences for the health, social, and financial situation of patients. Unfortunately, a treatment has not yet been developed that allows for the complete recovery of functions lost after SCI, which emphasizes the need to develop new effective therapeutic strategies. Apoptosis and autophagy are critical cell-death signaling pathways that occur after SCI, which therapeutic interventions must target to promote functional recovery. Tibolone (TIB) is a selective tissue estrogen activity regulator (STEAR) that has proven neuroprotective properties which could have an effect over cell-death mechanisms. Therefore, the aim of this work was to evaluate the effect of TIB on apoptotic and autophagic cell death after SCI and demonstrate if TIB administration can promote functional recovery. Male Sprague Dawley rats were used, to which a moderate contusion SCI was performed at the ninth thoracic vertebra (T9). Selected rats were treated daily with TIB (2.5 mg/kg) and sacrificed at 1-, 3-, 14- or 30-days post injury (dpi). Some autophagic and apoptotic markers were evaluated using western blot analysis and TUNEL assays, additionally, we evaluated spinal cord tissue preservation and motor function recovery using the BBB (Basso, Beattie and Bresnahan) scale. The obtained results demonstrate that TIB regulated autophagy marker expression in a time dependent manner, inhibiting their expression at 3 dpi while promoting their expression at 14 dpi. Furthermore, TIB consistently inhibited apoptosis, increased the amount of preserved tissue, and improved motor function recovery after SCI. Thus, TIB could represent a therapeutic alternative to promote motor function recovery after SCI.

2. Introducción general

La lesión traumática de la médula espinal (LTME) es producida por un daño en la médula espinal, lo que afecta la calidad de vida, la estabilidad emocional y la economía del paciente. La incidencia de la LTME es de 39 casos por cada millón en América del Norte[1] y se estima que el gasto por paciente con LTME es aproximadamente de 2.35 millones de dólares estadounidenses[2].

La fisiopatología de la LTME puede categorizarse en lesión primaria y lesión secundaria[3]. La lesión primaria, es el resultado de la fuerza física relacionada con el evento traumático inicial, producido ya sea por contusión, compresión, laceración o sección completa de la médula espinal[3,4], lo cual desencadena los mecanismos fisiopatológicos que generan la lesión secundaria, caracterizada por la activación de una cascada de eventos bioquímicos que expanden el área del tejido nervioso lesionado y exacerban el déficit neurológico[3]. En los humanos durante la etapa aguda de una LTME (48 h post lesión) se observa inflamación, hemorragia e isquemia que propician necrosis celular en el sitio de la lesión, y cuyos efectos se extienden hasta la fase subaguda (48 h -14 días post lesión (dpl))[5]. La necrosis favorece un ambiente pro-inflamatorio mediante la liberación de patrones moleculares asociados a daño (DAMPs)[6] que inducen la activación de la microglía y de los astrocitos, lo que resulta en su transición a fenotipos inflamatorios[7]. Posteriormente, se establece la fase intermedia (14 días - 6 meses post lesión) y crónica (>6 meses post lesión), en las que pueden aumentar las afectaciones neurológicas dado que persiste la muerte neuronal y de oligodendrocitos por procesos como la degeneración Walleriana, desmielinización, cicatrización glial, gliosis, etc., que inhiben los procesos de recuperación[1,8,9]. En la LTME se presenta muerte celular por apoptosis en neuronas, oligodendrocitos, microglía y astrocitos[10,11]. La muerte de oligodendrocitos continúa durante varias semanas después de la LTME lo que incrementa la desmielinización axonal observada en etapas posteriores[10].

En la apoptosis, o muerte celular programada tipo I, existen dos vías principales: la extrínseca y la intrínseca, que involucran la activación de enzimas zimógenos denominadas caspasas. Las caspasas son una familia de proteasas de cisteína que permanecen inactivas cómo procaspasas hasta que se presente un estímulo que las

active a caspasas. Una caspasa iniciadora (caspasas 2, 8, 9, y 10), induce la activación de una caspasa efectora (caspasas 3, 6 y 7), mientras la caspasa efectora inactiva proteínas esenciales para procesos celulares o activa proteínas que desestabilizan la integridad celular[12,13]. Las caspasas 8 y 9 son las principales iniciadoras de la vía extrínseca e intrínseca respectivamente, mientras que la caspasa 3 es la ejecutora indispensable para la apoptosis dependiente de caspasas[14].

La vía extrínseca es controlada por la activación de receptores de muerte celular localizados en la membrana citoplasmática, activados por ligandos de la familia del factor de necrosis tumoral (TNF). El acoplamiento de estos receptores con su ligando provoca un cambio conformacional en el dominio citosólico del receptor resultando en la formación del complejo de señalización inductor de muerte (DISC), que incluye a las procaspasas iniciadoras 8 o 10[12], las cuales activan a las caspasas ejecutoras.

Por otro lado, la vía intrínseca se activa debido a la disfunción mitocondrial que está regulada por la familia linfoma de células B2 (Bcl-2). Las proteínas Bcl-2 pro apoptóticas BAX (proteína X asociada a Bcl-2) y BAK (Bcl-2 homologous antagonist killer), forman poros transitorios de permeabilidad mitocondrial (PTPM) que permiten la liberación del citocromo C (Cyt-C) al citoplasma. El Cyt-C a su vez se une a Apaf-1 (factor apoptótico 1 activador de proteasas), y subsecuentemente ocurre la oligomerización de siete unidades Cyt-C-Apaf-1 formando el complejo apoptosoma. Por último, el apoptosoma recluta y activa a procaspasa 9 para posteriormente activar a procaspasas ejecutoras[12].

La apoptosis se caracteriza morfológicamente por encogimiento celular, condensación del material nuclear y formación de cuerpos apoptóticos[12,13].

La autofagia o muerte celular programada tipo II, se clasifica como macroautofagia, microautofagia y autofagia mediada por chaperonas que responden al estrés intracelular[15]. Debido a que el proceso macroautofágico es el más estudiado dentro de la fisiopatología LTME, este se usará de forma intercambiable con autofagia, en este texto.

La autofagia involucra la formación de vesículas membranales denominadas autofagosomas que encapsulan biomoléculas o estructuras celulares destinadas al reciclaje. Existen tres biomarcadores que se utilizan para medir el proceso autofágico:

beclina 1, la proporción de las proteínas II y I asociada a microtúbulos de cadena ligera 3 (LC3-II:LC3-I) y a p62 (o secuestrosoma 1). Estas biomoléculas tienen un rol importante en la formación y maduración de autofagosomas, y el reclutamiento de material celular a reciclar. La iniciación de la autofagia es regulada por el complejo fosfatidilinositol 3-cinasa (PI3K) clase III compuesto por beclina 1, VPS15 (proteína 15 de clasificación vacuolar) y VPS34 (proteína 34 de clasificación vacuolar)[16]. Este complejo promueve la síntesis de fosfatidilinositol 3-fosfato (PI3P), requerido para iniciar la formación del autofagosoma[16–18]. Por otro lado, durante la maduración del autofagosoma, la proteína LC3-I tiene cambios similares a la ubiquitina por los que se le une la fosfatidiletanolamina (PE), lo cual resulta en la formación de LC3-II, que a su vez se ancla a la membrana del autofagosoma para reclutar sustratos marcados para reciclaje, por lo que la proporción LC3-II:LC3-I denota la formación de autofagosomas[17,19,20]. Por su parte, p62 es una proteína que etiqueta sustratos a reciclar y su descomposición es usada para evaluar el flujo autofágico[17,21]. Inmediatamente después de una LTME por contusión en la rata, la expresión de beclina 1 se mantuvo constante, mientras que el nivel de las proteínas p62 y LC3-II se incrementó, alcanzando su punto máximo de expresión un día después de la LTME y disminuyó en el día 7 manteniendo una expresión basal al menos durante 5 semanas después de la LTME[22].

En diferentes modelos experimentales de LTME en rata, se ha descrito que la inhibición de la apoptosis mediante diferentes terapias o tratamientos produce beneficios sobre la recuperación funcional[23–30]. Un ejemplo de ello son los trabajos de Yune et al. realizados en 2004 y 2008, en los que se demostró que la administración del 17β-estradiol no solo reduce la muerte por apoptosis y el tamaño del área de lesión después de una LTME, sino que también favorece una mejor recuperación funcional[23,24].

En contraste, el papel de la autofagia en modelos de LTME en rata no es tan claro, ya que diferentes autores han evidenciado que la inhibición de la autofagia puede mejorar la recuperación funcional [31,32], mientras que otros han probado que la promoción o restauración de la autofagia mejora la recuperación funcional [33–36].

A pesar de que recientemente se ha demostrado que el tratamiento con hormonas sexuales (HS) puede mejorar el desenlace de la LTME[37,38], su administración produce

efectos secundarios significativos como el desarrollo de cáncer en tejidos de riesgo como mama y endometrio, así como complicaciones cardiacas[39–42].

Es por ello por lo que actualmente está siendo evaluado el efecto neuroprotector de diversos compuestos sintéticos con propiedades que imitan las acciones protectoras de ciertas HS, pero sin los efectos periféricos indeseables de estas[43–47]. Entre estos compuestos se encuentra la tibolona (TIB), un esteroide sintético, con actividad tejido-específica que tiene funciones estrogénicas, progestágenas y androgénicas[47–49].

En estudios *in vitro*, se ha probado la capacidad de la TIB para disminuir el estrés oxidativo y su actividad anti-inflamatoria[50], mientras que estudios *in vivo* realizados tanto en animales como en humanos, sugieren que la TIB puede ser útil contra cierto tipo de daño neuronal[50–54], ya que reduce la muerte neuronal generada por la exposición crónica a ozono en la región CA3 (*Cuerno de Amón 3*) del hipocampo de roedores[54], reduce el volumen del tejido infartado en el modelo de isquemia cerebral focal[53], y reduce la gliosis reactiva en un modelo de lesión cerebral[52].

Aunque algunos estudios, han demostrado que la administración de TIB a diferentes dosis, induce la expresión de receptores a estrógenos[55], actualmente se desconoce si la TIB produce algún efecto sobre otros mecanismos fisiopatológicos de la LTME, como la muerte celular, y si esto tiene algún efecto sobre la recuperación motora.

2.1   Planteamiento del problema

La LTME es un problema a nivel mundial, con una incidencia de 39 casos por cada millón en América del Norte[1], que provoca daño al tejido nervioso y con ello la pérdida de las funciones motoras, sensitivas y autónomas de forma parcial o total por debajo del sitio de la lesión[2]. Esta condición devastadora no solo afecta física, económica y emocionalmente a las personas que la padecen, sino también a sus familias y a la sociedad, e impacta directamente en los servicios de salud y la sociedad en su conjunto.

A la fecha no existe ningún tratamiento que permita la completa recuperación de las funciones perdidas después de una LTME, lo que hace necesario el desarrollo de nuevas alternativas terapéuticas[37]. Una alternativa para el tratamiento de la LTME podría ser la TIB, un esteroide sintético que tiene afinidad por los receptores estrogénicos, androgénicos y progestágenos, cuyos metabolitos tienen efectos neuroprotectores.

No obstante, a la fecha no se conocen todos los mecanismos de acción a través de los cuales la TIB ejerce sus efectos neuroprotectores, por lo que, considerando su potencial utilidad después de una LTME, en el presente proyecto se propone estudiar el efecto de la TIB sobre la muerte celular y la recuperación de la función motora, en un modelo de LTME por contusión moderada en rata.

2.2   Pregunta de investigación

¿Cuál es el efecto de la TIB sobre la muerte celular y la recuperación de la función motora después de una LTME en un modelo en rata?

2.3   Hipótesis

Si la tibolona disminuye la muerte celular después de una lesión traumática de médula espinal, entonces favorecerá la recuperación de la función motora en un modelo de lesión traumática de médula espinal en rata.

2.4   Objetivos

2.4.1   Objetivo general

Estudiar el efecto de la tibolona sobre la muerte celular apoptótica dependiente de caspasas, la muerte macroautofágica y, su relación con la recuperación funcional motora después de una LTME producida en la rata.

2.4.2   Objetivos particulares

2.4.2.1 Evaluar el efecto de la TIB sobre la muerte celular apoptótica después de una LTME.

2.4.2.2 Evaluar el efecto de la TIB sobre la autofagia después de una LTME.

2.4.2.3 Evaluar el efecto de la TIB sobre la cantidad del tejido preservado después de una LTME.

2.4.2.4 Evaluar el efecto de la TIB sobre la recuperación de la función motora después de una LTME.

3. Artículo científico de maestría

Article

Tibolone improves locomotor function in a rat spinal cord injury model by modulating apoptosis and autophagy

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Abstract: Spinal cord injury (SCI) can cause paralysis and harm patients' health, social, and financial well-being. Unfortunately, effective therapeutic strategies for this condition affecting millions worldwide have not yet been developed. Apoptosis and autophagy are critical cell-death signaling pathways after SCI, which should be targeted for early therapeutic interventions to mitigate their adverse effects and promote functional recovery. Tibolone (TIB) is a selective tissue estrogen activity regulator (STEAR) that has demonstrated neuroprotective properties in several models. This study aimed to investigate the effect of TIB on apoptotic and autophagic cell death after SCI and if it promotes functional recovery. In a model of SCI with a moderate contusion, rats were treated daily with TIB and sacrificed at 1-, 3-, 14- or 30-days post-injury. We evaluated motor function recovery and autophagic and apoptotic markers. Our results demonstrated that TIB increased the amount of preserved tissue, improved motor function recovery, and modulated the expression of autophagy markers in a time-dependent manner while consistently inhibiting apoptosis. Therefore, TIB could be a therapeutic alternative for recovering motor function after SCI.

1. Introduction

Spinal cord injury (SCI) results after direct damage to the spinal cord, devastating for patients' quality of life and their emotional and economic stability. SCI has a worldwide incidence of approximately 10.5 in 100,000 people [1], and the average treatment cost is around 2.35 million dollars per patient [2].

SCI can be classified as primary and secondary depending on the mechanism of injury [3,4]. The primary injury mechanism in SCI occurs immediately, derived from physical forces that may result from contusion, compression, laceration, or complete spinal cord segmentation [3,4]. Subsequently, secondary damage mechanisms activate diverse

biochemical events, including apoptosis and autophagy, expanding the area of damaged nerve tissue and aggravating neurological deficits [3,5,6].

Apoptosis and autophagy are central in SCI [2,7]. Although the role of apoptosis in SCI has been demonstrated, the exact contribution of autophagy remains controversial [8-12]. During the acute phase of SCI, autophagy biomarkers increase and autophagosomes accumulate [13,14], leading to neuronal death [12,15].

Apoptotic death occurs within hours to weeks after SCI and affects several cells, such as neurons, oligodendrocytes, microglia, and astrocytes [16]. Caspases are the most critical mediators of apoptosis, particularly caspase-3, which plays a crucial role in the spinal grey and white matter after SCI [17,18]. Generally, after SCI, caspase cleavage induces apoptotic phenotypic changes through skeletal degradation, DNA fragmentation, and disruption of cellular and DNA repair processes [19]. SCI treatment research has sought to develop early therapeutic interventions to mitigate the effects of apoptosis and autophagic mechanisms [15,20-22]. A growing body of experimental evidence has recently demonstrated the therapeutic benefits of estrogens (E2) in SCI [23-26] by decreasing apoptosis [24,27,28] and regulating autophagic mechanisms [20].

In a model of SCI, E2 treatment reduced caspase-3 activity and neuronal death and improved locomotor function [24,26,28]. Moreover, in traumatic SCI, E2 decreased the expression of autophagy-related proteins, such as Beclin-1 and LC3 II, improved motor function and reduced motor neuron loss by inhibiting the onset of autophagy [20].

The increased risk of breast and endometrial cancer associated with estrogen therapy has prompted the development of synthetic steroids, such as tibolone [29].

Tibolone exerts tissue-specific actions depending on its local transformation into active metabolites that exert estrogenic, progestogenic, and androgenic activities [29-33]. In addition, Tibolone has neuroprotective actions, providing an attractive alternative to traditional estrogen therapy 31,34-40].

Based on the above, we evaluated the neuroprotective effect of tibolone on neuronal death, particularly apoptosis and autophagy, and its action on motor function recovery in a rat model of SCI with a moderate contusion. The present study showed that tibolone improves locomotor function in a rat model of SCI by modulating apoptosis and autophagy.

2. Results

2.1. Morphometric analysis

After 14 days post-injury (dpi), histological analysis was performed to identify changes in the cytoarchitecture of the spinal cord. The epicenter of the injured spinal cord in animals receiving TIB was occupied primarily by tissue. In contrast, a cystic zone was formed around the injury site, and vacuolar structures containing fragments of degenerating axons and cellular debris were identified in the SCI group. Treatment with TIB markedly reduced structural nerve tissue damage in the rostral and caudal portions of the injured spinal cord compared to the untreated group. Moreover, in the group that received TIB, many neurons in the rostral zone of the spinal cord showed a conserved morphology (Figure 1). The morphometric analysis showed that nervous tissue is better preserved after SCI in animals that received TIB ($p < 0.05$) (Figure 1).
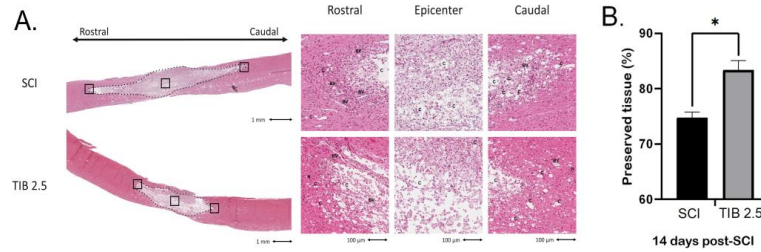
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Figure 1. Histological analysis of the spinal cord with no treatment or treated with tibolone after injury. **A.** Representative images of longitudinal sections of the spinal cord stained with hematoxylin-eosin one month after spinal cord injury: not treated (SCI); treated with tibolone at a dose of 2.5 mg/kg (TIB 2.5). Images correspond to the rostral area, the epicenter of the injury zone and the caudal area at magnifications of 2x (panoramic) and 40x. BV, blood vessels; C, cyst; N, neurons. Scale bar = 100 μm. **B.** Data show the percentage of preserved tissue 14 days after the traumatic spinal cord injury in rats not treated (SCI) or treated with tibolone (TIB 2.5). Values are the means ± SE of three histological sections per rat: SCI (n = 4), TIB 2.5 (n = 4). Data were analyzed with one-way ANOVA followed by Bonferroni's *post hoc*. *$p < 0.05$.

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2.2. Tibolone administration improves functional motor recovery

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Functional motor recovery was evaluated according to the BBB scale. The SCI group obtained a final average score of 8.2 ± 0.5, meaning that most animals exhibited hip, knee, and ankle movements in each displacement (Figure 2). Some performed a sweeping gait without body weight support, and others showed only plantar placement of the paw without body weight support.

Animals treated with TIB showed extensive movements of the three joints of the hindlimbs with occasional body weight-supported plantar steps but no forelimbs and hindlimbs coordination ($p < 0.05$), obtaining a score of 10.5 ± 0.3 at 30 days. TIB treatment allowed the animals to show a faster motor recovery than the SCI group (Figure 2).

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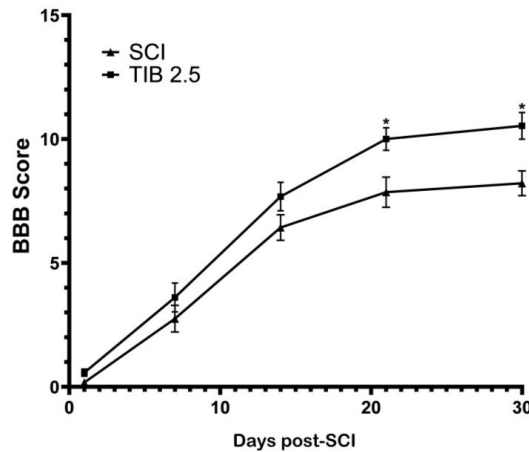104
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Figure 2. Tibolone administration promotes locomotor function recovery. Locomotor function recovery was evaluated in an open field with the Basso, Beattie and Bresnahan scale (BBB), as mentioned in the Methods section. Values are the mean ± SE of animals untreated (SCI, n = 8,

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triangles) or treated with tibolone (TIB 2.5, n = 8, squares). Data were analyzed with one-way 116
ANOVA followed by Bonferroni's *post hoc*. *$p < 0.001$ 117

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2.3. Tibolone regulates autophagic markers in a time-dependent manner after spinal cord injury 119

To determine the regulation of autophagic mechanisms with TIB treatment after SCI, 120
we analyzed the expression of Beclin-1 and p62 proteins and the LC3II/LC3I ratio by West- 121
ern blot assays in the laminectomy (LAM), SCI and TIB 2.5 groups at 1-, 3-, and 14-dpi. 122
The Beclin1-Vacuolar protein sorting 34-Vacuolar protein sorting 15 (Vps34-Vps15) 123
core complex is required in the pre-autophagosome structure; hence the expression of Be- 124
clin1 correlates closely to autophagosome activity [41]. When autophagy initiates, micro- 125
tubule-associated 1 protein light chain 3 (LC3I) undergoes ubiquitin-like changes and 126
binds to phosphatidylethanolamine (PE) on the surface of vacuole membranes of the au- 127
tophagosome, resulting in the formation of LC3II [42]. Therefore, LC3II expression or the 128
LC3II/LC3I ratio is a direct index that reflects the number of autophagic vacuoles [43]. 129
Finally, p62, also known as sequestosome 1 (SQSTM1), is incorporated into autophago- 130
somes through LC3-binding and degraded by the autophagic machinery. Hence, p62 pro- 131
tein levels can be used to assess autophagic flux [44]. 132
Figure 3A shows no significant changes in Beclin-1 levels at 1- and 3-dpi in the SCI 133
group compared to the LAM group. In contrast, Beclin-1 levels were significantly lower 134
in the SCI group than in the LAM group at 14 dpi. Interestingly, the expression of Beclin- 135
1 in the TIB group was also significantly lower than the LMA and SCI groups at 3 and 14 136
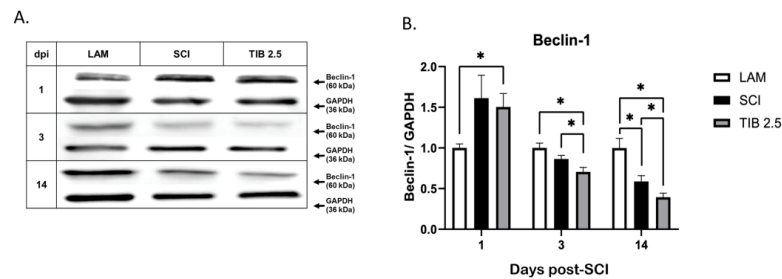dpi (Figure 3B). 137

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Figure 3. Tibolone regulates Becline-1 autophagic marker in a time-dependent manner after spinal 139
cord injury. **A.** Representative blots of Beclin-1 (~60 kDa) levels from the laminectomy (LAM), 140
spinal cord injury (SCI), and treated with tibolone 2.5 mg/kg (TIB 2.5) groups at 1-, 3-, and 14-days 141
post-injury (dpi). GAPDH (~36 kDa) was used as a loading control. **B.** Levels of Beclin-1 expressed 142
as fold change relative to GAPDH in the laminectomy (LAM), spinal cord injury (SCI), and treated 143
with tibolone 2.5 mg/kg (TIB 2.5) groups at 1-, 3-, and 14-days post-injury (dpi). Values are the 144
mean ± SE (n = 4). Data were analyzed with one-way ANOVA followed by Tukey's *post hoc*. 145
*$p < 0.05$. 146

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In the SCI group, the LC3-II:LC3-I ratio expression was significantly higher at 1 dpi, 148
remained with no significant changes at 3 dpi, and was considerably lower at 14 dpi than 149
the LAM group. Conversely, TIB treatment slightly decreased the LC3-II:LC3-I ratio at 1 150
and 3 dpi compared with the SCI group. However, TIB significantly increased the LC3- 151
II:LC3-I ratio at 14 dpi compared with the SCI and LAM groups. These results indicate 152
that TIB decreased lesion-induced autophagosome completion at 1- and 3-dpi while pro- 153
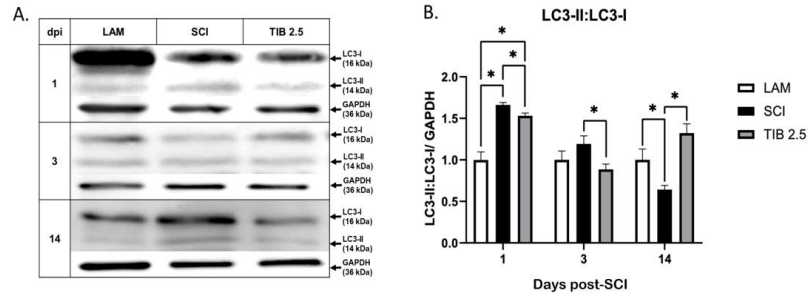moting autophagosome formation at 14 dpi (Figure 4). 154

Figure 4. Tibolone regulates the LC3-II:LC3-I ratio (an autophagic marker) in a time-dependent manner after spinal cord injury. **A.** Representative blots of LC3-I (~16kDa) and LC3-II (~14 kDa) from the laminectomy (LAM), spinal cord injury (SCI), and treated with tibolone 2.5 mg/kg (TIB 2.5) groups at 1-, 3-, and 14-days post-injury (dpi). GAPDH (~36 kDa) was used as a loading control. **B.** LC3-II:LC3-I ratio expressed as fold change relative to GAPDH in the laminectomy (LAM), spinal cord injury (SCI), and treated with tibolone 2.5 mg/kg (TIB 2.5) groups at 1-, 3-, and 14-days post-injury (dpi). Values are the mean ± SEM (n = 6). Data were analyzed with one-way ANOVA followed by Tukey's *post hoc*. *$p < 0.05$.

In the SCI group, p62 expression was significantly lower at 1 and 3 dpi but higher at 14 dpi than in the LAM group. Conversely, p62 expression significantly increased at 1 dpi but decreased at 3 and 14 dpi in the TIB 2.5 group compared to the SCI group (Figure 5). Based on these observations, we suggest a differential regulation of autophagic flux by TIB: TIB decreased autophagic flux at 1 dpi but increased autophagic flux at 3 dpi and 14 dpi.
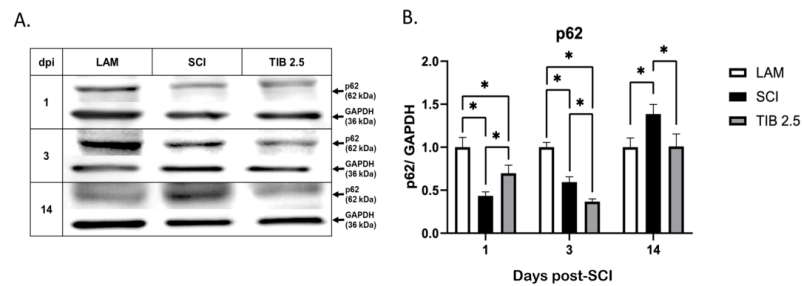

Figure 5. Tibolone regulates p62 (an autophagic marker) in a time-dependent manner after spinal cord injury. **A.** Representative blots of p62 (~62 kDa) levels from the laminectomy (LAM), spinal cord injury (SCI), and treated with tibolone 2.5 mg/kg (TIB 2.5) groups at 1-, 3-, and 14-days post-injury (dpi). GAPDH (~36 kDa) was used as a loading control. **B.** Levels of p62 expressed as fold change relative to GAPDH in the laminectomy (LAM), spinal cord injury (SCI), and treated with tibolone 2.5 mg/kg (TIB 2.5) groups at 1-, 3-, and 14-days post-injury (dpi). Values are the mean ± SE (n = 4). Data were analyzed with one-way ANOVA followed by Tukey's *post hoc*. *$p < 0.05$.

Our results indicate that TIB promotes autophagy even long-term after SCI. In contrast, TBI seems to partially decrease autophagy at shorter times after SCI (1- and 3-dpi). Therefore, these findings indicate that TIB modulates autophagy in a time-dependent manner.

2.4. Tibolone regulates apoptosis in spinal cord injury

We evaluated cell death by TUNEL assay and monitored active caspase-3 expression by Western blot analysis to determine whether TIB regulated apoptosis after SCI. Figure 6 shows the mean percentage of TUNEL-positive cells in the spinal cord's caudal and rostral regions of animals treated with TIB at 14 dpi. The rate of TUNEL-positive cells is lower in the rostral area than in the caudal region (10-15%). The percentage of TUNEL-positive cells in the spinal cord of animals treated with TIB was lower than that observed in the SCI group in the rostral and caudal regions ($p < 0.05$).
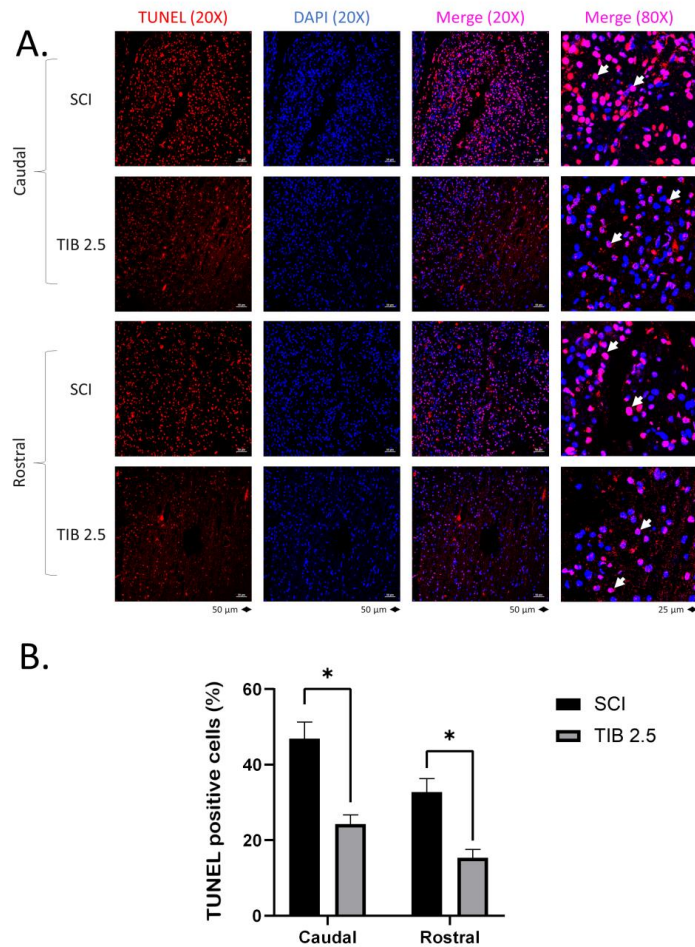

Figure 6. Figure 6. Tibolone reduced apoptosis after spinal cord injury. **A.** Representative images of the TUNEL assay 14 days after traumatic spinal cord injury (SCI) and treated with tibolone 2.5 mg/kg (TIB 2.5) in caudal and rostral regions. Arrows indicate TUNEL-positive cells. TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling (red), cellular nucleus (blue), caudal area at magnifications of 20x and 80x. **B.** Percentage of TUNEL-positive cells, 14 days post-injury in rostral and caudal regions. Data are expressed as mean ± SE of three independent experiments (n = 3). Data were analyzed with one-way ANOVA followed by Bonferroni's *post hoc*. *$p < 0.05$.

Caspase-3 expression was markedly higher in the SCI group than in the LAM group at 1, 3, and 14 dpi. In contrast, TIB treatment significantly decreased caspase-3 expression

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compared to the SCI groups at these times, similar to the levels observed in the LAM   205
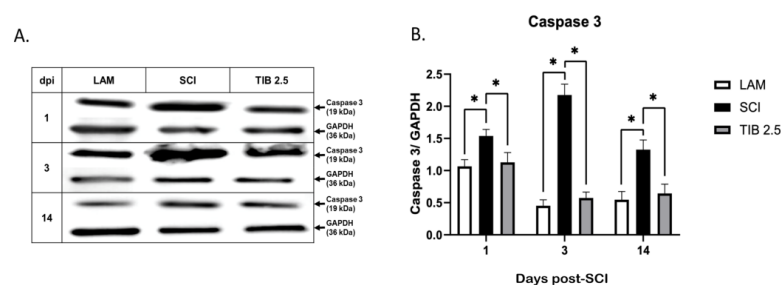group (Figure 7).   206

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Figure 7. Tibolone reduced active caspase-3 after spinal cord injury. **A.** Representative blots of active   209
Caspase-3 (~19 kDa) levels from the laminectomy (LAM), spinal cord injury (SCI), and treated with   210
tibolone 2.5 mg/kg (TIB 2.5) groups at 1-, 3-, and 14-days post-injury (dpi). GAPDH (~36 kDa) was   211
used as a loading control. **B.** Levels of active Caspase-3 are expressed as fold change relative to   212
GAPDH in the laminectomy (LAM), spinal cord injury (SCI), and treated with tibolone 2.5 mg/kg   213
(TIB 2.5) groups at 1-, 3-, and 14-days post-injury (dpi). Values are the mean ± SE (n = 4). Data were   214
analyzed with one-way ANOVA followed by Tukey's *post hoc*. *$p < 0.05$.   215

3. Discussion   216

Although there is still no effective treatment for SCI in humans, it has been demon-   217
strated that estrogens [20,24,45–50], progesterone [51], and selective-estrogen receptor   218
modulators (SERMs) [52–56] are effective in improving motor function scores in SCI ani-   219
mal models. However, hormone therapy has limitations regarding potential secondary   220
effects, as clinical studies in post-menopausal women have revealed that these hormones   221
pose a risk for developing cancer and cardiac complications, among other side effects [57–   222
60].   223

Tibolone is an attractive therapeutic alternative for treating the secondary injury of   224
SCI, as its active metabolites target specific tissues, particularly those in the central nerv-   225
ous system (CNS) over risk tissue (breast, endometrium) [61], and confer neuroprotective   226
properties against neuronal damage [31,34,62,63]. The present study shows that TIB treat-   227
ment promotes a better recovery of motor function after a concussion than untreated ani-   228
mals.   229

Immediate implementation of hormone therapy is crucial for optimal motor recovery   230
post-SCI. Colón et al. (2016) demonstrated that prompt administration of tamoxifen post-   231
SCI in rats significantly improved locomotor function over a group treated 24 hours after   232
injury [53]. Here, we treated rats with TIB 30 min after SCI, which was crucial for the   233
observed motor recovery. Besides, we administrated TIB continuously, which could also   234
be critical to maintaining its neuroprotective effect because, during the intermediate-   235
chronic phases of SCI, degenerative effects of SCI persist through the continuation of neu-   236
ronal and oligodendrocyte cell death by some events associated with the lesion, such as   237
Wallerian degeneration, demyelination, glial scar formation, and continued gliosis,   238
among others [64,65].   239

Moreover, apoptosis and autophagy are two types of programmed cell death that   240
generally inhibit each other; however, a dying cell can show apoptotic or autophagic pro-   241
files simultaneously or at different time points as diverse molecules or pathways can mod-   242
ulate both mechanisms [66,67]. Under low cellular stress levels, autophagy acts as a pre-   243
ventive mechanism and only becomes a cell death mechanism or precedes apoptosis as   244
cellular stress intensifies [66].   245

Several authors have demonstrated that different experimental treatments post-SCI, including sexual hormones, improve motor function and reduce apoptosis and autophagy [15,20,21,24,27,28,68]. These findings highlight the importance of regulating these processes for effective SCI treatment.

Other studies show that the autophagy flux changes after SCI depending on the severity and location of the injury [12]. Our results showed that autophagic expression increases only at 1 dpi, but at 14 dpi, autophagic expression decreases in a moderate contusion rat model of SCI. These results partially agree with those reported by Qin et al. (2014), who found that the LC3-II/LC3-I ratio in the spinal cord promptly increased on day 3, reached its expression peak on day 7, and reduced significantly 21 days after SCI [69].

Recent studies have shown the importance of modulating autophagy in neurological pathologies. Li et al. (2017) demonstrated that inhibiting autophagy by estradiol in central cerebral ischemia positively modulates neurological deficits [70]. Research on SCI has reached a similar conclusion, as both administration of insulin-like growth factor 1 (IGF-1) and estradiol post-SCI inhibited autophagy and improved motor recovery [20,71]. In the present study, no significant effect of TIB in the autophagic parameters was observed, except for a slight inhibition of the autophagic flux.

Interestingly, TIB demonstrated an ability to modulate autophagy at 3 dpi negatively. In contrast, although TIB partially inhibited beclin-1, the LC3-II:LC3I ratio increased, and the expression of p62 decreased compared to the SCI group, suggesting that TIB promotes autophagy at 14 dpi. The acute and subacute phases of SCI physiopathology—within the first 14 days post-SCI—are characterized by aberrant inflammation, excitotoxicity, ischemia, initiation of glial scarring, and cell death, including apoptosis and autophagy [2,65,67].

Several studies have assessed the effect of their respective therapies on autophagy at very early intervals [20,71]. In contrast, others have evaluated autophagy for extended periods [72–75], when several cell regeneration events occur after SCI, including remyelination and vascular and neuronal reorganization [2,65]. Therefore, promoting autophagy by TIB at 14 dpi to counteract apoptotic cell death could improve motor recovery, as observed in the BBB results. In this regard, our results demonstrate that TIB could differentially regulate autophagy in a time-dependent manner.

In this work, we did not evaluate pathways that could explain our results. However, other reports have suggested mechanisms of action of TIB during SCI. For example, PI3K/Akt and AMPK/mTOR pathways could explain the observed results related to autophagy. Some studies have shown that TIB can activate PI3K/Akt [76], and estrogens can activate the PI3K/Akt pathway in an SCI context to exert anti-apoptotic effects [46,77]. Similarly, PI3K/Akt acts as an inhibitor of autophagy in SCI when insulin is administered [71], which may explain how TIB can negatively modulate autophagy during the first 3 dpi. In addition, estrogens can activate the AMPK/mTOR pathway to promote autophagy in osteoblasts and chondrocytes and prevent apoptosis [78,79]. Upregulation of TFE3, intermittent fasting, and erythropoietin administration in SCI activated the AMPK/mTOR pathway [68,74,75], promoting autophagy and improving motor recovery. Given that TIB is a STEAR with estrogen-like mechanisms of action [30,80], it is feasible that the AMPK/mTOR pathway is partially responsible for the observed autophagic marker expression results at 14 dpi.

Previously, some authors have shown that inhibition of apoptosis has beneficial effects on motor recovery [81–83]. Some synthetic hormones, such as estradiol and other SERMs (bazedoxifene and tamoxifen), have shown promising improvements in motor recovery in murine models after SCI related to apoptotic inhibition [46,52,55,77,84,85]. Similarly, we observed a significant increase in SCI-induced apoptotic parameters and found that TIB significantly inhibited apoptosis. TIB reduced caspase-3 expression at all time points evaluated, and showed a lower percentage of TUNEL-positive cells in the caudal and rostral regions and a higher percentage of preserved tissue compared to the SCI group at 14 dpi. Other studies have demonstrated that estrogen therapy 1-7 dpi significantly

reduced the number of TUNEL-positive cells, consistent with previous findings with estrogens [47,50,77,85], bazedoxifene [52], or tamoxifen [55]. Therefore, our results are consistent with previous studies and support that TIB administration post-SCI decreases apoptotic markers and death.

Continued estrogen administration has been shown to attenuate neuronal and oligodendrocyte death, axonal degeneration, glial activation, and scarring [24,28,77,86,87]. Since the TIB group showed a lower percentage of TUNEL-positive cells in both the rostral and caudal regions, perhaps other cells, such as oligodendrocytes and astrocytes, could also have been protected by TIB. However, we did not evaluate these cells. Many articles have focused on how activation of the PI3K/Akt [72,77,81–83] or ERK [77] pathways can lead to the inhibition of apoptosis and improve post-SCI motor recovery. As previously mentioned, TIB activates PI3K and Akt [76]. Therefore, TIB could exert its beneficial effects by regulating these signaling pathways. Future research should be conducted to understand the impact of TIB on these signaling pathways after SCI.

4. Materials and Methods

4.1. Animals

Adult male Sprague Dawley rats weighing 250-320 g were used in this study. Animals were housed under standard conditions (12-h light/dark cycles, 22 °C) and randomly divided into three groups: laminectomy (LAM), spinal cord injury (SCI), and SCI treated with 2.5 mg/kg of tibolone (TIB 2.5) groups.

All surgical and experimental procedures were performed following the Regulation of the Mexican General Law of Health regarding research and science [88] and the Mexican Guidelines for Animal Care and Handling (NOM-062-ZOO-1999) [89] with the authorization of the National Committee for Scientific Research of the Mexican Institute of Social Security (protocol number R-2021-785-011). Every effort was made to minimize animal discomfort and reduce the number of animals used.

4.2. Surgical Procedure

Animals were anaesthetized intramuscularly with a mixture of xylazine (Xilasyn®2, Virbac) and zoletil (Zoletil®100, Virbac) at doses of 75 and 25 mg/kg body weight, respectively. A laminectomy was performed at the level of the thoracic vertebrae 9 (T9), and SCI was induced using the NYU stereotactic impactor. The presence of a hematoma at the site of injury was verified microscopically. Subsequently, muscle and skin were sutured in layers. An antibiotic (benzathine penicillin) was administered intramuscularly in a single dose (1 200,000 IU), and an analgesic in the drinking water (paracetamol, 5 ml/l of water) for 5 days. The animals were placed in individual boxes in the vivarium under the conditions previously described. The neurogenic bladder and bowel were manually emptied daily until the animal regained sphincter control. The surgical wound was checked daily, and the general health of each animal as well.

4.3. Treatments

Vehicle (water) or TIB (Livial©, 2.5 mg tablets) dissolved in water was administered orally. Rats in the TIB 2.5 group were given an initial dose of 2.5 mg/kg of tibolone orally 30 min after the injury, and after that, this same dose was administered daily. Animals were sacrificed at 1-, 3-, 5-, 7- or 14 days post-injury (dpi). Following the same scheme, other SCI and TIB 2.5 groups were treated up to 21 dpi and sacrificed at 14 or 30 dpi. These groups were used to obtain tissue for immunostaining assays, quantify preserved tissue, and assess motor function.

4.4. Tissue Collection

Once the treatments were completed, rats were sacrificed according to the Official Mexican Standard (NOM-033-SAG/ZOO-2014; NOM-033-ZOO1995) regarding the humane euthanasia of animals [90].

For Western blot analyses, euthanasia by decapitation was performed using a small animal guillotine (World Precision Instruments, Inc. Sarasota, FL USA; Model DCAP-M, serial 133708 9K). Euthanasia was performed by trained personnel in a room where one animal was placed at a time. Subsequently, the spinal cord, including the epicenter of the lesion plus 0.5 cm in the rostral direction and 0.5 cm in the caudal direction, was collected fresh and preserved at 4°C. Spinal cords were homogenized with protein extraction lysis buffer (150 mM NaCl, 20 mM Tris Base, 5 mM EDTA, 10% glycerol, Nonidet P-40 (Sigma Aldrich) and Complete (Roche)). The homogenate was centrifuged at 12,500 rpm for 30 min, and the supernatant was collected. Protein concentration was determined by the Bradford method (Quick Start Bradford 1X Dye Reagent, Bio-Rad).

For histological assays, rats were anesthetized with pentobarbital and perfused intracardially with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4) and 4% paraformaldehyde solution at a regulated rate of 30 mL/min using a peristaltic pump. After perfusion, a 2 cm segment of the spinal cord was removed with the epicenter of the lesion in the middle, maintaining an additional 1 cm from both cephalic and caudal orientations. Spinal cords were fixed in a 4% paraformaldehyde solution for 8 days, followed by dehydration with 15-minute treatments using a series of graded alcohols (70%, 96%, and 100% ethanol), xylol, and paraffin. Spinal cords were then embedded in paraffin blocks in a ventral-dorsal orientation. Spinal cord tissue sections 5 μm width were obtained using a Leica® RM2125 RTS microtome and mounted on poly-L-lysine coated slides. Slides were selected for each animal, using the epicenter of the lesion and the ependymal canal as references.

4.5. Morphometric Analysis

Three slides per group were rehydrated in 1-minute baths of paraffin, xylol, a series of graded alcohols (100%, 95%, 85%, 70%, and 50% ethanol), and water. The slides were then stained with hematoxylin-eosin and covered with Entellan®. Panoramic images were taken with a Leica Aperio® CS2 microscope slide scanner. Fiji software (NIH Image version 1.38x) was used to determine the total spinal cord area, damaged tissue surface and preserved tissue area of four sections per slide.

4.6. Evaluation of Functional Recovery

A double-blind scheme using the Basso, Beattie, and Bresnahan (BBB) locomotion scale evaluated motor function recovery in the open field. The BBB scale assesses the movement of hindlimb joints, plantar use of paws, weight-bearing by the limbs, and forelimb and hindlimb coordination during activity. The BBB scale is a 21-point rating scale, where a score of 0 indicates a total absence of limb movement, and a score of 21 indicates regular activity. The first assessment was performed 24 hours after injury to verify complete hindleg paralysis. Subsequently, weekly evaluations were performed for 4 weeks to assess functional recovery [91,92].

4.7. Western Blot

SDS-PAGE of the protein homogenates was performed. Subsequently, they were transferred to PVDF membranes (Millipore) and blocked in 5% milk Tris-buffered saline buffer (TBS; 100 mM Trizma, 150 mM NaCl, pH 7.5). Membranes were washed three times with TTBS (0.1% Tween-20 (polysorbate) TBS) for 5 min and incubated for 24 h with the respective antibodies: anti-caspase-3 (35, 19, 17 kDa; 1:1000; cat. 14220; Cell Signaling Technology ®), p62 (1:1000; cat. 88588; Cell Signaling Technology ®), beclin-1 (1:750; cat. 3495; Cell Signaling Technology ®) or LC3 I/II (1:2000; cat. NB100-2220; Novus-Biologicals). GAPDH antibody (1:1000; cat. Sc-32233; Santa Cruz Biotechnology®) was used as a loading control for these assays. Membranes were washed three times with TTBS for 5

min and then incubated with their corresponding secondary antibody: anti-mouse 399
(1:10,000; cat. 115-035-003; Jackson ImmunoResearch Laboratories, Inc) or anti-rabbit 400
(1:10,000; cat. 211-032-171; Jackson ImmunoResearch Laboratories, Inc) for 1 h. Mem- 401
branes were washed three times with TTBS for 5 min and then incubated for 5 min in 402
Immobilion® Crescendo Western HRP Substrate (Merck Millipore). Chemiluminescence 403
of the membranes was visualized with the Fusion Fx (Vilber Lourmat) imaging system 404
and its corresponding Fusion ® software. Band area analysis was performed with Fiji soft- 405
ware (NIH Image version 1.38x). 406

4.8. TUNEL Assay (terminal deoxynucleotidyl transferase dUTP nick end labeling) 407

Three slides per group were rehydrated with 5-min baths of paraffin/xylol, xylol, a 408
graded alcohol series (100%, 95%, 85%, 70%, and 50% ethanol) and PBS. The Abcam 409
ab66110 Brd-Red TUNEL kit was used according to the supplier's instructions. The slides 410
were then counterstained with DAPI and mounted with Vectashiled® (VectorLabs). A 411
Nikon TI eclipse confocal microscope was used to observe two spinal cord sections per 412
slide. Specific fluorescence was quantified in three random fields (500 x 500 microns) in 413
the cephalic and caudal regions. Total cells and TUNEL-positive cells were quantified by 414
analyzing the blue and red channels using Adobe Photoshop and FIJI software (NIH Im- 415
age version 1.38x). 416

4.9. Statistical Analysis 417

Data were analyzed using GraphPad Prism 8.0 (Dotmatics) and IBM SPSS software. 418
BBB scores were presented as means ± standard error (SE) and analyzed using re- 419
peated measures ANOVA followed by Bonferroni's *post hoc* analysis ($p < 0.05$). Protein 420
expression data were presented as means ± SE and analyzed using Student's t-test fol- 421
lowed by Tukey's *post hoc* ($p < 0.05$). TUNEL-positive cells and preserved tissue areas were 422
presented as means ± SE and analyzed using a one-way ANOVA followed by Bonferroni´s 423
post hoc ($p < 0.05$). 424

5. Conclusions 425

Tibolone attenuates cell death, improves the preserved tissue area post-SCI, and sig- 426
nificantly improves motor function, similar to what is observed with estrogen hormone 427
therapy. Additionally, tibolone modulates autophagy time-dependently and decreases 428
apoptosis in SCI. 429

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4. Discusión general y conclusiones

4.1 Discusión

Los estrógenos[23,32,56–61], la progesterona[62] y los moduladores selectivos de los receptores a estrógenos (SERMs)[25,27,63–65], han demostrado ser efectivos para mejorar la recuperación funcional en modelos animales con LTME. Sin embargo, el empleo de las hormonas esteroides es limitado debido a sus efectos secundarios, ya que diferentes estudios clínicos han demostrado que pueden elevar el riesgo de padecer cáncer, o presentar complicaciones cardiacas, entre otras enfermedades[39–42].

La administración de TIB es una propuesta terapéutica interesante para promover la recuperación funcional después de la LTME, ya que sus metabolitos actúan de forma tejido-selectiva, y se le han demostrado efectos neuroprotectores en el sistema nervioso central. Además de que tiene baja actividad en tejidos susceptibles (mama, endometrio, vagina, etc.) a desarrollar cáncer con el tratamiento hormonal[49,51,52,66]. Adicionalmente, de forma general, la administración de TIB ha demostrado generar un menor riesgo para el desarrollo de complicaciones secundarias[39]. Por lo tanto, la TIB presenta una ventaja competitiva sobre la terapia hormonal tradicional[39], siendo categorizada como un regulador selectivo de la actividad estrogénica tisular (STEAR).

A pesar de ser un potencial prospecto terapéutico, aún se desconoce el efecto de la TIB sobre la muerte celular y la recuperación funcional después de una LTME y, esto es relevante debido a que la muerte celular tiene un rol importante durante la lesión secundaria desencadenada por una LTME, donde son afectadas neuronas, oligodendrocitos, microglía y astrocitos, teniendo un efecto importante sobre las afectaciones motoras resultantes[10,11,67]. Debido a lo anterior, en el presente trabajo se evaluó el efecto de la TIB sobre la muerte celular apoptótica y autofágica.

Aunque la apoptosis y la autofagia son procesos de muerte celular programada que generalmente se inhiben entre sí, una célula en proceso de muerte puede presentar ambos procesos al mismo tiempo o en diferentes momentos, e interesantemente ambos procesos cuentan con biomoléculas y vías bioquímicas en común[13,67]. Sí la célula presenta estrés celular bajo, la autofagia actúa como un mecanismo citoprotector, pero se convierte en un mecanismo de muerte celular cuando la célula presenta niveles de

estrés celular elevado[13]. Por lo tanto, es importante hacer una distinción puntual entre la muerte celular autofágica y el concepto de flujo autofágico, en donde p62 es el marcador de flujo autofágico por excelencia, mientras que la proporción LC3-II:LC3-I es un indicador para la formación de autofagosomas[17,20].

Los resultados del presente trabajo muestran que durante los primeros dpl, se incrementó la expresión de algunos marcadores de muerte autofágica y apoptótica en el grupo SCI comparado con el grupo de laminectomía (LAM). A los 14 dpl podemos ver que persiste la expresión de los marcadores apoptóticos, mientras que la expresión de los marcadores autofágicos disminuye al comparar los grupos SCI y LAM. Por otro lado, la TIB disminuye la expresión de marcadores autofágicos durante los primeros dpl, y los regula positivamente a partir de los 14 dpl, mientras que inhibe la expresión de marcadores apoptóticos de forma constante. Estos resultados muestran que la TIB puede regular la muerte celular por autofagia de manera dependiente en el tiempo.

Li et al., han demostrado que la inhibición de la autofagia por parte de estradiol puede mejorar las alteraciones neurológicas generadas en un modelo de isquemia cerebral[68].

Diversas investigaciones han demostrado que la administración del factor de crecimiento 1 similar a la insulina (IGF-1) y el estradiol inhiben la autofagia y favorecen la recuperación de la función motora después de una LTME[31,32].

Por otro lado, investigaciones tanto *in-vitro*[69] como *in-vivo*[33–36,70] han demostrado que la inducción de la autofagia no solo inhibe la apoptosis, sino también favorece la recuperación funcional después de la LTME. Yuan et al., demostraron que el ayuno intermitente promueve la autofagia, disminuye la apoptosis y favorece la recuperación funcional motora después de una LTME[36].

Otros autores han demostrado que la alteración del flujo autofágico después de una LTME, se debe a la disfunción lisosomal generada parcialmente por la muerte apoptótica, producida por estrés del retículo endoplasmático [22,70]. Zhou et al., demostraron como la sobre regulación del factor de transcripción E3 (TFE3) después de la LTME restaura el flujo autofágico, al restablecer la función lisosomal, resultando en una mejora funcional [70]. Asimismo, la administración de ezetimibe y eritropoyetina después de la LTME mejoraron la funcionalidad motora al promover la autofagia[34,35].

De acuerdo con lo antes mencionado, los resultados del presente estudio indican que la TIB modula la autofagia de forma negativa durante los primeros tres dpl, mientras que 14 dpl promueve la autofagia. Las fases agudas y subagudas de la LTME ocurren durante los primeros 14 dpl, las cuales están caracterizadas por inflamación, excitotoxicidad, isquemia, cicatrización glial, y muerte celular aberrante[1,2,67].

La inhibición de cualquier tipo de muerte celular por parte de la TIB puede ser positivo para la recuperación funcional como lo reportaron previamente otros autores, que asocian la inhibición de la autofagia con una recuperación funcional post LTME[31,32]. Sin embargo, estos trabajos solo evalúan el efecto de las terapias aplicadas, inmediatamente después de la LTME, mientras que nuestro grupo de investigación y otros autores, evaluamos la autofagia a plazos más largos[33,34,36,70], permitiendo estudiar de forma más extensa el efecto de la TIB sobre la muerte celular más allá de la fase aguda o subaguda.

Durante las fases intermedia y crónica post LTME, persiste la muerte neuronal y de oligodendrocitos por procesos como la degeneración Walleriana, desmielinización, cicatrización glial, gliosis, etc.[1,8], sin embargo, la fase intermedia también está caracterizada por intentos de remielinización, reorganización neuronal y vascular, que se extienden hasta la fase crónica[1,2]. Por lo tanto, promover la autofagia para contrarrestar la apoptosis a los 14 dpl, puede resultar en una recuperación funcional al propiciar la sobrevivencia neuronal inhibiendo mecanismos degenerativos y auxiliando en los procesos de recuperación durante las fases intermedia-crónica.

Por otro lado, investigaciones realizadas con diversas terapias, han demostrado que la inhibición de la apoptosis favorece la recuperación funcional. Jung et al. demostraron que la rehabilitación con caminadoras reduce la apoptosis en la médula espinal después de una LTME, resultando en una mejor recuperación de la función motora [30].

Asimismo, terapias con fitoquímicos y agentes hormonales como estrógenos y SERMs, han demostrado una mejora funcional atribuida a la inhibición de la apoptosis después de una LTME[23–29,71]. Debido a la categorización de la TIB como un STEAR [72] en conjunto con los resultados de investigaciones *in-vitro* en las que se han demostrado

las cualidades anti apoptóticas de la TIB[73], se puede inferir que la TIB tiene un mecanismo de acción anti apoptótico similar al de los estrógenos después de una LTME.

Más aún, el ensayo de TUNEL y la evaluación del tejido preservado, corroboran la posible actividad anti apoptótica de la TIB a los 14 dpl. El grupo TIB 2.5 obtuvo un menor porcentaje de células positivas a TUNEL en la región caudal y rostral, así como un mayor porcentaje de tejido nervioso preservado en comparación con el grupo SCI. Investigaciones con ensayos de TUNEL evaluando el efecto anti apoptótico de estrógenos[24,28,57,61], del bazedoxifene [25] o del tamoxifen [27] administrados después de una LTME, demuestran como la terapia estrogénica puede reducir la cantidad de células positivas a TUNEL 1-7 dpl en el epicentro de la lesión, en la región rostral en la caudal de la médula espinal en la materia blanca y gris.

También los ensayos histológicos que evalúan la preservación del tejido medular por estrógenos[23,32,57,59,61], bazedoxifene [25] o tamoxifen [63,64] después de una LTME, han demostrado que la terapia hormonal puede incrementar la cantidad de tejido preservado o reducir la cantidad de tejido lesionado a los 18-42 dpl. Esto coincide con los resultados obtenidos en el presente trabajo, demostrando que la administración de TIB después de una LTME disminuye marcadores apoptóticos y la muerte celular, e incrementa la extensión del área de tejido preservado.

El grupo TIB 2.5 mostró una mejora funcional mayor a partir del día 14 después de la LTME comparado con el grupo SCI. Al finalizar los 30 días, las ratas en el grupo TIB 2.5 ocasional o frecuentemente soportaban el peso de sus miembros inferiores, pero sin coordinación, mientras que las ratas del grupo SCI no tenían soporte corporal de sus miembros inferiores. Este resultado liga la modulación de la muerte celular producida por la TIB con una mejora funcional.

Los resultados obtenidos en la recuperación funcional están limitados por el hecho que la TIB solo fue administrada hasta 21 dpl, mientras que el monitoreo funcional finalizó hasta el día 30. Aunque, evaluaciones realizadas con otros esquemas de tratamiento, usualmente no superan los 30 días y presentan una meseta en la recuperación funcional entre 14-28 dpl[23,25,32,57–60,63–65]. Por otro lado, García et al., demostraron como la administración continua de progesterona sigue mejorando la función motora en ratas después de 60 dpl[62].

Por otra parte, Colón et al., demostraron que la administración inmediata de tamoxifen después de una LTME en ratas, puede mejorar la función motora de forma significativa en comparación a un grupo tratado con tamoxifen pero 24 h después de la LTME[63]. Esto indica que el tratamiento agudo de la LTME es crítico para la recuperación de la función motora, por lo que nuestro grupo de investigación administró la TIB 30 min después de la LTME, y se obtuvo una mejor recuperación funcional, lo que demuestra la importancia de un tratamiento inmediato, después de la LTME.

4.2 Conclusiones

La TIB modula la autofagia de forma negativa y positiva de manera tiempo dependiente, y modula de forma negativa la apoptosis en un modelo de LTME en rata.

La TIB preserva una mayor cantidad de tejido después de una LTME al atenuar la muerte celular en un modelo en rata.

La administración de TIB mejora la recuperación de la función motora en un modelo de LTME en rata.

4.3 Perspectivas

En el presente estudio no se evaluó ninguna vía de señalización, sin embargo, se pueden plantear posibles mecanismos de acción de la TIB después de una LTME, considerando los resultados obtenidos y los procesos evaluados (Figura 1).

Las vías PI3K/Akt y AMPK/mTOR pueden explicar los resultados observados sobre la autofagia y la apoptosis. Estudios previos demuestran que la vía PI3K/Akt puede ser activada por la TIB[74] y por los estrógenos, además la activación de la vía PI3K/Akt inhibe la apoptosis[23,24].

En cuanto al efecto anti apoptótico, muchos autores han evaluado la activación de las vías PI3K/Akt[24,29,30,34,71,75] o ERK[24,75] y su papel en la inhibición de la apoptosis para favorecer la recuperación funcional, otros autores han encontrado que la TIB puede activar la vía PI3K/Akt, para mejorar la cognición en el envejecimiento y en algunas enfermedades neurodegenerativas[74]. Sin embargo, ninguna investigación ha reportado el efecto de la TIB sobre las vías PI3K/Akt, AMPK/mTOR o ERK después de una LTME,

por lo cual sería importante estudiar sí estas vías están involucradas en el mecanismo de acción de la TIB después de la LTME.

Por otro lado, la vía PI3K/Akt puede inhibir la autofagia en un modelo de LTME tratado con IGF-1[31], quizás la TIB podría activar dicha vía, lo que podría explicar cómo la TIB regula la autofagia de forma negativa durante los primeros 3 dpl.

Por otro lado, se ha demostrado que los estrógenos pueden activar la vía de AMPK/mTOR para promover la autofagia en condrocitos, previniendo así la apoptosis[76]. Otros estudios indican que después de una LTME, la regulación de TFE3, el ayuno intermitente, y la administración de eritropoyetina promueven la autofagia al activar la vía AMPK/mTOR, resultando en una mejora funcional[35,36,70]. Debido a que la TIB es un STEAR con un mecanismo de acción análogo al de los estrógenos[47,72], es posible que la vía AMPK/mTOR sea parcialmente responsable del incremento en expresión de los marcadores autofágicos a los 14 dpl, promoviendo la autofagia.
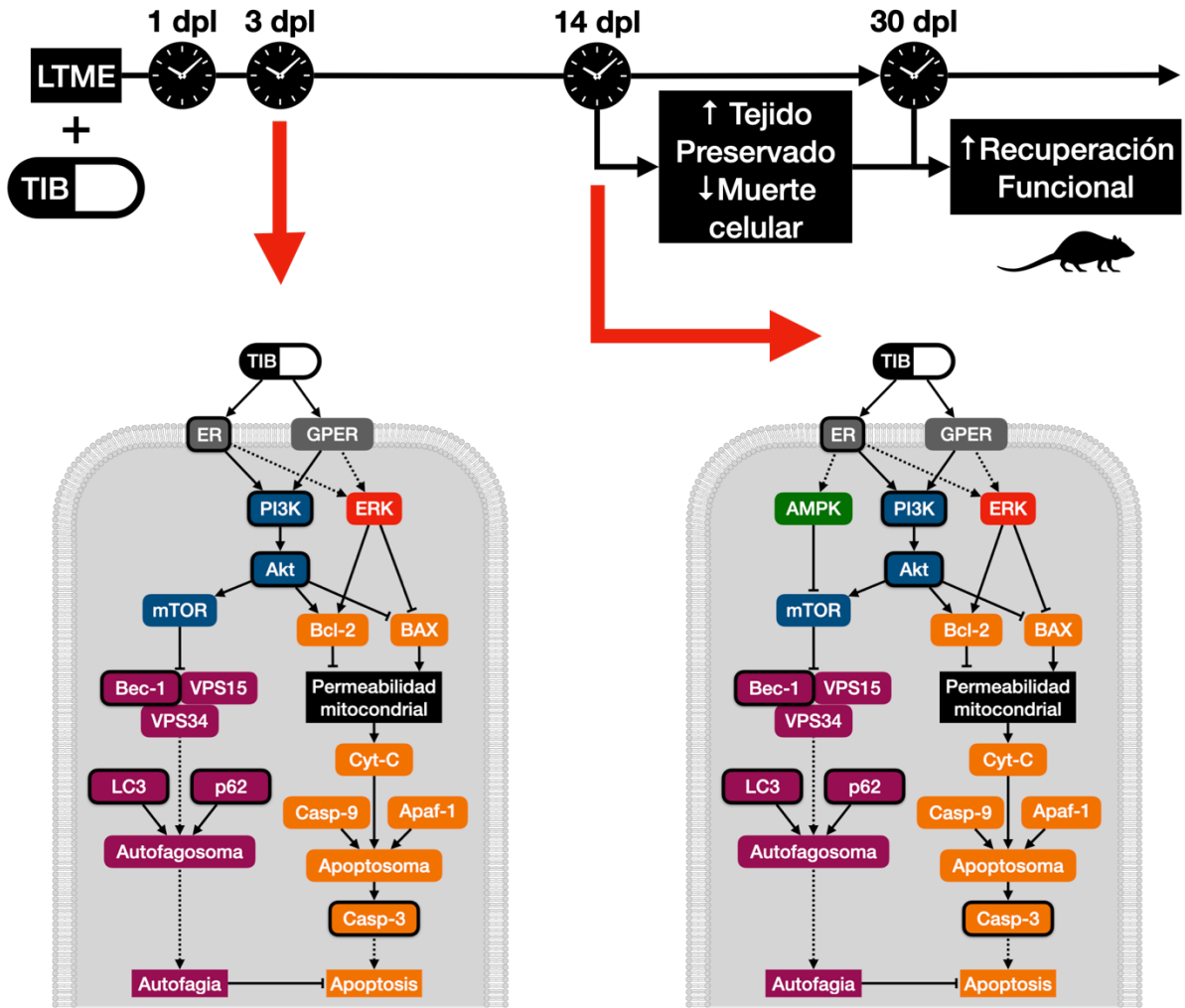
Figura 1. Posibles mecanismos de acción de la tibolona en la muerte apoptótica y autofágica en un modelo de lesión traumática de médula espinal en rata. Estos mecanismos se determinaron tomando en cuenta algunas vías de señalización celular moduladas por los estrógenos y considerando la acción estrogénica de la tibolona, así como los resultados obtenidos en el presente estudio (remarcadas con un perímetro negro). LTME: lesión traumática de médula espinal, TIB: tibolona, dpl: días post lesión, ER: estrogen receptors, GPER: G protein-coupled estrogen receptor 1, PI3K: phosphatidylinositol 3-kinase, ERK: extracellular signal-regulated kinase, AMPK: adenosine monophosphate activated kinase, Akt: protein kinase B, mTOR: mammalian target of rapamycin, Bcl-2: B-cell lymphoma 2, BAX: Bcl-2 associated X protein, Bec-1: Beclin-1, VPS15: vacuolar protein sorting 15, VPS34: vacuolar protein sorting 34, LC3: microtubule-associated protein 1 light chain 3, Cyt-C: cytochrome C, Casp-9: caspase 9, Apaf-1: apoptosis protease-activating factor 1, Casp-3: caspase 3, línea con flecha (⟶): estimulación bioquímica directa, línea con terminación plana (⟶): inhibición bioquímica directa, línea punteada con flecha (┈┈▶): estimulación bioquímica con pasos intermediarios, línea punteada con terminación plana (┈┈┤): inhibición bioquímica con pasos intermediarios.

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