Research Article

Low-dose metformin treatment in the subacute phase improves the locomotor function of a mouse model of spinal cord injury

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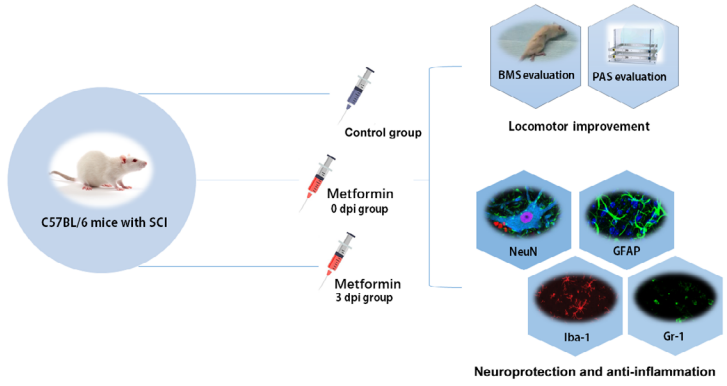
Graphical Abstract *Metformin treatment leads to better functional recovery after subacute spinal cord injury (SCI)*



Abstract

Metformin, a first-line drug for type-2 diabetes, has been shown to improve locomotor recovery after spinal cord injury. However, there are studies reporting no beneficial effect. Recently, we found that high dose of metformin (200 mg/kg, intraperitoneal) and acute phase administration (immediately after injury) led to increased mortality and limited locomotor function recovery. Consequently, we used a lower dose (100 mg/kg, i.p.) metformin in mice, and compared the effect of immediate administration after spinal cord injury (acute phase) with that of administration at 3 days post-injury (subacute phase). Our data showed that metformin treatment starting at the subacute phase significantly improved mouse locomotor function evaluated by Basso Mouse Scale (BMS) scoring. Immunohistochemical studies also revealed significant inhibitions of microglia/macrophage activation and astrogliosis at the lesion site. Furthermore, metformin treatment at the subacute phase reduced neutrophil infiltration. These changes were in parallel with the increased survival rate of spinal neurons in animals treated with metformin. These findings suggest that low-dose metformin treatment for subacute spinal cord injury can effectively improve the functional recovery possibly through anti-inflammation and neuroprotection. This study was approved by the Institute Animal Care and Use Committee at the University of Texas Medical Branch (approval No. 1008041C) in 2010.

Key Words: inflammation; locomotor function; metformin; microglia; mortality; neuroprotection; spinal cord injury; subacute administration

Chinese Library Classification No. R453; R744; R587.1

Introduction

Spinal cord injury (SCI) remains a serious neurological disorder without effective treatment strategies (Simpson et al., 2012). Severe SCI often causes motor dysfunction and loss of sensation (Krassioukov et al., 2009). There are an estimated 768,473 new cases of SCI annually worldwide (Singh et al., 2014; Kumar et al., 2018). The United States is one of the countries with the highest reported incidence of SCI (40.1 per million) (Singh et al., 2014).

The pathophysiology of acute SCI comprises both primary and secondary injury mechanisms (McDonald and Sadowsky, 2002) that attribute to neurological dysfunctions. Secondary damage is highly relevant to clinical intervention (Mautes et al., 2000; Beattie, 2004; Park et al., 2004; David and Kroner, 2011; Oyinbo, 2011). Inflammatory response is one of the major processes of secondary injury, which may lead to cell apoptosis and scar formation, ultimately inhibiting neuronal function. Many studies suggest that reducing inflammation

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can diminish secondary degeneration and functional defects after SCI (Blight, 1992; Beattie, 2004; Brambilla et al., 2005; Beck et al., 2010). On the other hand, inflammation is critical for the clearance of cellular debris by microglia. The formation of astroglia scar is beneficial in stabilizing the fragile tissues after injury, repairing the blood-brain barrier or blood-spinal barrier, preventing an overwhelming inflammatory response and limiting cellular degeneration (Kawano et al., 2012; Yuan and He, 2013). Whether inflammation is beneficial or deleterious depends not only on the nature of various cellular and soluble factors such as cytokines, but also on the timing of the inflammatory process. During the acute phase (1–3 days) after SCI, reactive astrocytes, via proliferating and sending processes around the lesion, separate the damaged area from healthy tissues and limit the spread of infiltrating inflammatory cells (Ohtake and Li, 2015). At the subacute phase (4–14 days after SCI in a mouse model), astrocytes migrate to the lesion site and scar formation can block neuronal regeneration (Pekny and Nilsson, 2005). The competition between the axonal regrowth and the inhibitory environmental cues in the adult central nervous system holds the key for structural and functional repair of the adult spinal cord (Hausmann, 2003; Donnelly and Popovich, 2008).

Anti-inflammation strategy to facilitate functional recovery after SCI was proposed decades ago (Wolf et al., 1996; Bethea et al., 1999; Gonzalez et al., 2003; Liu et al., 2019). Numerous candidates have been tested in preclinical animal models, including antagonists of inflammatory cytokines (Ahmed et al., 2007), synthetic (Lieber et al., 2004; Suwanna et al., 2014; Rong et al., 2017) or natural chemicals from plants or fruits, however, thus far only methylprednisolone sodium succinate, a corticosteroid, has been used as an anti-inflammatory treatment for SCI patients. Yet, its efficacy and side effects remain as concerns (Fehlings et al., 2017) and development of novel anti-inflammatory approaches is in demand.

Metformin is an oral hypoglycemic agent widely used as the first-line treatment for type 2 diabetes (Stumvoll et al., 1995; Hundal et al., 2000; Rowan et al., 2008; Tanokashira et al., 2019). Recent preclinical and clinical studies have shown that metformin not only mitigates chronic inflammation by improving hyperglycemia, insulin resistance and dyslipidemia, but also has a direct anti-inflammatory effect (Chakraborty et al., 2011; Koh et al., 2014; Wang et al., 2016). This led to the investigation of using metformin to treat SCI. While some preclinical studies reported that metformin can improve locomotor function after SCI (Zhang et al., 2017; Afshari et al., 2018), other study showed no beneficial effect (Lin et al., 2015). In above studies, the intervention regimens are different, which may be the reason for the difference in efficacy. Therefore, we intended to compare the effect of metformin, at various dosages and different starting times of administration, on histopathological outcomes and locomotor function recovery in a rodent model of SCI.

Materials and Methods

Animals and SCI model

Wild-type C57BL/6 mice of either gender, about 16 weeks old, weighing 20–25 g, were provided by Jackson Laboratory (Bar Harbor, ME, USA) and used in this study. They were subjected to spinal cord moderate contusion injury according to the procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. All surgery procedures were approved by the Institute Animal Care and Use Committee at the University of Texas Medical Branch (approval No. 1008041C) in 2010. Mice were anesthetized with 1.5% isoflurane (Piramal Enterprises Ltd., Telangana, India) inhalation. The absence of a flexor response to a noxious stimulus was taken as an indication of complete anesthesia (von Dincklage et al., 2012). After hair shaving and disinfection, the vertebral column was exposed by an incision, and a laminectomy was performed at T11 to expose spinal cord segment T13. The spinal column was secured with clamps. Precautions were taken to avoid unnecessary stretch that could influence the severity of injury. The spinal cord was exposed, and a moderate contusion was produced by using the Infinite Horizons impactor (IH-2002, Precision Systems and Instrumentation, Lexington, KY, USA) with a force of 50 kdyn (1 dyn = 10^{-5} N) and zero dwell time. After injury, muscle and fascia were sutured, and the skin incision was closed with autoclips. Animals received intraperitoneal (i.p.) injection of 1 mL lactated Ringer's solution, 1 mL 0.9% sodium chloride for rehydration and subcutaneous injection of 22,000 U/kg penicillin (Pfizer, New York, NY, USA) as antibiotic and 0.05 mg/kg buprenorphine (Indivior Inc., North Chesterfield, VA, USA) as analgesic. Mice were then allowed to recover from surgery on a thermal pad. Bladders of all injured mice were expressed manually twice daily until bladder function was restored within 2 to 4 weeks post-injury. Records of force of impact and tissue displacement indicated no significant differences in impact parameters among groups (Jakeman et al., 2000).

Metformin treatment

Previous studies started metformin treatment immediately after SCI with the dosage ranged from 50 mg/kg to 200 mg/kg (Sims and Yew, 2017; Zhang et al., 2017; Gaudet and Fonken, 2018), and reported no adverse reactions. To explore the optimal metformin dosage in this study, a pilot study was conducted. In this initial study, additional 40 C57BL/6 mice were randomly divided into four groups and ten in each group. The vehicle control group received intraperitoneal (i.p.) injection of phosphate-buffered saline (PBS) immediately after surgery. The low-dose and high-dose metformin groups received 100 mg/kg and 200 mg/kg metformin (Thermo Fisher Scientific, Sugar Land, TX, USA), respectively, immediately after surgery, once daily for 7 consecutive days. The subacute high-dose group received 200 mg/kg metformin starting 3 days after surgery, once daily for 7 consecutive days. We found that 200 mg/kg metformin administrated immediately after SCI lead to an increased mortality (shown in **Additional Figure 1**), which could be attributed to different animal species (mice in this study as compared to rats in others) (Wang et al., 2016), the acute administration of metformin, and/or high dosage treatment.

Given the high mortality of mice in this pilot study was associated with 200 mg/kg metformin administration, the rest of the study used the lower dose of metformin (100 mg/kg). Subsequently, additional 45 mice were randomly divided into three groups (n = 15 in each group): control, 0 day post-injury (dpi) and 3 dpi groups. The 0 dpi group received 100 mg/kg metformin by i.p. injection immediately after SCI for 7 days. The 3 dpi group started metformin administration at 3 dpi and continued for 7 days, while the control group used the same dose (1 mL) of PBS (i.p.) immediately after injury for 7 days. Two weeks after injury, fifteen mice (five mice randomly selected from each group) were sacrificed for spinal cord immunostaining. The study design of formal experiment is shown in **Additional Figure 2**.

The Basso Mouse Scale

The Basso Mouse Scale (BMS) is a validated test used to monitor the progress of hind-limb functional recovery following SCI in mice (Pajoohesh-Ganji et al., 2010). The scale ranges from 0 (no ankle movement) to 9 (complete functional recovery) points. BMS scores were recorded at 1, 4, 7, 14, 21, 28, 35, and 42 days after SCI by two independent examiners who were blind to the experimental conditions. The number and gender distribution of the mice for BMS score evaluation are shown in **Additional Figure 2**. Hind-limb motion was used to assess coordinated movement and stepping. When differences in the BMS score between the right and left hind limbs were observed, the average of the two scores was used.

Photobeam Activity System

Locomotor activity was also measured using the Photobeam Activity System (PAS) with FlexField software (San Diego Instruments, Inc., San Diego, CA, USA). Movements in the *X* and *Y* directions were recorded based on the obstruction of 16 photobeams in the "*X*" direction and 16 photobeams in the "*Y*" direction. These photobeams were 4 cm above the chamber floor. Obstruction of a third set of photobeams positioned 6 cm above the chamber floor allowed recording of movements along the "*Z*" axis and generated data as rearing events. The PAS monitors movements of a subject in an activity chamber (40 cm × 40 cm × 40 cm) by recording the number of times that the photobeams are obstructed in the *X*, *Y*, and *Z* axis-oriented grid system. Data were recorded only during the first 15 minutes for each animal and collected in consecutive 5-minute intervals. To eliminate olfactory stimuli such as urine from the previous test subjects, the chambers were cleaned with CaviCide (Metrex Research, LLC., Orange, CA, USA) and alcohol after each test.

Immunofluorescence staining

For tissue collection, mice were deeply anesthetized with ketamine (100 mg/kg, i.p., Akorn, Inc., Lake Forest, IL, USA) and xylazine (10 mg/kg, i.p., Akorn, Inc.), and then perfused transcardially with 5 mL PBS, followed by 30 mL of ice-cold 4% paraformaldehyde in PBS. Spinal cords were removed and postfixed for overnight at 4°C. Tissue was then cryoprotected in 15% sucrose at 4°C for 6 hours, and then changed to 30% sucrose at 4°C for 48 hours. Before tissue embedding, spinal cords were cut into 4 cm^3-sized blocks centered to the injury site. After submersion in optical coherence tomography compound (Fisher HealthCare, Houston, TX, USA), blocks were frozen, and longitudinal sections were cut at 30 µm thickness on a cryostat and mounted onto glass slides. Tissue sections were stored at –20°C until use. Slides were washed with PBS three times, each for 10 minutes, and blocked with 5% normal donkey/goat serum in PBS for 1 hour. The slides were then incubated with primary antibodies at 4°C overnight: mouse monoclonal anti-neuronal nuclear antigen (NeuN, a marker for neurons (Jessen and Mirsky, 1980; Wolf et al., 1996), 1:1000, Millipore, Waltham, MA, USA, Cat# MAB377), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP, a marker for astrocytes (Jessen and Mirsky, 1980); 1:2000, Chemicon, Temecula, CA, USA, Cat# PA1-10019), rabbit monoclonal anti-ionized calcium binding adaptor molecule 1 (Iba-1, a marker for microglia and macrophage (Ahmed et al., 2007); 1:500, Abcam, Cambridge, MA, USA, Cat# ab178847), rat monoclonal anti-Ly-6G/Ly-6C (a marker for neutrophils (Lieber et al., 2004), 1:50, eBioscience, Waltham, MA USA, Cat# 14-5931-82). The slides were washed with PBS and incubated with Alexa Fluor® 568 donkey anti-mouse IgG (1:500; Millipore , Cat# A-10037), Alexa Fluor® 488 goat anti-rabbit IgG (1:500; Millipore , Cat# A-11034), Alexa Fluor® 568 goat anti-rabbit IgG (1:500; Millipore , Cat# A11011), or Alexa Fluor® 488 goat anti-rat IgG (1:400; Millipore , Cat# A-11006) at 4°C for 2 hours. The slides were washed, sealed with a coverslip using 4′,6-diamino-2-phenylindole Fluoromount-G (Thermo Scientific, Waltham, MA, USA).

All sections used for lesion area quantification and NeuN/GFAP/Iba-1/Gr-1 quantification were obtained from similar locations according to the dorsoventral axis of spinal cord in each mouse. Data were collected from the same levels and areas of the injured spinal cord among groups. Representative images were selected to show the average treatment effects from multiple mice.

To determine the lesion area, spinal cord sections were immunostained with glial fibrillary acidic protein (GFAP) antibody and images of the injury site were taken on a Nikon D-Eclipse C1 laser scanning confocal microscope (Nikon Instruments Inc., Melville, NY, USA). Using ImageJ software (National Institutes of Health, Bethesda, MD, USA), a contour around the GFAP-negative area (lesion site) was drawn and the area of the enclosed region was measured.

For each immunostaining, two sections spanning a rostral-caudal distance of 480 µm per mouse were obtained. Five mice per group were used for measurement of NeuN and GFAP immunopositivities, four mice per group were used for measurements of Iba-1 and Gr-1 expression using the Image J software. For NeuN immunofluorescence staining in each spinal cord section image, six squares of 1.3 mm × 1.3 mm each were randomly chosen around the edge of the lesion site. The number of neurons in each square was counted and then averaged over six squares. For GFAP and Iba-1 staining, six 1.3 mm × 1.3 mm square areas were randomly taken around the lesion site, and the intensity of GFAP or Iba-1 expression in each square was measured by the ImageJ software.

Statistical analysis

To analyze the mortality rate between groups, Kaplan-Meier survival analysis was employed. The Wilcoxon test was used to detect the overall differences between groups, and the Tukey-Kramer *post hoc* test was used for multiple comparisons to determine the difference between each two groups. One-way analysis of variance (ANOVA) with Bonferroni multiple comparisons test was used to determine statistical significance in locomotor acticity and immunopositivity among groups. BMS scores were analyzed by two-way repeated measures ANOVA with Tukey's *post hoc* test adjustments. A *P*-value less than 0.05 was considered to be statistically significant. Statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA).

Results

Metformin administration in the subacute phase reduces mortality post-SCI

In a pilot study, doses and time points of initiating metformin administration were selected based on previous animal studies (Anson et al., 2003; Jeong et al., 2011) with some modifications. At 1 dpi, the mortality rates were 0 in control, low-dose and subacute high-dose metformin groups. However, a surprisingly high mortality of 60% was found in the high-dose metformin group (**Additional Figure 1**). At 5 dpi, the mortality rates were 50% in the control group, 10% in the low-dose metformin group, 20% in subacute high-dose metformin group, and 100% in the high-dose metformin group. The overall difference among four groups was significant ($P < 0.0001$). The mortality rate in the high-dose metformin group was significantly higher than that in the other three groups ($P = 0.0236$, *vs*. control group; $P < 0.0001$, *vs*. low-dose metformin group; $P = 0.0001$, *vs.* subacute high-dose metformin group). Thus, our pilot data indicated that high dose of metformin (200 mg/kg) and acute phase administration (immediately after injury) increased the mortality of mice. Based on these findings, the dose of metformin was then reduced to 100 mg/kg per day throughout the rest of the study.

Following SCI, the survival of injured mice in three groups (control, 0 dpi and 3 dpi groups) was recorded daily. No death occurred in any of the three groups on the first day post-injury. At 3 days after SCI, the mortality rates were 20% in the control group, 0% in the 0 dpi group, and 10% in the 3 dpi group. There were additional deaths in the following weeks in each of the groups, but the mortality rates were stabilized by 10 dpi, i.e. 50% in the control group, 30% in the 0 dpi group, and 20% in the 3 dpi group (**Figure 1**). No further death was observed in each group after 14 dpi. The difference in survival rates of control animals between the pilot study (10%) and the later experimental study (50%) was likely due to different batches of animals. While the nonsignificant difference in mortality among different treatment groups indicated that the

use of 100 mg/kg metformin in the subacute phase of severe SCI tended to reduce mortality.

Metformin treatment in the subacute phase improves the locomotor function of SCI mice

BMS locomotor function tests were scored as baseline for all experimental mice before injury, and all mice had normal motor function (9 points). On 1 dpi, BMS scores of mice in all groups fell to 0–1 point, and then began to recover overtime. On 7 dpi, BMS scores of all animals recovered to 1–4 points, but there were no statistical differences among the three groups ($P > 0.05$). On 14 dpi, the BMS score in the 3 dpi group began to be higher than that of the control group ($P = 0.0055$). On 28 dpi, the BMS score in the 3 dpi group was significantly higher than that in the 0 dpi ($P < 0.05$) and control ($P < 0.01$) groups. By the end of the experiment (42nd day post-injury), the BMS score in the 3 dpi group was significantly higher than that in the control group ($P < 0.05$), and kept a trend slightly, but not significantly, better than that in the 0 dpi group ($P = 0.17$; **Figure 2**).

Four weeks after SCI, PAS open-field locomotor function test was conducted to determine rearing activity, rest time, average speed and travel distance of mice. **Figure 3A** shows the chamber for performing the PAS open-field locomotor function test. The 3 dpi group tended to have slightly, but not significantly better improvements in rearing activity compared to the control group (**Figure 3B**) ($P = 0.063$). No differences in the rest time were observed among the three groups ($P > 0.05$; **Figure 3C**). The 3 dpi group tended to have slightly, but not significantly, better improvement in average speed ($P = 0.083$; **Figure 3D**). However, the 3 dpi group has significantly longer travel distance compared to the control group ($P < 0.05$; **Figure 3E**). Taken together, our results demonstrated that metformin treatment in the subacute phase had a better functional recovery compared with treatment at other time points studied.

Metformin treatment in the subacute phase reduces the spinal cord lesion size

Reactive astrogliosis occurs after SCI. The primary benefits of glial scars include confining the inflammatory injury area and minimizing the extent of secondary damage following SCI (Gaudet and Fonken, 2018). Several studies demonstrated that elimination of astrogliosis early after SCI resulted in greater lesion area and worse functional outcomes (Sims and Yew, 2017). To compare the treatment effect of metformin at different phases, the area of the lesion site in each treatment group was measured. GFAP immunofluorescence staining was employed to outline the lesion site and GFAP-positive astrocytes were considered the boundary of the glial scar at the lesion site in mice (Wanner et al., 2013). Since our data showed that metformin treatment significantly improved motor function as early as 14 dpi, we performed morphological studies in the spinal cord tissues collected at this time point. Spinal cord sections were immunostained with the GFAP antibody. As shown in **Figure 4A**, in the control group, obvious tissue atrophy around the lesion center was observed. In the 0 dpi group, the overall morphology of the spinal cord tissue was relatively preserved (**Figure 4B**). The area of injury around the lesion center was significantly reduced in the 0 dpi group compared to the control group ($P < 0.01$). In the 3 dpi group, the morphology of the spinal cord was well preserved (**Figure 4C**). The white matter and gray matter were well arranged without a large amount of glial hyperplasia around the epicenter. Quantitative analyses showed that the average area of the GFAP-defined lesion site in the 3 dpi group was significantly smaller than that of the control group ($P < 0.001$; **Figure 4D**).

Metformin treatment in the subacute phase reduces the pathological injury of the spinal cord of SCI mice

Activated astroglia in the injured spinal cord produce a variety of pro-inflammatory cytokines, including interleukin-1β, tumor necrosis factor-α, proteases and other cytotoxic factors (Klusman and Schwab, 1997). Excessive glial cell proliferation can further induce apoptosis, enlarge the area of the injured area and lead to excessive glial scar formation, thus hinder the regeneration of functional neurons and functional recovery (Tran et al., 2018). In this study, the spinal cord tissues of mice in each group were obtained at 14 dpi, and NeuN/GFAP double-labeled staining was performed to investigate the activation of glial cells and the preservation of neurons. The NeuN staining without typical cell morphology (**Figure 5**) in the epicenter is most likely related to dying neurons. In the control group, only a few neurons could be observed around the lesion site (**Figure 5A** and **C**). A high magnification revealed a large amount of astroglial cells intermingled with a very small number of red neurons (**Figure 5D**). In the 0 dpi group, more neurons were observed around the lesion site with a reduced number of astrocytes (**Figure 5E–H**). In the 3 dpi group (**Figure 5I–L**), the intensity of GFAP immunoreactivity was lower compared to both control and 0 dpi groups, and a large number of neurons were observed around the lesion site. Statistical analyses indicated that the number of neurons around the lesion site in the 3 dpi group was significantly higher than that of the control group ($P = 0.0207$; **Figure 5M**). The immunopositivity of GFAP around the lesion site of spinal cord in both 3 dpi group ($P = 0.0017$) and 0 dpi ($P = 0.0156$) groups were significantly lower than that in the control group (**Figure 5N**).

Metformin treatment in the subacute phase reduces the microglia/macrophage hyperplasia in the spinal cord of SCI mice

Ibal-1 immunofluorescence staining was performed to observe the hypertrophy, proliferation or infiltration of microglia/macrophages at 14 dpi (Nakamura et al., 2013). As shown in **Figure 6A–C**, in the control group, Iba-1 immunoreactivity around the lesion epicenter was strong, and a larger number of hypertrophic microglia were observed under a higher magnification (**Figure 6C**). No significant changes of Iba-1 immunoreactivity were observed in 0 dpi group (**Figure 6D–F**). In the 3 dpi group, the immunoreactivity of Iba-1 at the lesion site was drastically reduced (**Figure 6G–I**). Quantitative analysis confirmed that Iba-1 immunofluorescence intensity in the 3 dpi group was significantly lower than that in the control group ($P = 0.0271$).

Metformin treatment in the subacute phase reduces neutrophils infiltration in the spinal cord of SCI mice

Neutrophils start to infiltrate into the lesion site about 2 hours after injury. Infiltration of neutrophils at the early stage of injury can have a positive influence on the pathophysiological process after SCI, and depletion of neutrophils can worsen neurological outcome (Stirling et al., 2009). However, a previous study has shown that activated neutrophils can cause inflammation by releasing various inflammatory factors (Smith, 1994), are involved in ischemia/reperfusion and induce further secondary injury (Kaminski et al., 2002). Here, we chose Ly-6G/Ly-6C (Gr-1) to identify infiltrated neutrophils since Gr-1 is predominantly found on neutrophils in the peripheral blood (Conlan and North, 1994). Thus, immunofluorescence staining with the Gr-1 antibody was performed on the spinal cord tissue at 14 dpi. As shown in **Figure 7A–C**, Gr-1-labeled neutrophils accumulated in a large number at the lesion center in the control group. In the 0 dpi group (**Figure 7D–F**), the number of neutrophils in the epicenter tended to decrease without statistical significance ($P = 0.184$) compared to the control group (**Figure 7J**). In the 3 dpi group, the number of neutrophils was significantly reduced compared to the control group ($P = 0.0396$; **Figure 7G–J**).

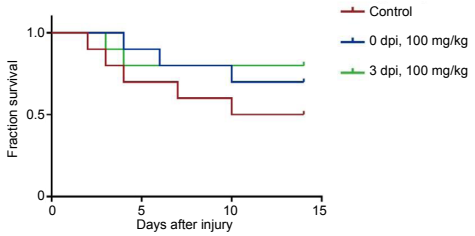

Figure 1 | **Effect of metformin on the survival of SCI mice.**
All deaths were recorded within the first 2 weeks after SCI surgery. *n* = 10 per group. The mortality rate between groups was analyzed by the Kaplan–Meier survival analysis, and the overall differences among groups were analyzed by the Wilcoxon test followed by Tukey-Kramer *post hoc* test. Control: Vehicle treated; 0 dpi or 3 dpi: the first dose of metformin given immediately or 3 days post SCI, respectively. SCI: Spinal cord injury.

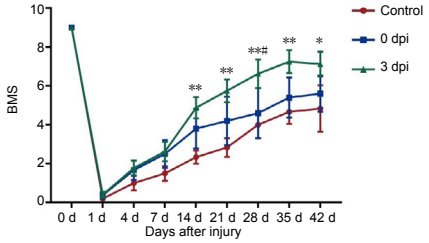

Figure 2 | **Effect of metformin on the hind limb motor function of SCI mice.**
Progress of hind limb functional recovery was evaluated by BMS scoring. Data are presented as the mean ± SEM (*n* = 10 in each group at the start of the experiment; *n* = 5, 7, or 8 survived in the control, 0 dpi or 3 dpi groups, respectively, at 14 dpi and beyond). **P* < 0.05, ***P* < 0.01, *vs*. control group; #*P* < 0.05, *vs*. 0 dpi group (two-way repeated measures analysis of variance followed by Tukey's *post hoc* test adjustments). Control: Vehicle treated; 0 dpi or 3 dpi:, the first dose of metformin given immediately or 3 days post SCI, respectively. BMS: Basso Mouse Scale; SCI: spinal cord injury.

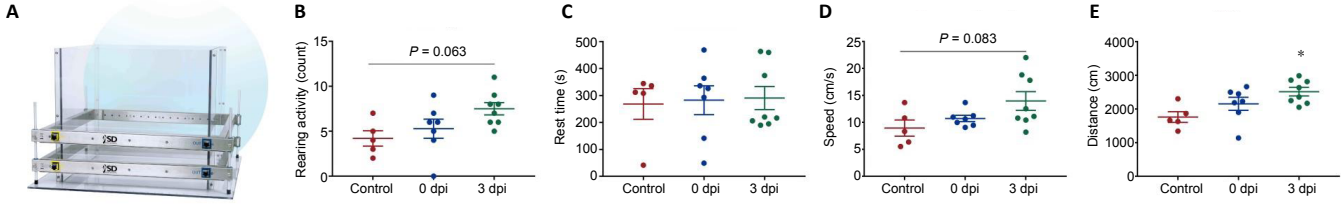

Figure 3 | **Effect of metformin on the open-field locomotor activity of SCI mice.**
Photobeam Activity System open-field locomotor function test was performed 4 weeks post injury. (A) The test chamber of the PAS system. (B–E) Rearing activity (B), rest time (C), average speed (D) and travel distance (E) were shown by scatter plots. Data are presented as the mean ± SEM (*n* = 5 in control, *n* = 7 in 0 dpi, *n* = 8 in 3 dpi). **P* < 0.05, *vs*. control group; #*P* < 0.05, *vs*. 0 dpi group (one-way analysis of variance followed by Bonferroni *post hoc* test). Control: Vehicle treated; 0 dpi or 3 dpi: the first dose of metformin given immediately or 3 days post SCI, respectively. SCI: Spinal cord injury.

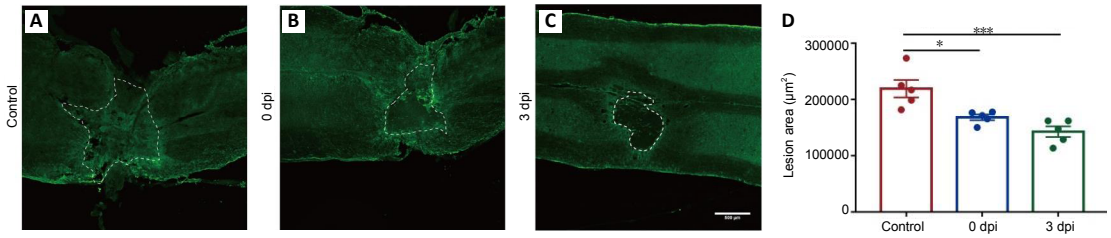

Figure 4 | **Effect of metformin on the spinal cord lesion size in SCI mice at 14 days post-injury.**
(A–C) Longitudinal sections of mouse spinal cords were immunostained with an antibody against GFAP (green, stained by Alexa Fluor® 488), which outlines the boundary of the lesion site. Scale bar: 500 μm. The cavity areas in the 3 dpi group and the 0 dpi group are significantly smaller than those in the control group. (D) Quantitation of the lesion area (delineated by GFAP astroglial scar). Data are presented as the mean ± SEM (*n* = 5). **P* < 0.05, ****P* < 0.001 (one-way analysis of variance followed by Bonferroni *post hoc* test). Control: Vehicle treated; 0 dpi or 3 dpi: the first dose of metformin given immediately or 3 days post SCI, respectively. GFAP: Glial fibrillary acidic protein; SCI: spinal cord injury.

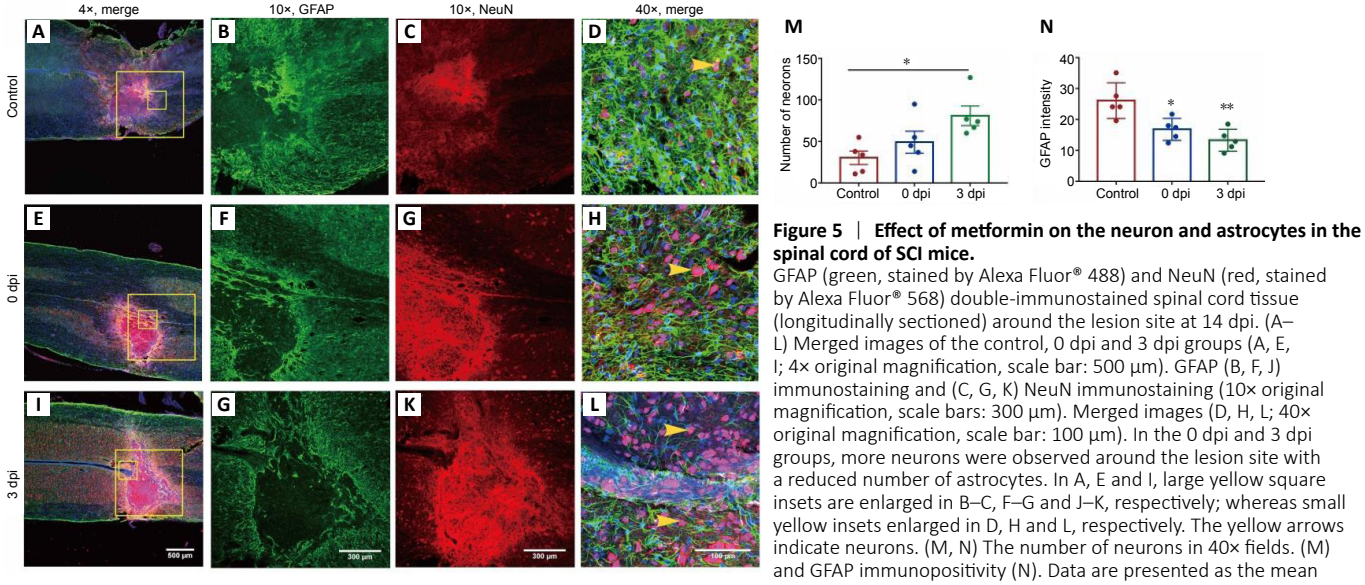

Figure 5 | **Effect of metformin on the neuron and astrocytes in the spinal cord of SCI mice.**
GFAP (green, stained by Alexa Fluor® 488) and NeuN (red, stained by Alexa Fluor® 568) double-immunostained spinal cord tissue (longitudinally sectioned) around the lesion site at 14 dpi. (A–L) Merged images of the control, 0 dpi and 3 dpi groups (A, E, I; 4× original magnification, scale bar: 500 μm). GFAP (B, F, J) immunostaining and (C, G, K) NeuN immunostaining (10× original magnification, scale bars: 300 μm). Merged images (D, H, L; 40× original magnification, scale bar: 100 μm). In the 0 dpi and 3 dpi groups, more neurons were observed around the lesion site with a reduced number of astrocytes. In A, E and I, large yellow square insets are enlarged in B–C, F–G and J–K, respectively; whereas small yellow insets enlarged in D, H and L, respectively. The yellow arrows indicate neurons. (M, N) The number of neurons in 40× fields. (M) and GFAP immunopositivity (N). Data are presented as the mean ± SEM (*n* = 5) **P* < 0.05, ***P* < 0.01 (one-way analysis of variance followed by Bonferroni *post hoc* test). Control: Vehicle treated; 0 dpi or 3 dpi: the first dose of metformin given immediately or 3 days post SCI, respectively. GFAP: Glial fibrillary acidic protein; NeuN: neuronal nuclear antigen; SCI: spinal cord injury.

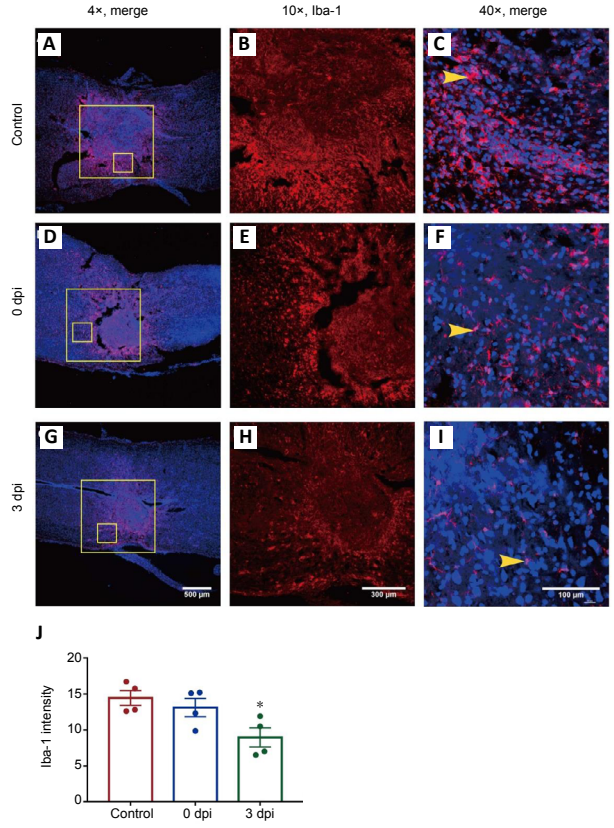

Figure 6 | **Effect of metformin on the microglia/macrophage activation in the spinal cord of SCI mice.**
Iba-1 (red, stained by Alexa Fluor® 568) immunostained (longitudinally sectioned) spinal cord tissue around the lesion site at 14 dpi. Iba-1 immunofluorescence intensity in the 3 dpi group was significantly lower than that in the control group (A, D, G). Merged images (4× original magnification, scale bar: 500 μm). The large yellow square insets are enlarged in B, E and H showing Iba-1 staining (10× original magnification, scale bar: 300 μm). The small insets are enlarged in C, F and I (40× original magnification, scale bar: 100 μm). The yellow arrows indicate microglia/macrophage. (J) Quantitation of Iba-1 immunoreactivity. Data are presented as the mean ± SEM (n = 4). *P < 0.05, *vs*. control group (one-way analysis of variance followed by with Bonferroni *post hoc* test). Control: Vehicle treated; 0 dpi or 3 dpi: the first dose of metformin given immediately or 3 days post-SCI, respectively. Iba-1: Ionized calcium-binding adapter molecule 1; SCI: spinal cord injury.

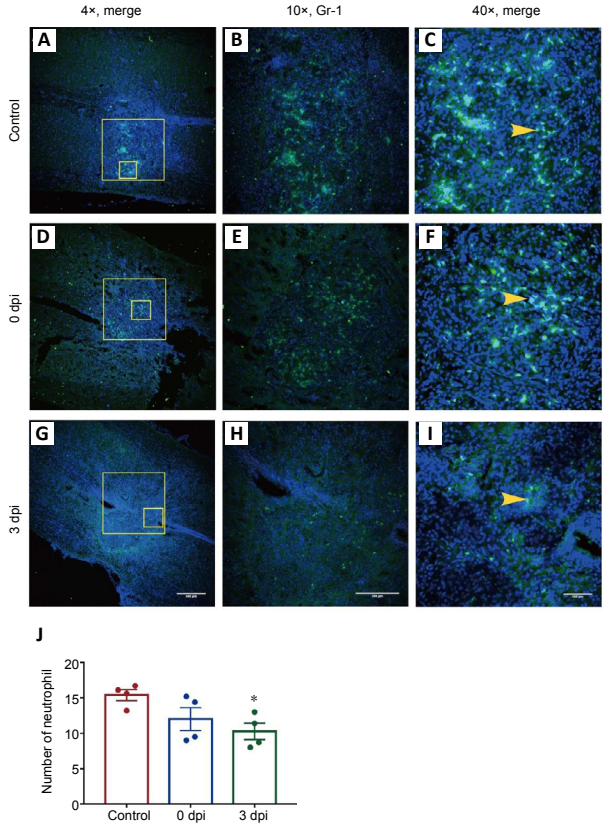

Figure 7 | **Effect of metformin on the neutrophil infiltration in the spinal cord longitudinal sections around the lesion site of SCI mice.**
Gr-1 (green, stained by Alexa Fluor® 488) immunostained spinal cord longitudinal sections around the lesion site at 14 dpi. (A, D, J) Merged images (4× original magnification, scale bar: 100 μm). The large yellow square insets are enlarged in B, E and H (10× original magnification, scale bar: 100 μm), and the small insets enlarged in C, F and I (40× original magnification, scale bar: 100 μm). In the 3 dpi group, the number of neutrophils was significantly reduced compared to the control group. Blue: Nuclear counterstain with DAPI. (J) Number of neutrophils. Data are presented as the mean ± SEM (n = 4) *P < 0.05, *vs*. control group (one-way analysis of variance followed by Bonferroni *post hoc* test). Control: Vehicle treated; 0 dpi or 3 dpi: the first dose of metformin given immediately or 3 days post-SCI, respectively. DAPI: 4′,6-Diamidino-2-phenylindole; Gr-1: monoclonal anti-Ly-6G/Ly-6C; SCI: spinal cord injury.

Discussion

The current study demonstrated that metformin, administrated at a dose of 100 mg/kg starting at the subacute phase after injury, significantly improved the locomotor function in mice and inhibited the activation of microglia/macrophage and astrogliosis at the lesion site. Metformin treatment in the subacute phase also significantly reduces neutrophil infiltration. The pathological changes were in parallel with the increased survival rate of spinal neurons in animals treated with metformin. This study suggests that the therapeutic effects of metformin on SCI are dose- and time-dependent, and metformin is a promising therapeutic alternate to restore locomotor function after SCI.

We found that starting from 14th day after injury, mice underwent metformin treatment at the subacute phase exhibited better locomotor functional recovery when compared with other groups. The time course of the functional recovery in our study is consistent with the previously published work (Zhang et al., 2017). However, the therapeutic effects of different dose and time windows for metformin administration have not been carefully studied for SCI before. According to our pilot study, administration of high dose metformin immediately after SCI can be toxic. Therefore, it is necessary to explore the dosage and the time window for metformin administration. Previous studies started metformin treatment immediately after SCI with the dosages ranged from 50 mg/kg to 200 mg/kg (Sims and Yew, 2017; Zhang et al., 2017; Gaudet and Fonken, 2018), and found no adverse reactions. However, we found that 200 mg/kg metformin immediately after SCI lead to increased mortality, which could be attributed to different animal species (mice in this study as compared to rats in others) and the severity or level of the injury. In terms of metformin doses, a previous study identified a biphasic dose-response curve, indicating that the efficacy of metformin decreases at both very low and very high doses. Accordingly, we applied 100 mg/kg metformin (i.p.) in SCI mice, and compared the effect of immediate administration (acute phase) to that of 3 days post-injury (subacute phase). The goal was to find out the optimal timing and dosage of metformin administration to balance the benefit and harm of neuroinflammation following SCI.

The beneficial effects of metformin on functional recovery after SCI may be explained through various mechanisms (Conlan and North, 1994; Singh et al., 2014; Wang et al., 2016; Guo et al., 2018). Previous studies have shown that metformin inhibited the inflammatory response by inhibiting nuclear factor kappa B via adenosine monophosphate-activated protein kinase (AMPK)-dependent and independent pathways (Hirsch et al., 2013; Zhu et al., 2015). It has been

reported that metformin increases nitric oxide synthesis by activating AMPK and reduces reactive oxygen species (ROS) production by inhibiting NADPH oxidase and mitochondrial oxidative respiratory response chains, and thus reduce ROS production (Batandier et al., 2006; Morales et al., 2010; Lu et al., 2016). The anti-inflammatory effect of metformin has been found to attenuate the tumor necrosis factor-α induced inflammatory response after SCI (Wanner et al., 2013). In agreement with these studies, we found that treatment with metformin (100 mg/kg) at the subacute phase (3 dpi) after SCI significantly inhibited the inflammatory response. In an effort to find the optimal time window for metformin treatment, we discovered that the same dose of metformin administration at the subacute phase achieved better treatment efficacy than administration at the acute phase, in terms of reducing postoperative mortality and promoting the recovery of impaired locomotor function.

After SCI, astrocytes, a type of neuroinflammatory cells that surround the lesion, become reactive and typically undergo hypertrophy and process extension (Mukhamedshina et al., 2019). At the acute phase (1–3 days after SCI), reactive astrocytes migrate centripetally to the lesion epicenter and aid in tissue repair processes (Ohtake and Li, 2015). However, at the subacute phase (4–14 days after SCI in mice), they help the formation of glial scar that produces axonal growth inhibitors and prevents axonal regeneration (Tysseling-Mattiace et al., 2008; Pajoohesh-Ganji et al., 2010). We showed here that starting metformin administration at the beginning of the subacute phase can prevent glial scar formation and minimize the area of lesion site. Thus, subacute metformin administration seems better than acute treatment in terms of minimizing the negative effects of astrocyte reactivation.

Microglia and macrophages are critical players in the inflammatory response following SCI. They play an important role in promoting neuronal survival and axonal growth (Neumann et al., 2009; David and Kroner, 2011). Studies have shown that these cells can effectively remove cell debris and proteases that inhibit axon growth. At the same time, microglia and macrophages can secrete a variety of soluble factors to promote cell survival and axonal regeneration (Neumann et al., 2009; Loane and Byrnes, 2010). There is evidence that after activation of local macrophages, the recovery of myelin phagocytosis and hindlimb function in nerve-damaged rats is accelerated (Takeuchi et al., 1999; Toshchakov et al., 2002; Gensel and Zhang, 2015). In contrast, inhibition of macrophage recruitment compromises myelin phagocytosis and delays nerve regeneration. Microglia plays a dual role in SCI (Gomes-Leal, 2012; Kawabori and Yenari, 2015). On one hand, microglia promote tissue recovery, but on the other hand, they could cause neurodegeneration. Different stages after injury and internal environmental factors may influence the activation characteristics of microglia and their effects. Previous studies have shown that microglial activation peaks on 2–3 days after SCI. In this study, we chose to start metformin treatment at both acute phase (0 dpi) and subacute phase (3 dpi) to reduce inflammatory reaction, and compared the effect of these two time window treatments on microglia inhibition. Our data showed that metformin administration at the subacute phase was more effective in inhibiting microglia/macrophages proliferation, or led to reduced microglia/macrophages activation and reduced monocyte derived macrophage infiltration into the injury site. Together, this may help to reduce inflammatory factors while preserve the beneficial effect of microglia in promoting tissue repair and locomotor function recovery.

In the present study, neutrophil infiltration after metformin treatment at both acute and subacute phase was studied. After metformin treatment at the subacute phase, the number of neutrophils in the injury site was significantly reduced. Neutrophils release proteases and ROS, which are detrimental to the recovery after SCI. A sharp decrease in neutrophils in the 3 dpi group may be associated with the effect of metformin to inhibit local inflammation caused by secondary damage. However, the exact mechanism remains to be further studied.

At the molecular level, metformin works through the inhibition of mitochondrial respiratory complex I and ATP synthase to reach therapeutic effects as demonstrated previously (Owen et al., 2000). As an AMPK agonist, metformin elicits a neuroprotective effect through autophagy enhancement (Tomic et al., 2011; Shi et al., 2012; Wang et al., 2016). Moreover, AMPK is an important regulator of mitochondrial biogenesis. The activation of AMPK by metformin may improve the mitochondrial biogenesis after SCI. On the other hand, metformin overdose may induce mitochondrial dysfunction and ATP synthesis inhibition. Consequently, accumulation of ROS and ATP shortage could lead to further cell damage. This may help to explain the high mortality in the group with high dosage of metformin (200 mg/kg) administered at the acute phase of SCI in the pilot study. Furthermore, the serotonergic neural pathway plays an important role in functional recovery. Further studies are needed to determine the therapeutic effect of metformin on 5-hydroxytryptamine axon regeneration after SCI.

The discovery of time- and dose-dependent metformin treatment affecting locomotor improvement is exciting, and sensory and other functional recoveries after metformin treatment deserve further investigation. It may also be worth to evaluate the metformin-mediated functional improvement by additional tests such as Horizontal Ladder or CatWalk analyses. Another limitation of the current study is the lack of blood glucose monitoring, which is mainly due to the routine care procedure of rodents by receiving lactated Ringer's solution and high protein-containing liquid diet immediately after injury to promote recovery after surgery. Glucose metabolic alteration (Anson et al., 2003) and calorie restriction (Jeong et al., 2011), however, were known to benefit neuronal protection and functional recovery after SCI, and metformin in theory may intervene this process. Thus, an appropriate design to monitor blood sugar levels at different periods after injury and metformin treatments may be a strategy to better understand the dose- and time-dependent effect of metformin.

In conclusion, metformin, acting as an anti-inflammatory and neuroprotective agent, improves both functional and pathophysiological outcomes after SCI in mice. This therapeutic effect for SCI is both dose- and time-dependent. Our study demonstrates an innovative treatment strategy for SCI using metformin, which was traditionally used for diabetes treatment. And this summary fits perfectly with our study. We think no modification is needed here.

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Additional files:
Additional Figure 1: *Survival of mice after SCI and metformin treatment in pilot study.*
Additional Figure 2: *Study design of the main experiment.*
Additional file 1: *Open peer review reports 1 and 2.*

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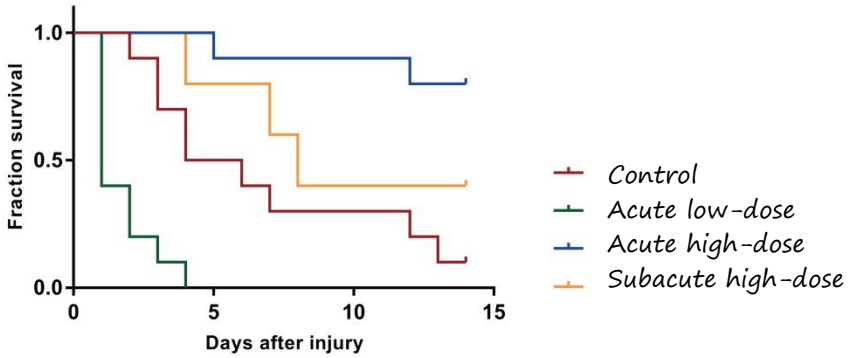
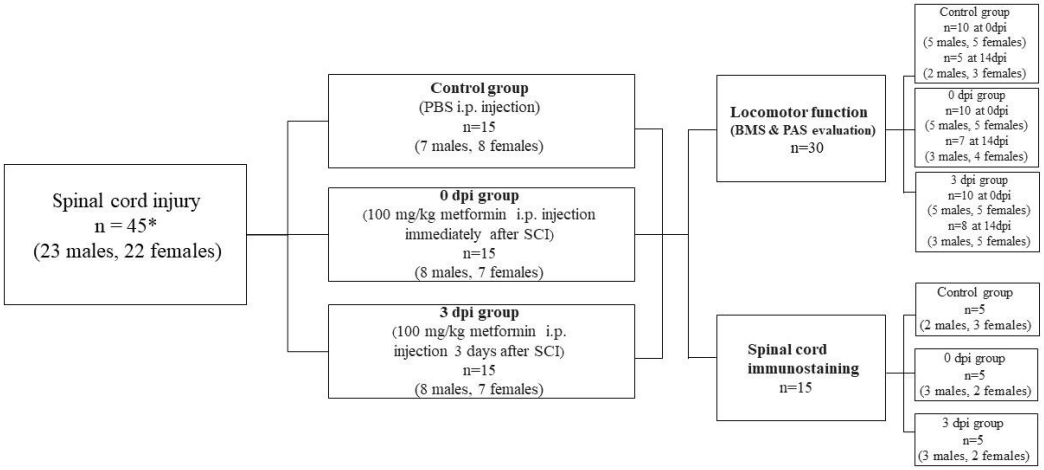


Additional Figure 1 Survival of mice after SCI and metformin treatment in pilot study.

All deaths are recorded within the first two weeks after SCI surgery (n = 10 per group). Mortality rates were determined by Kaplan–Meier survival analysis. Wilcoxon test was used to detect the overall differences between groups, and Tukey-Kramer post-hoc test was used for multiple comparisons to determine the difference between each two groups. dpi: day(s) post injury; SCI: spinal cord injury.



Additional Figure 2 Study design of the main experiment.

The number and gender distribution of the mice for behavioral evaluation and immunostaining are shown here. Forty-five animals were subjected to contusion spinal cord injury. The number of subjects in each group was declining due to death within two weeks post injury. No further loss of animals after 14 days post injury. BMS: Basso motor score; dpi: day(s) post injury; PAS: photobeam activity system for open field locomotion test; SCI: spinal cord injury.