Low-energy Extracorporeal Shock Wave Therapy Promotes VEGF Expression and Angiogenesis and Improve Locomotor and Sensory Functions after spinal cord injury

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Introduction: Extracorporeal shock wave therapy (ESWT) is widely used for various human diseases. The low-energy ESWT increases the expression of vascular endothelial growth factor (VEGF) in cultured endothelial cells [1]. VEGF stimulates not only endothelial cells to promote angiogenesis but also neural cells to induce neuroprotective effects [2]. Our previous study demonstrated the low-energy ESWT promoted mRNA expression of VEGF in damaged neural tissue and improved locomotor function after spinal cord injury (SCI) [3]. However, it has been still unknown about cell specificity of VEGF protein expression and angiogenesis induced by the low-energy ESWT. It has been also unclear about the neuroprotective mechanism in the injured spinal cord and therapeutic effect on sensory function produced by the low-energy ESWT. The purpose of this study was to investigate the effect of the low-energy ESWT on neural cell death and the cell specificity of VEGF expression and to examine the neuroprotective functions after SCI.

Methods: Animals
Adult female SD rats were divided into SCI group (SCI only), SCI-SW 1W group (ESWT applied for 1 week after SCI) and SCI-SW 3W group (ESWT applied for 3 weeks after SCI).

Surgical procedure
A SCI was induced by New York University Impactor. A 10 g rod was dropped from 12.5 mm onto the T10 segment.

Extracorporeal shock wave therapy
The low-energy ESWT was applied to the injured spinal cord 3 times a week for 3 weeks in SCI-SW 3W group and for one week in SCI-SW 1W group after SCI. The condition of the shock wave was 0.1 mJ/mm², 4 Hz, 200 shots/spot with two spots for each treatment [3]. Based on the effectiveness on locomotor recovery, which group (1W or 3W) to be performed histological analysis was determined.

Locomotor function
Locomotor function was evaluated using the BBB open field locomotor score for 6 weeks after SCI.

Mechanical allodynia
To evaluate mechanical sensitivity in the hindpaw, withdrawal threshold was measured using a von Frey filament (0.25-15 g) applied to the plantar surface. A modification of the “up-down” method was used to determine the value at which paw withdrawal occurred 50% of the time.

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Thermal sensitivity was assessed by measuring the withdrawal latency of the hindpaws from an infrared heat stimulus. An automatic plantar test instrument was used based on Hargreaves’ method.

**Immunohistochemistry**
Immunohistochemical stainings for VEGF and CD31 were performed using transverse sections of the spinal cord centered at the lesion epicenter obtained at 7 or 42 days after SCI.

**Immunodensity analysis of VEGF staining**
To investigate protein expression of VEGF, the immunodensity of VEGF staining was analyzed in the spinal cord sections at 7 days. Using the ImageJ analysis system, the immunodensity of VEGF staining was quantified and compared between SCI and SCI-SW groups.

**Double staining for VEGF and various cell type markers**
To examine the expression of VEGF in a specific population of cells in the injured spinal cord, the transverse sections in the SCI-SW group at 7 days were double-stained for VEGF and various cell type markers: NeuN for neurons, GFAP for astrocytes and Olig2 for oligodendrocytes.

**Immunodensity analysis of CD31 staining**
To investigate angiogenesis, the immunodensity of CD31 staining was analyzed in the spinal cord sections at 42 days, as described above.

**Counting of TUNEL-positive cells**
To detect DNA fragmentation caused by cell death in the injured spinal cord, TUNEL staining was performed using the sections at 7 days. The numbers of TUNEL-positive cells in the sections were counted.

**Results: Locomotor function**
The SCI-SW 3W group had significant locomotor improvement than SCI group from 35 days to 42 days (p < 0.05) (Fig. 1A). The SCI-SW 1W group had relatively but not significantly higher BBB score than SCI group. Based on the results, the histological analyses were performed using animals in SCI-SW 3W group.

**Mechanical allodynia**
The mechanical allodynia in SCI-SW 3W group was significantly improved than that in SCI group from 28 to 35 days (p < 0.05) (Fig. 1B). There was no significant improvement in SCI-SW 1W group.

**Thermal allodynia**
The SCI-SW 3W group showed significantly better improvement in the withdrawal latency compared to SCI group from 35 days to 42 days (p < 0.05) (Fig. 1C). There was no significant improvement in SCI-SW 1W group.

**The expression of VEGF**
In the immunostaining of VEGF, the VEGF-positive cells were more frequently observed in the SCI-SW group than in the SCI group (Fig. 2A, B). The immunodensity of VEGF staining in the SCI-SW group was significantly higher than that in the SCI group at the epicenter (p < 0.01) (Fig. 2C).

**Double staining for VEGF and various cell type markers**
In the double staining, the expression of VEGF was observed in NeuN, GFAP and Olig2 labeled cells (Fig. 2D-I). The double staining demonstrated the VEGF protein expression to be observed in neurons, astrocytes and oligodendrocytes.

**Immunodensity analysis of CD31 staining**
In the immunostaining of CD31, the CD31-positive cells were more frequently observed in the SCI-SW group than in the SCI group (Fig. 3A, B). The immunodensity of CD31 staining in the SCI-SW group was significantly higher than that in the SCI group at 1500 μm rostral to the epicenter, epicenter and 1000 μm rostral to the epicenter (p < 0.05) (Fig. 3C).

**The number of TUNEL-positive cells**

In the TUNEL staining at 7 days, the TUNEL-positive cells were obviously decreased in the SCI-SW group compared with the SCI group (Fig. 3D-G). The number of TUNEL-positive cells was significantly lower in the SCI-SW group compared to the SCI group at the epicenter and at 1000 μm rostral and caudal to the epicenter (p < 0.05) (Fig. 3H).

**Discussion:** Previous studies demonstrated that VEGF has neuroprotective effect in SCI [4] [5]. VEGF can enhance angiogenesis to restore blood supply [1] and promote neuronal survival and repair [6]. The present study demonstrated that the low-energy ESWT significantly increased VEGF protein expression in various neural cells and promoted CD31 expression in the injured spinal cord. These results suggested the low-energy ESWT enhances angiogenesis that is regulated by VEGF and may contribute to neuroprotection following SCI.

The present study showed that the low-energy ESWT applied for 3 weeks significantly improved not only locomotor function but also mechanical and thermal allodynia after SCI. Interestingly, the low-energy ESWT significantly reduced the number of TUNEL-positive cells in the injured spinal cord. These results suggested that the neuroprotective effect of VEGF may suppress the cell death in damaged neural tissue and improved locomotor and sensory functions following SCI.

**Significance:** The low-energy ESWT promoted the VEGF expression to induce neuroprotective mechanisms such as reduction of cell death and enhancement of angiogenesis and provided significant improvement of locomotor and sensory functions after SCI. Therefore, the low-energy ESWT can be a novel therapeutic strategy for the treatment of SCI.
Fig. 1. (A) The locomotor function evaluated using BBB score for 6 weeks. The SCI-SW 3W group demonstrated significantly better locomotor improvement in BBB scoring than the SCI group from 35 days to 42 days after injury (p = 0.011, n = 10 per group). (B,C) Mechanical and thermal alldynia for 42 days after SCI. (B) In the assessment of mechanical alldynia, SCI-SW 3W group were significantly improved than the SCI group from 28 days to 35 days (p = 0.028, n = 5). (C) In the assessment of thermal alldynia, SCI-SW 3W group were significantly improved than the SCI group from 35 days to 42 days (p = 0.028, n = 5). The values are mean ± SD (*p < 0.05, n = 5 per group).
Fig. 2. The immunodensity analysis of VEGF staining. (A-D) The VEGF-positive cells in the section at epicenter were more frequently observed in the SCI-SW group than SCI group. Scale bar = 100 µm. (E) The immunodensity of VEGF staining in the SCI-SW group was significantly higher than that in the SCI group at the epicenter ($p = 0.0086$). The values are means ± SD (**$p < 0.01$, $n = 4$ per each group). (D-I) In the double staining, the expression of VEGF was observed in NeuN, GFAP and Olig2 labeled cells (Arrows). Scale bars = 50 µm.
Fig. 3. The immunodensity analysis of CD31 staining. (A, B) The CD31-positive cells in the section were more frequently observed in the SCI-SW group than SCI group. Scale bar = 100 μm. (C) The immunodensity of CD31 staining in the SCI-SW group was significantly higher than that in the SCI group in the section at 1500 μm rostral to the epicenter, epicenter, and 1000 μm rostral to the epicenter ($p = 0.016, 0.021, 0.027$). The values are means ± SD (*$p < 0.05$, $n = 8$ per each group). Immunohistochemical staining of TUNEL in the SCI and SCI-SW groups at 7 days after SCI. (D-E) Representative sections at the epicenter showed there were more TUNEL-positive cells in the SCI (D, E) than in the SCI-SW group (F, G). Scale bars = 200 μm. (H) The number of TUNEL-positive cells in the SCI group was significantly higher than those in the SCI-SW at the epicenter and 1000 μm rostral, caudal from epicenter ($p = 0.021, 0.021, 0.043$). The values are means ± SD (*$p < 0.05$, $n = 4$ per group).