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Low-energy extracorporeal shockwave therapy improves locomotor functions, tissue regeneration, and modulating the inflammation induced FGF1 and FGF2 signaling to protect damaged tissue in spinal cord injury of rat model: an experimental animal study

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Background: Spinal cord injury (SCI) is a debilitating condition that results in severe motor function impairments. Current therapeutic options remain limited, underscoring the need for novel treatments. Extracorporeal shockwave therapy (ESWT) has emerged as a promising noninvasive approach for treating musculoskeletal disorders and nerve regeneration.

Methods: This study explored the effects of low-energy ESWT on locomotor function, tissue regeneration, inflammation, and mitochondrial function in a rat SCI model. Experiments were performed using locomotor function assays, CatWalk gait analysis, histopathological examination, immunohistochemical, and immunofluorescence staining.

Results: The findings demonstrated that low-energy ESWT had a dose-dependent effect, with three treatment sessions (ESWT3) showing superior outcomes compared to a single session. ESWT3 significantly improved motor functions [run patterns, run average speed, and maximum variation, as well as the Basso, Beattie, and Bresnahan score] and promoted tissue regeneration while reducing inflammation. ESWT3 significantly decreased levels of IL-1β, IL6, and macrophages (CD68) while increasing leukocyte (CD45) infiltration. Additionally, ESWT3 upregulated NueN and mitofusin 2 (MFN2), suggesting enhanced neuronal health and mitochondrial function. Moreover, ESWT3 modulated the expression of fibroblast growth factor 1 (FGF1), FGF2, their receptor FGFR1 and phosphorylation of ERK, aiding tissue repair, and regeneration in SCI.

Conclusions: This study highlights the potential of low-energy ESWT as an effective noninvasive treatment for SCI, demonstrating significant improvements in motor recovery, tissue regeneration, anti-inflammatory effects, and mitochondrial protection. These findings provide valuable insights into the mechanisms of ESWT and its therapeutic application for SCI recovery.

Keywords: extracorporeal shockwave therapy, FGF signaling, locomotor function, spinal cord injury, tissue regeneration

Introduction

Spinal cord injury (SCI) occurs due to traumatic injury to the spinal cord, resulting in complete or incomplete impairment of neural functions, affecting mobility and sensory function[1]. The damages caused by SCI is categorized into primary and secondary injury[2]. The primary injury refers to the immediate mechanical trauma to spinal cord. It typically involves bruising of the spinal cord, followed by compression caused by dislocated bone and soft tissues[3]. As the injury develops, the spinal cord may become rotated, lacerated, hyper-bent, and over-stretched; however, the white matter is usually spared[2,3]. The secondary damage occurs after the initial trauma, driven by the inflammatory response that regulates numerous cellular and molecular interactions[4]. This response activates and recruits peripheral and resident

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inflammatory cells, including monocytes, astrocytes, microglial cells, neutrophils, and T lymphocytes to further promoting the development of secondary damage following SCI[5]. The ultimate changes after SCI, including neuronal apoptosis, syrinx formation, and glial scar formation, can result in permanent loss of sensorimotor functions, often unresponsive to therapy[6]. Effective SCI treatment requires stabilizing the spine and managing inflammation to prevent further damage. Given the complexity of secondary injury cascades, a detailed understanding of these processes is crucial for selecting the optimal time-point for therapeutic interventions[7]. To date, researchers have explored various approaches to SCI treatment, including stem cell transplantation, bioengineering methods, exoskeletons, brain-computer interfaces, and functional electrical stimulation[8,9]. These advanced methods offer promising prospects for the future treatment of SCI.

One emerging noninvasive treatment option that has gained attention for its therapeutic potential in SCI is extracorporeal shockwave therapy (ESWT). ESWT has shown effects in treating orthopedic disorders, including tendinopathies and nonunion of long-bone fractures, as well as soft tissue for over 30 years[10]. Many studies have demonstrated that ESWT stimulated subperiosteal callus formation by inducing micro-fraction in the cortex[11]. Unlike many existing treatment methods that require invasive procedures or carry risks of severe side effects, ESWT offers a less intrusive approach. Additionally, ESWT has been reported to promote the expression of growth factors, including bone morphogenetic protein and vascular endothelial growth factor (VEGF)[12–14]. It also improves blood supply, stimulates cell proliferation, and promotes tissue regeneration positions it as a promising solution for addressing the multifaceted pathophysiology of SCI[13,15,16]. According to these characteristics, ESWT is potential for reducing inflammation and promoting nerve regeneration makes it a valuable addition to the range of therapeutic options for SCI.

Studies have shown that ESWT (2000 impulses, 0.18 mJ/mm²) can stimulate new bone growth and significantly increased the stiffness of spinal fusion segments in flexion and extension in animal model. These results support the efficacy and safety of ESWT in promoting spinal fusion in animals[17–19]. Additionally, although ESWT has been shown to induce microscopic alterations in myelin sheaths in treated spinal cords, it does not result in neurological symptoms[20,21]. Furthermore, studies on the effects of ESWT on the sciatic nerve in rats have demonstrated that high-energy shockwaves (2000 impulses, 0.49 mJ/mm²) can lead to demyelination and a decrease in motor nerve conduction velocity. In contrast, low-energy shockwave treatments (2000 impulses, 0.08 mJ/mm²) showed no significant differences in nerve structure or function, indicating a safer profile[22]. Additionally, ESWT has been reported to promote recovery in cases of sciatic nerve crush injury by enhancing peripheral nerve regeneration[23,24]. Importantly, all observed changes following low-energy ESWT were reversible, suggesting a low risk of long-lasting or harmful complications in peripheral nerves, further supporting its safety for therapeutic use[22].

The effects of ESWT on different cell types and tissues can influence both intact or damaged nerve cells and tissue. Most studies have focused on the palliative effects of low-energy ESWT in both clinical and experimental applications[25–27]. When applied to the skin at effective doses, ESWT has been shown to induce analgesia; however, it may also result in localized nerve injury, which stimulates the expression of regeneration and transcription-related factors in dorsal root ganglion neurons[28]. Several studies have indicated that an appropriate dose of ESWT can promote cell proliferation, differentiation, and tissue regeneration. For example, a single treatment of 300 impulses at 0.1 mJ/mm² did not induce axonal degeneration after 1 week, whereas treatments with 900 or 1500 impulses caused moderate to severe degeneration[23]. Recently, Yamaya *et al*.[29] demonstrated that low-energy ESWT is a safe and effective therapeutic strategy for SCI. ESWT was found to enhance the expression of VEGF and Flt-1 in the spinal cord, reduce neuronal loss in injured neural tissue, and improve locomotor function following SCI[29]. However, further research is needed to determine the optimal dosage and to clarify the mechanisms of action of ESWT in the treatment of SCI. ESWT has been demonstrated as a noninvasive treatment method across various fields. Given the structural similarities between the spinal cord and the peripheral nervous system in both rats and humans, low-energy ESWT may provide similar beneficial effects in SCI treatments. In this study, a rat clip compression model was used to induce SCI, and the efficacy of low-energy ESWT was investigated with one or three treatments. The study also explored the motor function, pathological changes associated with SCI in neuron and mitochondria, as well as assessed the effects of low-energy ESWT on inflammation-induced fibroblast growth factor 1 (FGF1), FGF2, FGFR1, and phosphorylation of ERK (pERK) levels to protect the damaged spinal cord.

HIGHLIGHTS

- Extracorporeal shockwave therapy (ESWT) with three treatments is better than once for spinal cord injury.
- ESWT promotes locomotor function in rats with spinal cord injury.
- ESWT increases the expression of NueN and MFN2 in the cells of spinal cord injury.
- ESWT reduces inflammation induced FGF1 and FGF2.

Materials and methods

Animals

A total of 40 with body weights ranging from 250 to 300 g of Sprague-Dawley (SD) rats (BioLASCO) were used in the experiments. The detailed experimental protocol of the animal study was approved by the Animal Care Committee of the hospital. The animals were maintained at the Laboratory Animal Center for 1 week before experiments. The rats were housed at 23 ± 1°C with a 12 h light and dark cycle and given food and water. The work was reported in accordance with the Animals in Research: Reporting In Vivo Experiments guidelines[30].

Spinal cord injury (SCI)

The 250–300 g of SD rats were anesthetized with Zoletil (25 mg/kg), along with Xylazine (10 mg/kg). Before surgery, the animals received a subcutaneous injection of 3 ml of saline. They were then placed on a heating pad set to 37°C during the surgical procedure, which involved performing a laminectomy at the T9 level to expose the spinal cord. Dissection of the extradural plane

between the dura and adjacent vertebrae was carried out using a dissecting hook with a curvature and thickness similar to that of the clip. The SCI was induced using an applicator to apply a twice (once per minute) pressure of 60 g via a clip, which was then rapidly released to create acute impact compression injury[31]. The clip was allowed to compress the spinal cord for 1 min before being removed using the applicator. Postsurgery, ampicillin (25 mg/kg) and ketorolac (1 mg/kg/day) were administered for 5 days to prevent infection and alleviate pain.

Study design

Thirty two rats were randomized into four groups (eight rats for each group), as shown in Figure 1A. Sham group was that rats did not undergo surgery or receive treatment. The purpose of the Sham group is to differentiate between the effects of surgery-related stress and the actual injury, ensuring that any observed changes in the experimental groups are specifically due to the SCI and not external procedural factors. SCI group was that rats received clip compression on the T9 spinal cord to create injury. This group allows us to observe the natural progression of SCI and serves as a comparison to determine the effectiveness of the treatments in the other groups. It provides a baseline to assess the impact of ESWT on SCI recovery. In the ESWT group, the SCI rats were treated with shockwaves (0.13 mJ/mm^2 with 500 impulses, 4 Hz), which were modified and from a shockwave treatment apparatus (DUOLITH SD1, STORZ MEDICAL AG, Swiss) post-one week surgery[32]. The purpose is to investigate whether a single treatment can reduce inflammation, promote nerve regeneration, and improve functional recovery following SCI. This group helps assess the short-term effects of ESWT on SCI. In the ESWT3 group, the SCI rats received shockwaves at 1, 2, and 3 weeks postsurgery. The objective is to determine whether multiple sessions of ESWT lead to enhanced therapeutic effects compared to a single treatment. By comparing this group with the ESWT group, we aim to explore whether repeated applications of ESWT provide additional benefits in terms of reducing inflammation, improving tissue repair, and enhancing recovery outcomes. All rats were sacrificed at 7 weeks and the specimens were collected for further analysis.

Locomotor function assay

The limb motor functions were assessed at 2, 3, 5, and 7 weeks using the open-field locomotor test developed by Basso, Beattie, and Bresnahan (BBB)[33]. The score of each hind limb was recorded and the averages were calculated.

CatWalk gait analysis

The walking patterns of the rats were acquired and analyzed to measure the conditioned locomotion using the CatWalk Device (Noldus Information Technology Inc.). The four paws of rats were captured in the location of the locomotion by CatWalk Device. After an acclimatization period, baseline performance was assessed for the animals in the Sham, SCI, SW, and SW3 groups. A measurable run was defined as the ability of the animals to take three or more consecutive steps without interruption while traversing the walkway, and average values were subsequently calculated for analysis. The label paw prints of rats were

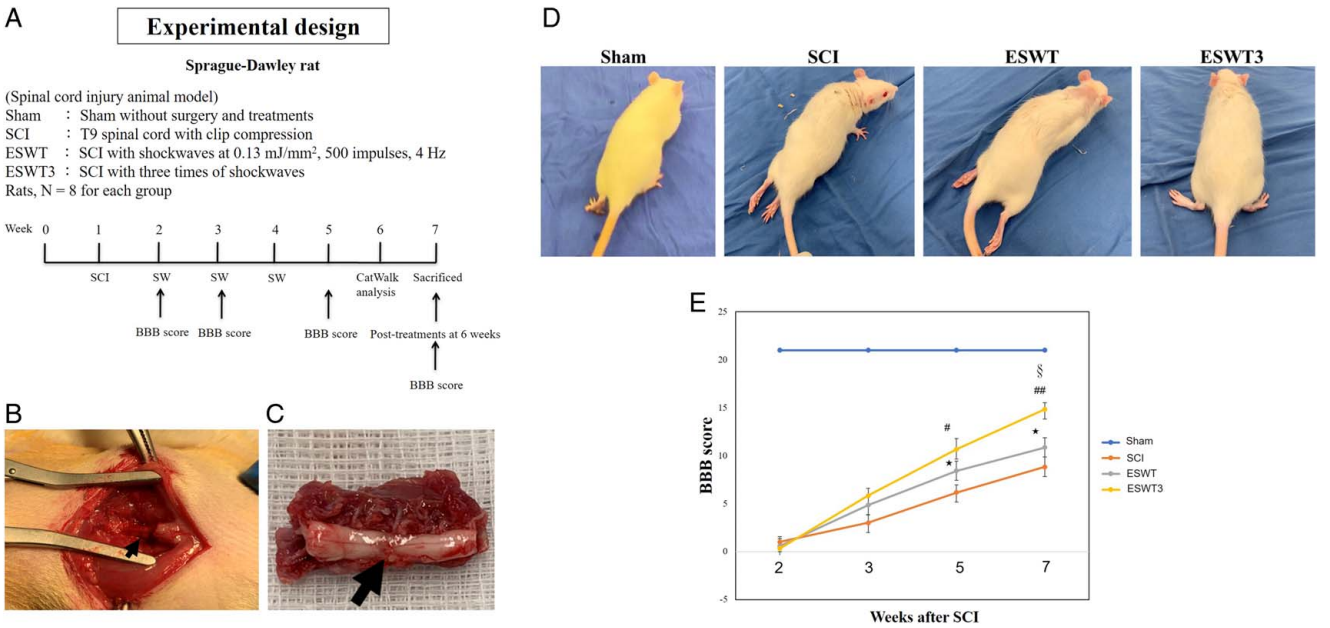

Figure 1. The study design and locomotor function assay. (A) The graph presents the study design of the experiment including Sham, SCI, ESWT, and ESWT3 groups. ESWT is that shockwaves applied at 1 week postsurgery and ESWT3 is that shockwaves applied at 1, 2, and 3 weeks postsurgery, respectively. The sacrifice of animals are at 6 weeks postsurgery. Eight rats are used for each group. (B) The injury is created at T9 of the spinal cord in the rat as indicated by the black arrow. (C) The damage region of the spinal cord is indicated by the black arrow. (D) The hind legs of the rat drag in the SCI and ESWT groups. Some of the rats have their paws turned upwards in the ESWT3 group at 6 weeks postsurgery. (E) The BBB scores are presented in the Sham, SCI, ESWT, and ESWT3 groups at 2, 3, 5, and 7 weeks. $^{*}P < 0.05$ compared between ESWT and SCI groups. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ are compared between ESWT3 and SCI group. $^{\S}P < 0.05$ compared between ESWT and ESWT3 group. $N = 8$; BBB, Basso, Beattie, and Bresnahan; ESWT, extracorporeal shockwave therapy; ESWT3, three ESWT treatments; SCI, spinal cord injury.

adjusting the intensity threshold and autoclassifying the prints by CatWalk software (ver. 10.1). All steps of rats were detected between these two points.

Histopathological examination

All animals were sacrificed at 6 weeks postsurgery. A 2 cm segment of the spinal cord, centered on the injury site, was harvested and postfixed in 4% neutral buffered formalin for 48 h. Subsequently, all tissues underwent alcohol processing before being embedded in paraffin blocks. The sections were then cut into 5 μm thick slices and stained with hematoxylin and eosin to cover the entire injury site.

Immunofluorescence staining

For immunofluorescence staining of CD45, NeuN, and MFN2, the tissue slides were deparaffinized with xylene, hydrated by a graded ethanol solution, and treated with 0.2% Triton-X in PBS for 25 min and a protein-blocking reagent for 1 h. Added primary antibody of CD45 (50165365, Thermo Fisher Scientific Inc.), MFN2 (9482, Cell Signaling Technology) at 1:100 dilution and incubated for 48 h at 4°C, and then added the red-fluorescent conjugated secondary antibody (Invitrogen) at 1:500 dilution for 2 h at room temperature. The sections then added the primary antibody of NeuN (MAB377, Merck Millpore) at 1:500 dilution and incubated for overnight at 4°C, and then added the green-fluorescent conjugated secondary antibody (Invitrogen) at 1:500 dilution for 1 h at room temperature. Cells were counterstained with DAPI (Vector Laboratories) to detect nuclei. Images were analyzed under a florescence microscope (Carl Zeiss).

Immunohistochemistry staining

IHC staining was used with a horseradish peroxidase-diaminobenzidine (HRP-DAB) staining kit (R&D Inc.) as described previously[34]. The samples were performed from the spinal cord of Sham, SCI, ESWT, and ESWT3. Polyclonal or monoclonal antibodies against IL1-β, IL6, FGF1, FGF2, and FGFR from Santa Cruz, USA; pERK from Cell Signaling, USA; were used as the primary antibodies. The specific binding of the secondary antibodies to the primary antibodies was visualized by employing HRP to catalyze the enzymatic conversion of the chromogenic substrate 3,3'-DAB, resulting in the formation of a brown precipitate. Following mounting and clearing, the sections were cover-slipped and examined using a Zeiss fluorescence microscope (Zeiss). Images were analyzed using Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring). The percentage of immunolabeled positive cells out of the total cells in each area were counted, and the average of each specimen was used as the results.

Statistical analysis

The statistical analysis of the data were performed using SPSS software (version 26.0, SPSS Inc.). Statistics were performed using one way ANOVA and Dunn-Bonferroni nonparametric comparison for post-hoc which the Kruskal–Wallis test was significant. The results were summarized as the mean ± SD, and $P < 0.05$, $P < 0.01$, and $P < 0.001$ were considered statistically significant.

Results

The study design and SCI rat model

In the current experiments, the study design was presented in Figure 1A. The experimental groups were divided into four groups such as Sham, SCI, ESWT, and ESWT3. In the experiments, we compared the effects of one treatment of ESWT and three treatments of ESWT for SCI rat and established the SCI rat model. SCI rat model was established by twice clip compressions with jaw pressure of 60 gravity on T9 spinal cord (Fig. 1B). The damage of the spinal cord was observed as indicated by the black arrow (Fig. 1C). The dosage of ESWT (0.13 mJ/mm^2, 500 impulses, 4 Hz) was referenced as previous studies and our experiences.

The Basso, Beattie, and Bresnahan score of ESWT3 is better than ESWT for treatment of SCI rat

In addition, we measured the motor function of the SCI rats after treatments. In the Figure 1D (7 weeks), the hind legs of the rat dragged in the SCI and ESWT groups. However, in the ESWT3 group, some of the rats had their paws turned upwards. The BBB score was used to assess the locomotor function of the hindlimbs on a 21-point scale in Sham, SCI, ESWT, and ESWT3 groups from 2, 3, 5, and 7 weeks (Fig. 1E). The maximum of 21 points in the BBB score was in the Sham group and the BBB score was dropped near to zero after surgery (SCI group). The BBB score was improved in ESWT ($P < 0.05$ and $P < 0.05$) and ESWT3 ($P < 0.05$ and $P < 0.01$) as compared with the SCI group at 5 and 7 weeks and ESWT3 ($P < 0.05$) was better than ESWT group at 7 weeks. The results indicated that ESWT could improve the locomotor function of SCI rat and three times treatment of ESWT3 was better than once treatment of ESWT.

The dosage-effect of shockwave treatment to improve motor function in SCI rat

Further, the CatWalk gait analysis was performed for behavioral tests in Sham, SCI, ESWT, and ESWT3 groups before rat sacrificed. Every run and the results of each group were showed in the Figure 3. In the SCI group, there was no print intensity (Fig. 2A) and print length (Fig. 2B) parameters in the hindlimbs (pink as right hind and green as left hind) compared to the Sham group after the injury. After shockwaves treatment, the print intensity and print length were improved in the ESWT and ESWT3 groups as compared with SCI as well as ESWT3 was better than the ESWT group.

The CatWalk gait parameters were measured, including run average speed and maximum variation in Sham, SCI, ESWT, and ESWT3 (Figs 2C and D). The run average speed and maximum variation were significantly improved in ESWT ($P < 0.05$ and $P < 0.01$) and ESWT3 ($P < 0.05$ and $P < 0.001$) than SCI. The improvement of the ESWT3 group was better than the ESWT group in run average speed ($P < 0.01$) and maximum variation ($P < 0.01$).

Shockwave treatment promoted the histological structure, anti-inflammation as well as improved neuron and mitochondria function in the injury spinal cord of the rat

In the pathological analysis of SCI rats after treatments, the gray (red arrow) and white (yellow arrow) matters were destroyed in the lesion center of the SCI group and repaired in the ESWT and

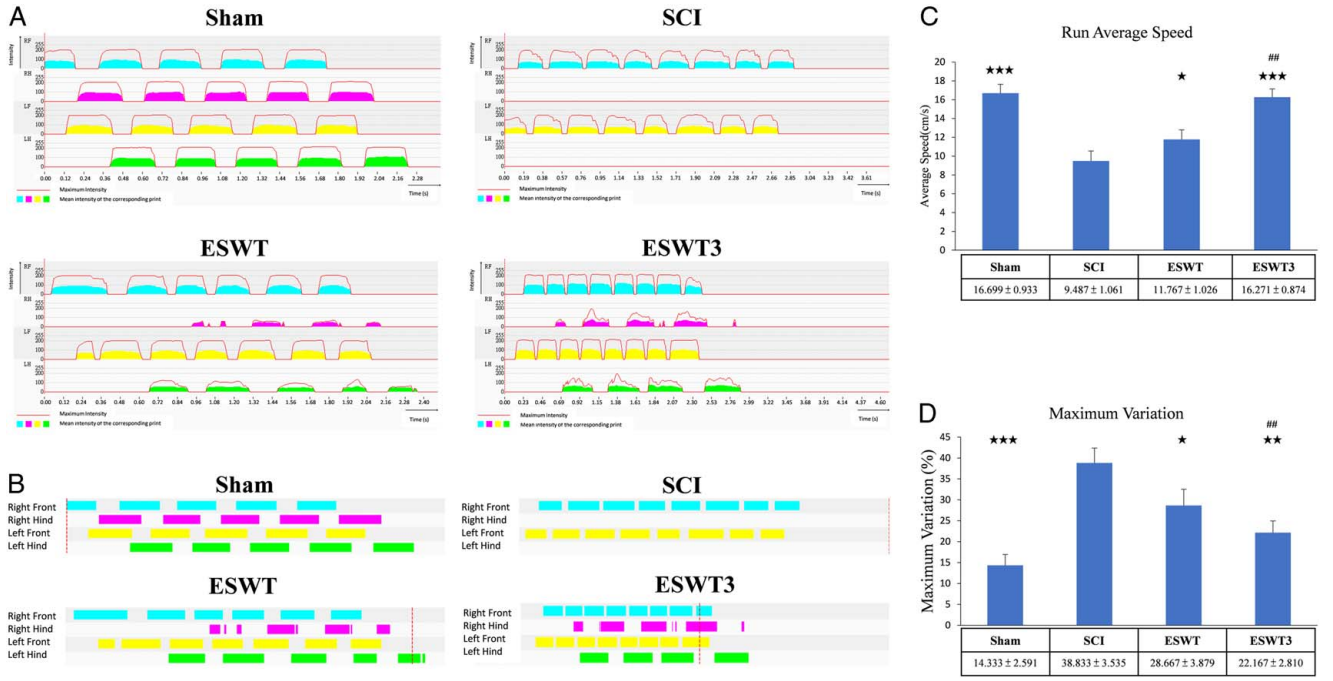

Figure 2. The graphical print view and data for each group by gait analysis from CatWalk. (A) The intensity of the run patterns. (B) Print view of the run pattern. (C) Run average speed. (D) Maximum variation. The $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ are compared with the SCI group. The $^{\#\#}P < 0.01$ are compared between ESWT and ESWT3 groups. $N = 8$. ESWT, extracorporeal shockwave therapy; ESWT3, three ESWT treatments; SCI, spinal cord injury.

ESWT3 groups, as well as the ESWT3 group, was better than ESWT group (Fig. 3A) showing from HE staining.

In addition, inflammatory markers, macrophages, leukocytes, and mitochondrial proteins were measured in the SCI group and treatment groups (Figs 3 and 4). The inflammatory markers IL-1β, IL-6 (Figs 3B and C), and macrophage (CD68, Fig. 3D) were increased in the SCI group, as well as leukocyte (CD45, Figs 3E and F) levels, while neuronal cells (NeuN) and the mitochondrial function marker mitofusin 2 (MFN2) were decreased (Fig. 4). In contrast, the treatment groups, ESWT ($P < 0.05$, $P < 0.001$, and $P < 0.05$) and ESWT3 ($P < 0.01$, $P < 0.001$, and $P < 0.001$), reduced IL-1β, IL-6, and macrophage (CD68) levels, increased leukocyte (CD45) levels ($P < 0.05$, $P < 0.01$), and enhanced the expression of NeuN (ESWT3, $P < 0.05$) and MFN2 (ESWT3, $P < 0.05$) in cells. Furthermore, the ESWT3 group exhibited superior effects compared to the ESWT group in promoting histological structure, reducing inflammation, and improving neuronal cell and mitochondrial function in SCI rats.

Shockwave treatment modulated the expression of inflammation-induced FGF1, FGF2, their FGFR1 receptor and phosphorylation of ERK in SCI rat

Additionally, we know that FGF1 and FGF2 play dual roles in tissue repair and inflammation. Endogenous FGF1 and FGF2 are highly expressed in inflammatory tissues through the pERK pathway to induce fibrosis, where they stimulate the expression of cytokines. However, exogenous administration of FGF1 and FGF2 can assist in tissue regeneration. However, the homeostasis of FGF1 and FGF2 are important for the tissue repair. In the results, the expression levels of endogenous FGF1, FGF2, their FGFR1 receptor and pERK were highly elevated in the SCI group. These levels were reduced to those observed in the Sham group after treatments in the ESWT ($P < 0.01$, $P < 0.001$, $P < 0.01$, $P < 0.001$) and ESWT3 ($P < 0.001$, $P < 0.001$, $P < 0.01$, and $P < 0.001$) groups (Fig. 5). In addition, ESWT3 ($P < 0.05$) was better than ESWT group to inhibit the activity of phosphorylation of ERK. The results indicated that ESWT protected the damaged spinal cord by modulating inflammation-induced FGF1, FGF2, and pERK signaling.

Discussion

In the study, we demonstrated the dose-dependent effects of low-energy ESWT on motor function recovery, tissue regeneration, anti-inflammatory responses, and mitochondrial function in a rat model of SCI. We found that three treatments of ESWT (ESWT3) were more effective than a single treatment (ESWT) in improving motor function, as evidenced by run patterns, run average speed, maximum variation, and the BBB score. Additionally, ESWT3 significantly reduced the inflammatory markers IL-1β, IL-6, and macrophages, while increasing the levels of leukocytes, NueN, and MFN2 compared to the SCI and ESWT groups. Furthermore, ESWT3 was superior in modulating inflammation-induced FGF1, FGF2, FGFR1, and pERK signaling to support tissue repair after SCI.

Motor function recovery

SCI leads to severe motor impairment, and restoring locomotor function is a critical challenge in SCI treatment[35]. Emerging treatment modalities such as ESWT have shown promise in promoting tissue repair and enhancing motor function recovery

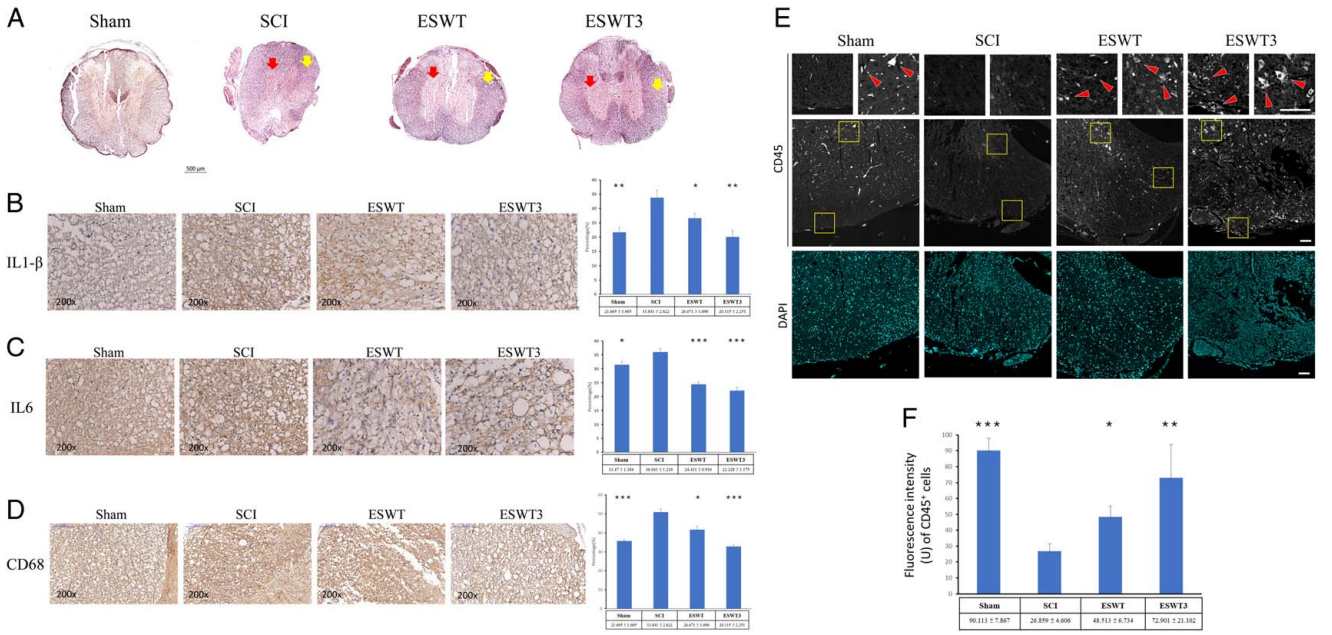

Figure 3. The histological structure, inflammation cytokines and immunocells measurement in the spinal cord injury after treatments. (A) The morphological observation is performed in the Sham, SCI, ESWT, and ESWT3 groups. The scale bar is 500 μm. The red arrow is indicated gray matter and yellow arrow is indicated white matter in the spinal cord. (B) The immunohistochemistry staining of IL1-β in the Sham, SCI, ESWT, and ESWT groups. The right panel is the expressed percentage of IL1-β. (C) The immunohistochemistry staining of IL6 in the Sham, SCI, ESWT, and ESWT groups. The right panel is the expressed percentage of IL6. The images are 200x magnification. (D) The immunohistochemistry staining of CD68-positive macrophages in the Sham, SCI, ESWT, and ESWT groups. The right panel is the expressed percentage of CD68-positive macrophages. The images are 200x magnification scale bar is 100 μm. (E) The immunofluorescence staining of CD45-positive leukocyte in the Sham, SCI, ESWT, and ESWT groups. Red arrows: increased leukocyte infiltration. Scale bar: 100 μm. (F) The panel is the fluorescence intensity of CD45-positive leukocyte. The $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ are compared with the SCI group. N = 4. ESWT, extracorporeal shockwave therapy; ESWT3, three ESWT treatments; SCI, spinal cord injury.

in animal model with SCI[36]. ESWT is a noninvasive therapeutic technique that utilizes low-energy shockwaves to stimulate tissue regeneration and repair[37]. It has been successfully used in various musculoskeletal conditions, such as tendinopathies and fractures, but its application in SCI is relatively novel[10,38]. The fundamental principle of ESWT involves the delivery of

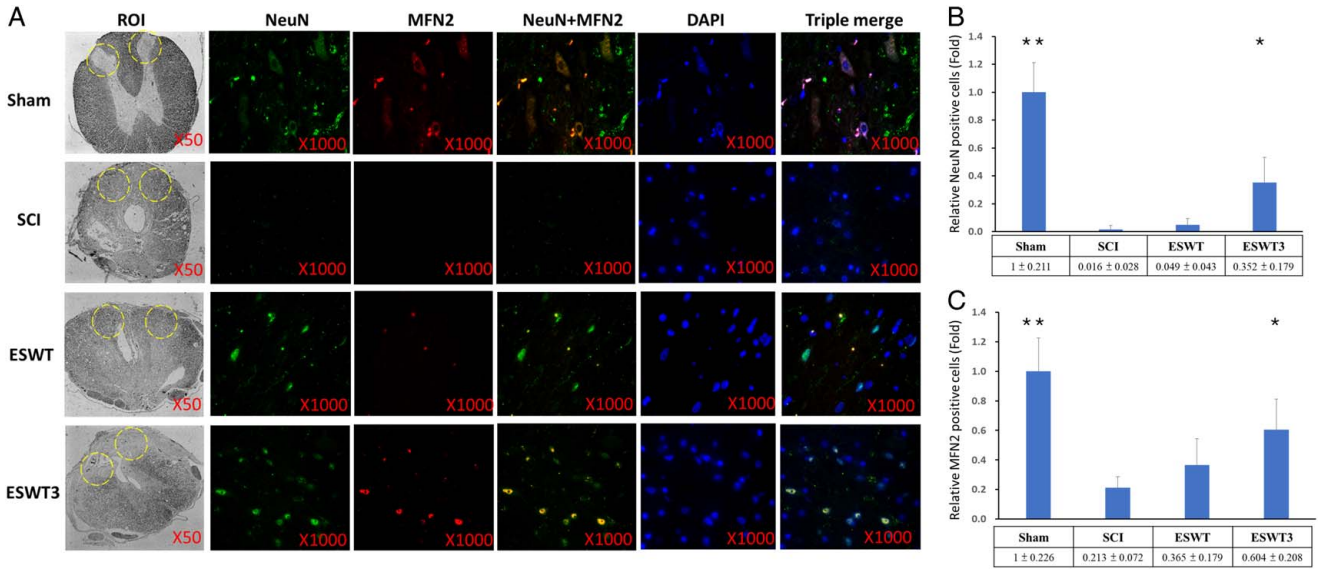

Figure 4. The NeuN and MFN2 measurements in the spinal cord injury after treatments. (A) The immunofluorescence staining of NeuN and MFN2 in the Sham, SCI, ESWT, and ESWT3 groups. (B) The related NeuN positive cells. (C) The related MFN2 positive cells. The $^{**}P < 0.01$ and $^{*}P < 0.05$ are compared with SCI group. N = 8. ESWT, extracorporeal shockwave therapy; ESWT3, three ESWT treatments; ROI, region of interest; SCI, spinal cord injury. The bright field of the images is 50x magnification. The images of immunofluorescence staining is 1000x magnification.

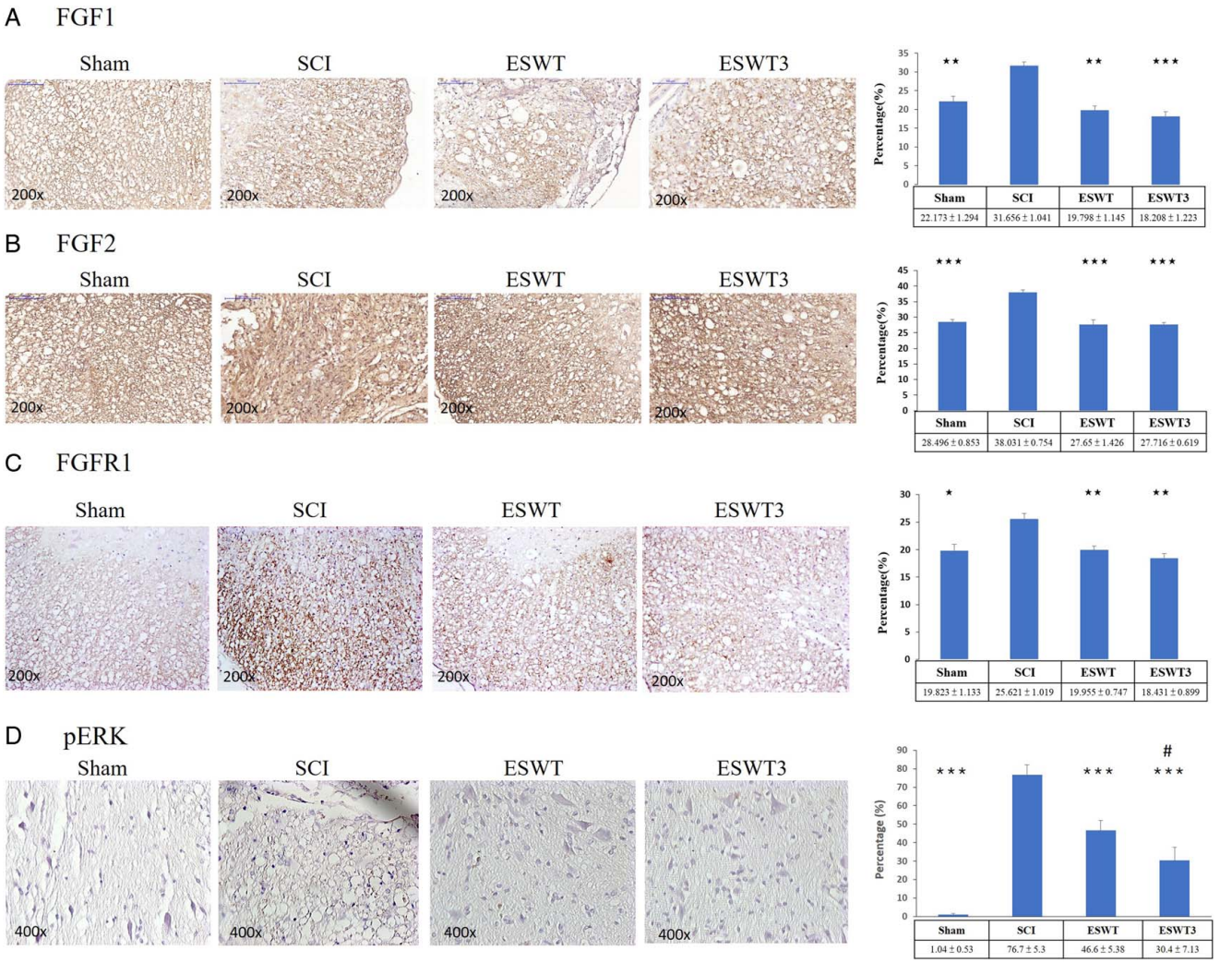

Figure 5. The expression of inflammation induced FGF1, FGF2, and FGFR1 receptor in the spinal cord injury after treatments. (A) The immunohistochemistry staining of FGF1-positive cells in the Sham, SCI, ESWT, and ESWT groups. The right panel is the expressed percentage of FGF1-positive cells. The images are 200x magnification. (B) The immunohistochemistry staining of FGF2-positive cells in the Sham, SCI, ESWT, and ESWT groups. The right panel is the expressed percentage of FGF2-positive cells. The images are 200x magnification. (C) The immunohistochemistry staining of FGFR1-positive cells in the Sham, SCI, ESWT, and ESWT groups. The right panel is the expressed percentage of FGFR1-positive cells. The images are 200x magnification. (D) The immunohistochemistry staining of pERK-positive cells in the Sham, SCI, ESWT, and ESWT groups. The right panel is the expressed percentage of pERK-positive cells. The $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ are compared with the SCI group. $^{\#}P < 0.05$ is compared with the ESWT group. N = 8. ESWT, extracorporeal shockwave therapy; ESWT3, three ESWT treatments; SCI, spinal cord injury.

mechanical energy to the affected area, triggering a cascade of cellular responses that promote healing and tissue regeneration[37]. Our results demonstrated that ESWT, particularly with three treatments (ESWT3), improved motor function significantly compared to the SCI and ESWT groups, as shown in the BBB score and CatWalk gait analysis (Figs 1, 2 and 3A). The improved run patterns, average speed, and reduced variability in gait further indicate the effectiveness of repeated ESWT treatments. This finding aligns with the growing evidence that ESWT promotes neuroprotection and recovery in SCI. Recently, ESWT is used for spinal cord ischemia and is demonstrated with safety and feasibility in patients[39]. In addition, a clinical trial protocol of ESWT for acute traumatic SCI on motor and sensory function is reported and one section of ESWT is used for the treatment[40]. However, the optimal dosage of ESWT for human SCI patients is yet to be precisely determined, and further research is needed to establish standardized protocols.

Anti-inflammatory effects and mitochondrial protection

SCI triggers an intense inflammatory response that can exacerbate tissue damage. In our study, ESWT3 significantly reduced the levels of IL-1β and IL-6, key inflammatory cytokines, as well as macrophage infiltration in the injured spinal cord (Fig. 3). These results suggest that repeated ESWT treatments provide better control of inflammation, potentially preventing secondary injury. These results suggest that repeated ESWT treatments provide better control of inflammation, potentially preventing secondary injury. Accumulating evidence reveal that the intrinsic dynamicity of the mitochondria plays a central role in regulating

the inflammatory response[41]. Here, we observed that SCI significantly reduced MFN2 expression, which is crucial for mitochondrial function. ESWT3 restored MFN2 levels, further supporting its role in reducing inflammation and protecting mitochondrial integrity (Fig. 4). This is consistent with previous findings that mitochondrial dysfunction exacerbates inflammation, and MFN2 plays a central role in maintaining mitochondrial dynamics during injury[42]. The recovery of MFN2 expression by ESWT3 indicates its potential to preserve mitochondrial function, which is essential for neuronal survival and tissue repair. It is possible that ESWT3 administration not only reduces inflammatory response but also facilitates mitochondrial transportation in axons by maintaining the level of MFN2.

Immune response and leukocyte infiltration

The immune response plays a crucial role in regulating various pathological processes and has a major impact on the prognosis of SCI[43]. In SCI, the immune response coordinates the activation of different immune cells and the release of immune factors. Injury can activate T cells and/or B cells, triggering an autoimmune response in the nervous system and maintaining their activation for an extended period of time[44]. During the repair phase, numerous cell adhesion molecules appear on the surface of activated T cells, facilitating their adhesion to vascular endothelial cells and enabling their entry into the central nervous system, which inhibits axonal death and promotes neuroprotection[45]. Our study demonstrated that ESWT3 increased leukocyte infiltration in the injured spinal cord compared to the SCI and ESWT groups (Fig. 3E). This increase in immune cell infiltration likely contributes to the enhanced regenerative capacity observed in the ESWT3 group. Leukocytes, particularly T and B cells, play an essential role in modulating inflammation and promoting neuroprotection[46]. The ability of ESWT to stimulate immune cell involvement in tissue repair highlights its therapeutic potential in modulating the immune response in SCI recovery. However, further studies are needed to explore whether ESWT promotes cell adhesion molecule expression, facilitating leukocyte infiltration.

Modulation of FGF1, FGF2, and FGFR1 signaling

Abnormal expressions of FGFs/FGFRs involve the progression of fibrosis formation[47]. Elevated expression of FGF1 and FGFR1 in idiopathic pulmonary fibrosis. However, FGF1 and FGF2 play dual roles in tissue repair and inflammation[48]. The homeostasis of FGF1 and FGF2 is crucial for inflammatory tissue response and repair. In various experimental models, these growth factors have been demonstrated to enhance angiogenesis, accelerate wound healing, and facilitate the regeneration of damaged tissues[49]. This regenerative effect is attributed to their ability to stimulate cell proliferation, differentiation, and survival in the context of tissue injury. Therefore, while endogenous FGF1 and FGF2 may contribute to fibrosis under inflammatory conditions, their therapeutic administration can potentially override these fibrotic pathways and promote the restoration of normal tissue architecture. In this study, SCI rats exhibited elevated levels of FGF1, FGF2, and their receptor FGFR1, which were reduced after ESWT treatment (Fig. 5). The modulation of these growth factors by ESWT suggests a mechanism through which ESWT may limit fibrosis and support tissue repair. The reduction of FGF1 and FGF2 expression and signaling in the ESWT and ESWT3 groups further underscores the role of shockwaves in balancing inflammation and promoting regeneration. The ability of ESWT to modulate these growth factors offers promising insights for its application in clinical settings.

Limitations

While our study shows promising results regarding low-energy ESWT for SCI recovery, several limitations exist. First, the rat model used may not fully replicate the complexity of human SCI, limiting the direct translational potential. Further studies in large animal models or clinical trials are necessary. Second, the optimal ESWT treatment regimen (frequency, intensity, and duration) remains undetermined, requiring further research to standardize protocols. Third, the 6-week observation period does not address the long-term effects of ESWT, including the potential for chronic complications such as scarring and fibrosis. Lastly, the molecular mechanisms by which ESWT modulates FGF1, FGF2, FGFR1, and pERK signaling require more investigation to clarify their roles in tissue repair.

Conclusion

In conclusion, our study demonstrates that repeated low-energy ESWT (ESWT3) is more effective than a single treatment in improving motor function, reducing inflammation, and promoting tissue repair in a rat model of SCI. ESWT3 also enhances mitochondrial function and modulates the expression of inflammation-related growth factors. These findings provide new insights into the therapeutic potential of ESWT for SCI treatment, suggesting that optimal dosing is crucial for maximizing its benefits. Future research is needed to explore the underlying mechanisms of ESWT and determine standardized protocols for clinical applications in SCI patients.

Ethical approval

This study was subjected to the approval of the Institutional Animal Care and Use Committee (IACUC) at KCGMH (Approval number: 2019120603).

Consent

Not applicable.

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Author contribution

C.-C.H.: funding acquisition, methodology, writing – original draft, and writing – review and editing; K.L.H.W.: methodology and writing – original draft; J.-M.P.: data curation, supervision, validation, and writing – original draft; Y.-N.W.: formal analysis, investigation, and writing – original draft; H.-T.C.: data curation, methodology, and validation; M.-S.L.: methodology and writing – original draft; J.-H.C.: conceptualization, investigation,

methodology, supervision, validation, writing – original draft, and writing – review and editing.

Conflicts of interest disclosure

The authors declare no conflicts of interest.

Research registration unique identifying number (UIN)

Not applicable.

Guarantor

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Data availability statement

The dataset used and analyzed in this study were obtained from the corresponding authors upon reasonable request.

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