Local application of MDL28170-loaded PCL film improves functional recovery by preserving survival of motor neurons after traumatic spinal cord injury

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ABSTRACT
Neuronal death and organization degeneration can happen inordinately after spinal cord injury (SCI), which lead to nerve dysfunction. We aimed to determine whether local application of a cell permeable calpain I inhibitor (MDL28170) can promote SCI recovery by increasing neuronal cell viability. MDL28170-loaded poly-caprolactone (PCL) film was fabricated. Scanning electron microscopy showed the surface of PCL film was smooth with holes (diameter at μM level). The PCL film was non-toxic, biological compatibility, and had good neuron adhesion and slow release characteristic. MDL28170 increased VSC4.1 motor neurons’ viability under tunicamycin (an endoplasmic reticulum stress) induced injury. In a traumatic SCI rat model, MDL28170-loaded PCL film reduced the area of lesion cavity, and promoted recovery of locomotor behavior. Moreover, the expression of GAP-43 was upregulated after MDL28170-loaded PCL film treatment. Thus, our findings demonstrated that localized delivery of MDL28170 could promote SCI recovery by inhibiting endoplasmic reticulum stress, preserving survival of the motor neurons, which may point out a promising therapeutic target for treating SCI patient.

1. Introduction
Spinal cord injury (SCI) is pernicious to spinal cord with limited functional recovery. During SCI, prolonged endoplasmic reticulum (ER) stress results in neural apoptosis. Previously, we reported that ER stress was involved in cell death after SCI [1,2]. ER stress induced apoptosis and cell death are primarily mediated by calpains (calcium-activated cysteine proteases) [3]. Calpains are activated within a few minutes following SCI and their elevated activity persists for 24 h, which contributes to the tissue damage and impaired locomotor function [4,5]. Calpain I knockdown improves tissue sparing and functional outcomes after SCI in rats [6], providing a reasonable window of opportunity for post-injury inhibition by pharmacological inhibitor in clinics.

MDL28170, a specific cell permeable calpain I inhibitor, can inhibit ER stress and cell death [7]. It has demonstrated positive efficacy on SCI after intravenous infusion or a daily i.p. dose [8]. In addition, MDL28170 could improve the survival of Schwann cells and stem cells after transplantation in the spinal cord and promote the motor function recover in SCI rats [9,10]. To avoid adverse side effects caused by high dose of MDL28170, direct microinjection of MDL28170 with low dose is proven to be more effective to reduce the activity of calpain than intravenous injection [11,12]. However, the process of microinjection might cause secondary traumatic injury in the spinal cord. We hereby hypothesize that local delivery of MDL28170 in a biodegradable

Abbreviations: ER, endoplasmic reticulum; PCL, polycaprolactone; BSA, bovine serum albumin; H&E, hematoxylin and eosin; GFAP, glial fibrillary acidic protein; GAP43, growth associated protein 43; SCI, spinal cord injury
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material can promote SCI repair by reducing neuronal cell death.

Polycaprolactone (PCL), a frequently-used biomaterial with biodegradability, biocompatibility and benign drug permeability, has been widely adopted in a variety of biomedical applications, such as the delivery of bioactive drugs for spinal cord regeneration [13].

In the present study, we evaluated the potential efficacy of MDL28170 in a rat model of SCI, and determined that local delivery of MDL28170 in PCL film can promote SCI recovery by inhibiting ER stress and preserving the surviving of the motor neurons.

2. Materials and methods

2.1. Manufacture of PCL film

The biodegradable PCL film was manufactured with a modified protocol as previously reported [13]. We dissolved PCL (MW = 55 kDa, Sigma-Aldrich, St. Louis, MO, wt/vt = 15%) in trifluoroethanol (TFE, Aladdin, China) by the vortex shock to get a homogeneous of PCL and TFE solution. MDL28170 (100 μM) in dimethyl sulfoxide (DMSO) was added into the homogeneous solution. The mixed solution was poured onto a glass dish (diameter 9 mm), and dried in fume hood to allow slow evaporation of solvent for at least one week at room temperature. The concentration of MDL28170 used for intraspinal microinjection in SCI model was 9.5 μg [12]. In present study the final concentration of MDL28170 was set as 2.85 μg or 11.4 μg in each small film (2 mm × 2 mm). The vehicle DMSO-loaded PCL film was used as control film.

2.2. Drug release

As previous described, in vitro assessment was performed to determine the kinetics of drug release of PCL film [13]. In brief, 10 mg/mL bovine serum albumin (BSA) in phosphate buffered solution (PBS) was loaded in the PCL film, then immersed in 10 mL of PBS at 37°C. We examined the BSA concentration in PBS within 7 days by a Pierce BCA Protein Assay kit.

2.3. VSC4.1 motor neuron culture and treatment

To evaluate the biocompatibility of PCL film, the ventral spinal cord (VSC4.1) motor neuron cells (1 × 10⁶ cells/well) were cultured with normal culture medium (control group), 5% DMSO (toxicity group), or PCL film (2 mm × 2 mm) for 24 h in 96-well plates, respectively. Five parallel wells were designed for each treatment.

To evaluate the protective effects of MDL28170 on motor neurons in vitro, VSC4.1 motor neurons (1 × 10⁴ /well) were exposed to MDL28170 (1–100 μM) for 24 h in 96-well plate. Next, with or without MDL28170 (1–10 μM) pretreatment for 1 h, VSC4.1 motor neurons were exposed to an ER stress inducer tunicamycin (1, 3, 10 μg/mL, Sangon Biotech, Shanghai, China) for 24 h, respectively.

2.4. Scanning electron microscopy (SEM)

The morphology of PCL film was examined with SEM. VSC4.1 motor neurons were seeded on the surface of PCL film and cultured for 48 h. PCL film was fixed in 2.5% glutaraldehyde solution overnight, then immersed in OsO4 for 40 min and dehydrated in alcohol solution (30%-100%, v/v). Finally, PCL films were mounted on aluminum stubs and coated with gold. They were photographed under a Hitachi Se3000 N SEM.

2.5. CCK-8 assay

The viability of VSC4.1 motor neurons were evaluated by CCK-8 assay [14]. Experiments were performed at least in triplicate. Data were expressed as percentages of control values.

2.6. SCI model

Twenty-four adult Sprague–Dawley female rats (200–220 g) were divided into 3 groups (n = 8/group): vehicle-loaded PCL film group, low dose of MDL28170-loaded PCL film group (2.85 μg/μL) and high dose of MDL28170-loaded PCL film group (11.4 μg/μL). After rats were anesthetized with intraperitoneal injection of 1% sodium pentobarbital, SCI model was conducted as described previously [15]. In brief, an aneurysm clamp (70 g force, Medicon, Germany) was used to clamp spinal cord at T10 and was left for 1 min to produce an injury. After the clamp was removed, the spinal cord was then covered with a piece of PCL film (loaded with MDL28170 or vehicle). The muscle and skin were sutured layer by layer. The bladders were manually voided twice daily until the reflexive urination returned. The experimental procedures were approved by the Animal Ethics Committee of Zhejiang University and carried out in accordance with institutional guidelines.

2.7. Motor functional scale

The locomotor function was assessed by the Basso, Beattie and Bresnahan (BBB) open field locomotor scores once per week within 8 weeks after operation [14].

2.8. Tissue processing, H&E staining and Masson Trichrome staining

After 8 weeks, rats (n = 3/group) were sacrificed by overdose of sodium pentobarbital (60 mg/kg, i.p.). About 4 mm of the spinal cord in lesion epicenter was removed and embedded by paraffin. Longitudinal sections (5 μm) were prepared for hematoxylin-eosin (H&E) staining, Masson staining and immunohistochemistry. Slides were photographed with a BX41 Olympus microscope (Olympus Inc., Tokyo, Japan). Quantitative analysis was performed blindly by calculating data from sections using IPP software.

2.9. Immunohistochemistry

The continuous sections (5 μm) from above samples (n = 3/group) were conducted with immunohistochemical staining [16]. The primary antibodies were used: anti-glial fibrillary acidic protein (GFAP, sc-33673; 1:500; Santa Cruz Biotechnology, USA) and rabbit anti-growth associated protein 43 (GAP43, sc-33705, 1:500, Santa Cruz Biotechnology, USA).

2.10. Western blot analysis

A segment (4 mm length) of spinal cord at the contusion epicenter was removed after 8 weeks and homogenized for protein assay. The primary antibodies used were anti-GFAP (sc-33673; 1:100; Santa Cruz Biotechnology, USA), rabbit anti-GAP43 growth associated protein 43 (sc-33705, 1:500, Santa Cruz Biotechnology, USA) and β-actin antibody (1:5000; Sigma, USA). Band intensity was analyzed with Image Studio Ver 5.2 system. β-actin was used as internal control.

2.11. Statistical analysis

We used two-way analysis of variance (ANOVA) with Bonferroni post hoc test for BBB score. One-way ANOVA with Student’s-Newman-Keuls test was used for statistical comparison when appropriate. All data are presented as mean ± SEM. P < 0.05 was accepted as significant.

3. Results

3.1. Characterization of PCL films

The fabrication process of PCL film was shown in Fig. 1A. PCL film...
is soft and transparent as shown in light photograph (Fig. 1B). The surface of the PCL films is smooth and has a porous structure under SEM. The diameter of pores varies, and on the micron level. It may facilitate the infiltration of tissue fluid to promote drug release (Fig. 1C).

The fabricated PCL film was sterilized by incubation with 75% (v/v) alcohol for 10 min and washed thoroughly by PBS for three times. PCL film exhibited neuron adhesion characteristic as evidenced by SEM (Fig. 1D). Moreover, CCK-8 assay confirmed the viability of the VSC4.1 motor neuron was not altered by PCL film (Fig. 2A). The BSA loaded-PCL film showed relatively continuously release for 7 days in vitro as previously reported [13].

3.2. MDL28170 attenuated ER stress induced neuronal cell death in vitro

We explored whether MDL28170 could enhance the survival of motor neurons under condition of ER stress injury. The cell viability was significantly decreased by tunicamycin in a dose dependent manner \( p < 0.01 \), Fig. 3A). We confirmed that tunicamycin (3 μg/ml, 24 h) was sufficient to induce the death of VSC4.1 motor neurons in vitro. Next, we found that low concentration of MDL28170 (100 nM~10 μM) had no significant effect on cell viability, while high concentration of MDL28170 (50 μM, 100 μM) markedly decreased cell viability \( 65.34 \pm 0.17\% \), \( 58.76 \pm 0.32\% \), \( p < 0.01 \) vs control, Fig. 3A). We then tested whether pretreatment with low concentration of MDL28170 could enhance the viability of motor neurons against ER stress damage. Pretreatment with MDL28170 significantly increased the viability of VSC4.1 motor neurons dose dependently. 10 μM MDL28170 exhibited the maximal effect, and the viability was elevated from 48.71 \( \pm \) 2.49% to 71.73 \( \pm \) 4.61% \( p < 0.01 \), Fig. 3C). These results support the potential of MDL28170 to protect VSC4.1 motor neurons from cell death induced by ER stress.

3.3. Local delivery of MDL28170-loaded film reduced cavity formation and promoted locomotor functional recovery in vivo

Rat spinal cord at T10 was clamped by an aneurysm to produce a SCI model (Fig. 4A). The area of rough surface on the dorsal side of spinal cord in vehicle-loaded PCL film alone group was larger than those in other two groups, while no difference was observed in the areas of rough surface on the ventral side of spinal cord among three groups (Fig. 4B).

To explore the role of the MDL28170 film on cavity formation in vivo, H&E staining was performed on the longitudinal sections after SCI. In vehicle-loaded PCL film groups, there was the maximal size of cavum in epicenter of injury sites, which was remarkably reduced in the high dose of MDL28170-loaded PCL film treated group (Fig. 4C). The mean cavity area was 65.06 \( \pm \) 6.43% in the vehicle-loaded PCL film group, while that in the high dose of MDL28170-loaded PCL film treated group was 31.24 \( \pm \) 8.23% \( p < 0.01 \), Fig. 4D). Although the mean cavity area in the low dose of MDL28170-loaded PCL film treated group was slightly decreased (54.24 \( \pm \) 9.37%), there was no difference between low dose of MDL28170-loaded PCL film treated group and vehicle-loaded PCL film group \( p > 0.05 \), Fig. 4D). These data suggest that localized delivery of MDL28170 could reduce cavity formation after SCI.

Behavioral performance was assessed by BBB locomotor scores. After surgery, the score dropped from 21 to zero at day 1. Low dose MDL28170-loaded PCL film group and vehicle-loaded PCL film group had the similar BBB scores \( p > 0.05 \), Fig. 4E). BBB score in high dose MDL28170-loaded PCL film treated group was higher than those in vehicle-loaded PCL film treated group at week 2 and 8 week \( 3.20 \pm 1.39 \) vs \( 0.83 \pm 0.17 \); \( 8.40 \pm 1.60 \) vs \( 3.20 \pm 0.92 \), \( p < 0.01 \), Fig. 4E). These results indicate that high dose of MDL28170-loaded PCL film could improve functional recovery of SCI rats within 8 weeks.

![Fig. 1. Fabrication and characterization of PCL film. (A) The schematic diagram illustrating the fabrication process of PCL film. (B) Light photograph of MDL28170-loaded PCL film. (C) SEM on the surface of MDL28170-loaded PCL film. (D) SEM on the morphology of VSC4.1 motor neurons on MDL28170-loaded PCL film.](image_url)
3.4. Effect of MDL28170 on GAP43 in the late stage of SCI in vivo

Longitudinal sections of spinal cord were stained by immunohistochemistry for GAP43 expression at 8 weeks after SCI. There was less expression of GAP43 in vehicle-loaded PCL film group, which was slightly increased in high dose of MDL28170-loaded film group ($p > 0.05$, Fig. 5A). Likewise, Western blot showed SCI also induced an upregulation of GAP43 protein expression, which was not reversed by neither doses of MDL28170-loaded films treatment ($p > 0.05$, Fig. 5B, C).

3.5. Effect of MDL28170 on GFAP expression and astrogliosis in the late stage of SCI in vivo

SCI induced a stronger positive staining of GFAP in the injured spinal cord. This staining was slightly attenuated in high dose of MDL28170-loaded film group, but was similar with SCI group or low dose of MDL28170-loaded film treated group ($p > 0.05$, Fig. 6A). However, high dose of MDL28170 film treatment decreased astrogliosis, which might contribute to the protective effect on the surrounding neurons at the lesion site. These data suggest that local delivery of MDL28170 in PCL film can provide innovative strategy for treatment of SCI.

4. Discussion

Our study proved that localized administration of MDL28170 could promote locomotor function recovery in rat SCI model. The PCL film we fabricated showed no cytotoxic and the drug release kinetics of drug delivery systems was sustainable. MDL28170 preserved motor neurons survival induced by tunicamycin in vitro. MDL28170-loaded PCL film reduced cavity formation in vivo, which might contribute to the protective effect on the surrounding neurons at the lesion site. These data suggest that local delivery of MDL28170 in PCL film can provide innovative strategy for treatment of SCI.

ER stress and calpain activation mediated cell death in the initial lesion are the leading causes of secondary spinal cord injury [10,17,18]. Here we proved that calpain I inhibitor, MDL28170, protected ER stress-induced neurotoxicity in motor neurons. At a low concentration range (<10μM), MDL28170 restored the survival of motor neurons injured by tunicamycin. We found that if the concentration of MDL28170 was beyond 10μM (for example, at 50μM or 100μM), MDL28170 itself showed toxic effect on motor neurons. This may provide an explanation for those studies that abandoned the systemic application of MDL28170 to avoid the risk of side effects from high-dose applications throughout the body in vivo.

MDL28170 arouses the most attention amongst various calpain inhibitors because it antagonizes calpain I. The best characterized calpains in CNS are the ubiquitous μ- and m-calpains (calpain I and calpain II). The activation of calpain I requires μM Ca$^{2+}$ while calpain II requires mM Ca$^{2+}$ [19]. Both calpain I and calpain II are involved in...
Fig. 4. Local delivery of MDL28170 reduced cavity formation and promoted locomotor functional recovery in SCI rats. (A) Process of SCI model; Blue arrow indicated the site of the injury epicenter in the spinal cord; Yellow arrow indicated the MDL28170-loaded PCL film; (B) General morphology of spinal cord isolated from SCI rats after 8 weeks. Arrows indicated the areas of the rough surface around the injury site in the spinal cord; From left to right: SCI + vehicle-loaded PCL film group, SCI + MDL28170-loaded PCL film group (2.85 μg), SCI + MDL28170-loaded PCL film group (11.4 μg). b1: Dorsal view; b2: Ventralis view. (C) Longitudinal H & E stained section at the injury epicenter depicts the loss of gray matter and white matter. Scale bar = 500 μM. (D) Quantification of the cavity areas of spinal cords. Data are mean ± SEM. **p < 0.01, vs vehicle-loaded PCL film groups. (E) BBB (Basso, Beattie and Bresnahan) score were evaluated within 8 weeks after SCI. Data are mean ± SEM. *p < 0.05, vs vehicle-loaded PCL film groups.

Fig. 5. Local administration of MDL28170 on the expression of GAP43 in the injured spinal cord after 8 weeks of SCI. (A) Immunohistochemistry staining of GAP43 expression. Scale bars: 500 μM. (B) Western blotting analysis of GAP43 protein expression in the injured spinal cord. (C) Densitometric quantification of GAP43 protein expression normalized to β-actin level. Data are mean ± SEM (n = 3) and expressed as fold increase relative to control value.
diverse functions of neurons in CNS [20,21]. Studies have proven that calpain I was activated under CNS injury condition [22,23], suggesting that knocking down or inhibiting calpain I might limit the damage due to excessive calpain activity. Here we confirmed the protective effects of MDL28170 on VSC4.1 motor neurons. Similarly, MDL28170 was also reported to enhance the survival of Schwann cells in the injured spinal cord [9]. It is supposed that the protective effect of MDL28170 on SCI might be a combined effect on various cells in the spinal cord.

We further explored the potential role of MDL28170 in a rat SCI model. PCL film was invisible at 8 week after SCI, indicating that it has been completely degraded in vivo. Locomotor function recovery was significantly improved in high dose MDL28170-loaded PCL film group, which meant the MDL28170 delivery system was effective. Studies have already elucidated that MDL28170 has protective effect in rat SCI model when it was applied as a single postinjury i.v. bolus (20 mg/kg), a daily i.p. dose (1 mg/kg), or a combination of i.v. and daily i.p. treatment [8]. The high dosage that we used (11.4 μg) is far less than that used in systemic administration. Moreover, one-off delivery of MDL28170 by PCL film is quite convenient. Since our study mainly focused on the function recovery in the late stage of SCI, the effect of MDL28170 on apoptosis and neuronal cell death in the early stage of SCI was not examined. Previously, we have revealed that SCI activated calpains and ER stress in rats [10]. In the present study, we observed that MDL28170 administration significantly reduced the cavity formation, as well as slightly increased the expression of GAP43 in neurons, which will accelerate the recovery of SCI. As a result, we speculate that MDL28170 could inhibit the activation of calpain I, suppress ER stress, and protect the neurons against the injury in the spinal cord. These findings need to be fully illuminated in further research.

On the other hand, reactive gliosis and scar formation after SCI injury can inhibit the locomotor function recovery process [24]. MDL28170 delivery reduced the collagen deposition in the lesion site, indicating an inhibition on the gliosis scar formation in the late stage of SCI. We noticed that MDL28170 slightly decreased the expression of GFAP, suggesting that MDL28170 inhibited astrocyte-mediated astrogliosis on a small scale. However, it proposed a possibility that MDL28170 may also inhibit microglia-mediated glial scar formation because both microglial and astrocyte are jointly involved in scar formation in SCI [25,26]. The combination of these two mechanisms may contribute to the significant less of collagen deposition in high dose of MDL28170 group.

5. Conclusion

To sum up, we demonstrated that MDL28170 could reduce cell death in VSC4.1 motor neurons in vitro. Local delivery of MDL28170-loaded PCL film could reduce cavity formation and promote rat locomotor functional recovery in vivo. Our findings indicated that local application of MDL28170 in PCL film can promote SCI recovery by preserving neuronal cell survival, thus to provide a new convenient and safe therapeutic strategy for SCI treatment.

Conflict of interest

The authors indicated no potential conflicts of interest.

Author contributions

Dongling Shi, Teng He, Weijian Tang, Heyangzi Li and Chao Wang performed the experiments; Yueliang Shen, Bingzhi Zheng conducted data acquisition and assembly of data; Xinghui Song, Yuemin Ding and Ying-Ying Chen: data analysis and interpretation; Hongfeng Jin and Lin-Lin Wang: conception and design, manuscript revising and final approval of manuscript, and financial support.

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