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Enhancing neuroprotective effect of aminosalicylic acid-grafted chitosan electrospun fibers for spinal cord injury

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ABSTRACT

The hyperinflammation microenvironment after spinal cord injury (SCI) remains a great challenge for neural regeneration. Methylprednisolone has been used to reduce the inflammatory response after SCI, but it is controversial due to side effects associated with off-specific targeting effects. In this study, we synthesized in situ 5-ASA grafted chitosan electrospun fibers (ASA-EF) with excellent injectable and self-healing properties to reprogram nerve cells via displaying biological distribution, gene expression, and functional changes. With the support of ASA-EF, the downregulation of inflammatory cytokines expression and the upregulation of anti-inflammatory and regenerative gene expression were found in vitro studies. Moreover, ASA-EF administration polarized macrophages toward proregenerative phenotypes in the injured lesion, and significantly reduced cavity area. In addition, ASA-EF administration increased myelination and regenerating axons and improved motor function (score of 5 versus 2 for SCI group). These results illustrate that the neuroprotective effect of this artificial nanoplatform will facilitate the clinical treatment of traumatic-related diseases via forming a recycled microenvironment that supports regeneration and functional recovery. These particles may be applied to trauma and potential other inflammatory diseases.

1. Introduction

Spinal cord injury (SCI) causes a variety of detrimental effects, including neuroinflammation, leading to chronic functional impairment and death [1,2]. In spite of the protective nature of their early response, macrophages soon became pro-inflammatory and started a series of reactions that led to the entry of peripheral innate immune cells [3]. Neruoinflammation is mainly induced by innate immune cells (including inflammatory microglia and monocytes) [4]. These cells infiltrate the injured lesion within a few minutes to hours, and secrete the inflammatory cytokines and reactive oxygen species (ROS), which cause neuron death, demyelination and dysfunction [5,6]. Methylprednisolone has considered as a standard of the treatment for acute SCI due to its anti-inflammatory effect, these drugs are also related to side-effects, such as femoral head necrosis, gastrointestinal bleeding, and thromacbolic, indicating that comprehensive strategies of the injured spinal cord are critical to SCI treatment [7]. Monocytes-derived cells are necessary for tissue regeneration after injury, reprogramming immune response may be a more effective strategy, which can minimize functional loss and promote repair [8]. In SCI, recent studies have shown that hematological infiltrating immune cells are mainly responsible for secondary axis death, which indicates that the infiltration of blood related immune cells may indirectly reduce tissue by reducing secondary events that can be discovered by inflammation [9–11]. Reprogramming immune cells to accumulate and present the regenerative phenotype can provide a direct regulating damage environment to promote regeneration.

Chitosan is a deacetylated form of chitin that exhibits favorable affinity toward metal ions and small molecule compound due to its abundant polar groups [12]. Because of these properties, chitosan has attracted the attention of researchers' that are focused on the development of unique fibrous materials, especially for chitosan electrospun nanofibers, due to their biodegradability, cell affinity, high porosity and high surface area [12,13]. Among these advantages, chitosan electrospun nanofibers have utility in key areas of importance in biomedical applications. Herein, three objectives are addressed: (i) To synthesize 5-aminosalicylic acid (ASA) grafted chitosan and to prepare its electrospun

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fibers (EF); (ii) To carry out a structural study of the electrospun fibers and (iii) To investigate therapeutic efficacy of chitosan electrospun nanofibers (ASA-EF) in order to demonstrate the effects of 5-aminosalicylic acid on chitosan-based nanomaterials. In this work, we assessed ASA-EF that reprogram inflammatory cells and rebulid microenvironment in the injured spinal cord, thereby enhancing regeneration and functional recovery after SCI. The inflammation responses are tested by historical and genetic analysis. The regeneration after ASA-EF treatment was evaluated through the number of axons and remyelination of axons with motor function performing. ASA-EF administration targeted innate immune cells to reprogram their function, representing the novel neuroprotective strategy for SCI.

2. Results and discussion

2.1. Preparation and characterization of EF

The ASA-EF was synthesized by adding deacetylated chitosan and 5-ASA to 4-toluenesulfonyl chloride mixture in a dropwise manner, in which the triethyl amine was used to promote the formation of EF with better water dispersity (Fig. 1a). The EF was readily injected using a injection syringe, forming within 5s (Fig. S1). The morphology of EF and ASA-EF was characterized by scanning electron microscope (SEM), showing uniform fibrous shape (Fig. 1b). The prepared EFs displayed a characteristic absorbance peak by Fourier transform infrared spectrum (FT-IR) (Fig. 1c). Solid-state ^{13}C NMR spectra are displayed in Fig. S2. To

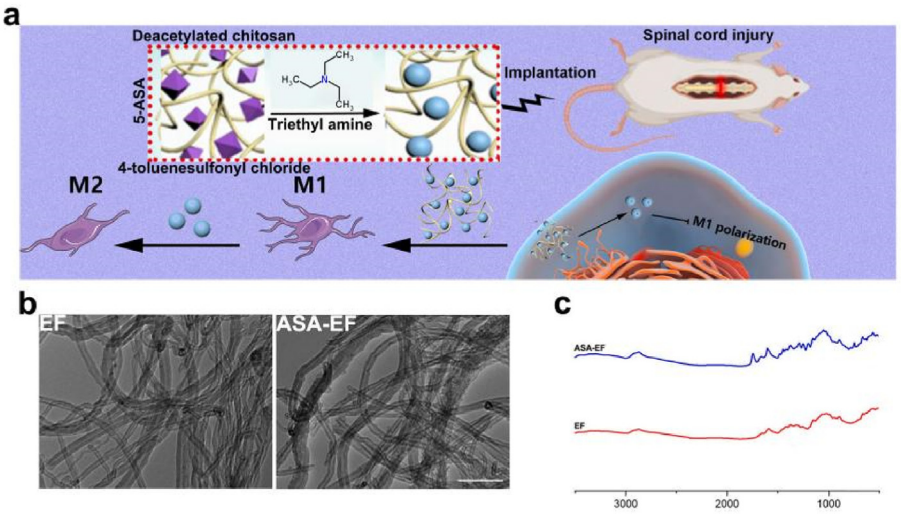

Fig. 1. Characterization of the EFs. (a) Schematic illustration of EFs synthesis and EFs based treatment for SCI in vivo. (b) TEM images of EF and ASA-EF. (c) FT-IR image of EFs.

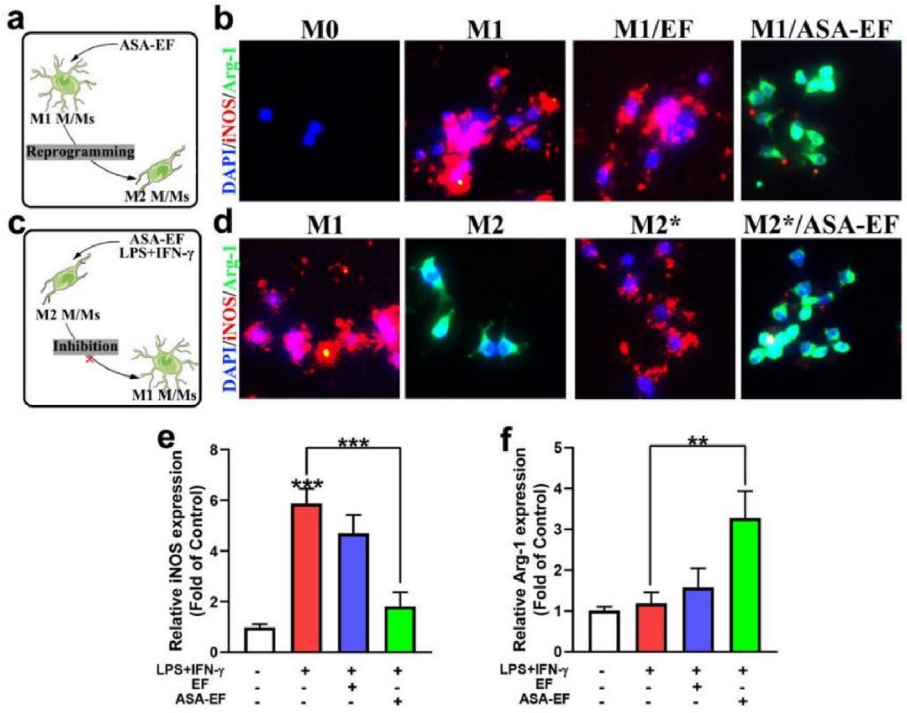

Fig. 2. In vitro macrophage polarization by EFs. (a) Schematic illustration for M1-M2 macrophage polarization by ASA-EF. (b) Representative images of iNOS- and Arg-1-labeled cells. (c) Schematic illustration for inhibition of M2-M1 macrophage polarization by ASA-EF. (d) Representative images of iNOS- and Arg-1-labeled cells. (e, f) Representative quantification of iNOS and Arg-1 mRNA expressions in vitro. **P < 0.01; ***P < 0.001.

simulate the drug release of ASA-EF at physiological condition, in vitro drug release was performed in PBS (0.01 M, pH 7.4) with or without H_2O_2. At 0.5 mM H_2O_2, about 96% of ASA was released from ASA-EF within 24 h, while only 37% of total drug was released in the absence of H_2O_2 (Fig. S3). These results indicated that ASA could be released from ASA-EF at the high level of H_2O_2.

2.2. Macrophage polarization by ASA-EF in vitro

To investigate the macrophages reprogram of the ASA-EF in vitro polarization of macrophages by labeling inducible nitric oxide synthase (iNOS) (red, M1 marker) and Arg-1 (green, M2 marker) was observed in Fig. 2a. According to Fig. 2b, the ASA-EF-treated group had lower red intensity and higher green intensity than the EF and M1 groups. Moreover, ASA-EF-treated cells had a much lower iNOS fluorescence and a much higher Arg-1 fluorescence compared to M2* cells. These results showed that ASA-EF significantly suppressed the polarization from M2 toward M1 in vitro (Fig. 2c–d). To further verify the genes expressions of M1-M2 macrophage polarization by ASA-EF, we found that in the treatment of ASA-EF, RAW264.7 cells had much lower iNOS (Fig. 2e) and much higher Arg-1 (Fig. 2f) mRNA expressions as compared to LPS + IFN-γ-treated and EF-treated cells, indicating that M1-M2 macrophage polarization was promoted by ASA-EF administration.

2.3. In vivo therapeutic efficacy of ASA-EF

As shown in Fig. 3a, the spinal cord of C57 mice was semi transected with a gap length of 2–3 mm. Through different treatments (Fig. 3b), SCI mice were divided into three groups, including simple SCI, EF and ASA-EF. Then, 28 days after surgery, the spinal cord was dissected and stained for histological evaluation. First, evaluate the cyst cavity and cell infiltration of the whole tissue by H&E staining. As shown in Fig. 2B, T9 – T11, there are large cavities in the injured spinal cord of SCI mice, and only a few cells are scattered around the injury. In the EF and ASA-EF groups, the cavity was significantly reduced, and a large number of cells filled the injured spinal space. In the quantized figure, the cavity areas of EF and ASA-EF were reduced to 47% and 24% of SCI group respectively (Fig. 3c). The data is consistent with the image, indicating that the implantation of hydrogel can help to eliminate cavities. The capsule generated during SCI is a barrier for cell infiltration and trans

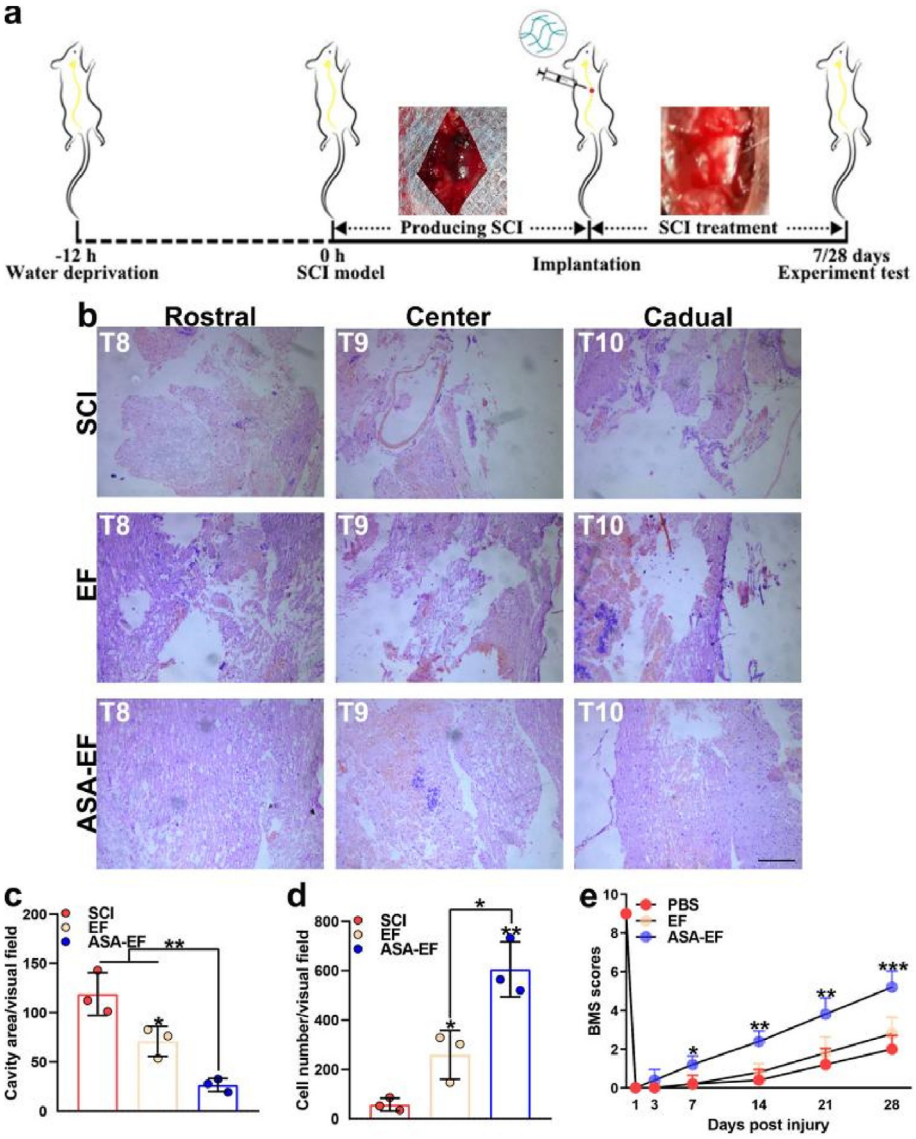

Fig. 3. In vivo neuroprotection of ASA-EF on Day 28 after SCI. (a) Schematic illustration of the establishment and treatment schedule of SCI mice. (b) HE staining images of spinal cord tissues in SCI, EF, and ASA-EF groups. (c) Representative quantification of cavity area. (d) Representative quantification of cell numbers. (d) Representative quantification of BMS scores. *P < 0.05; **P < 0.01; ***P < 0.001.

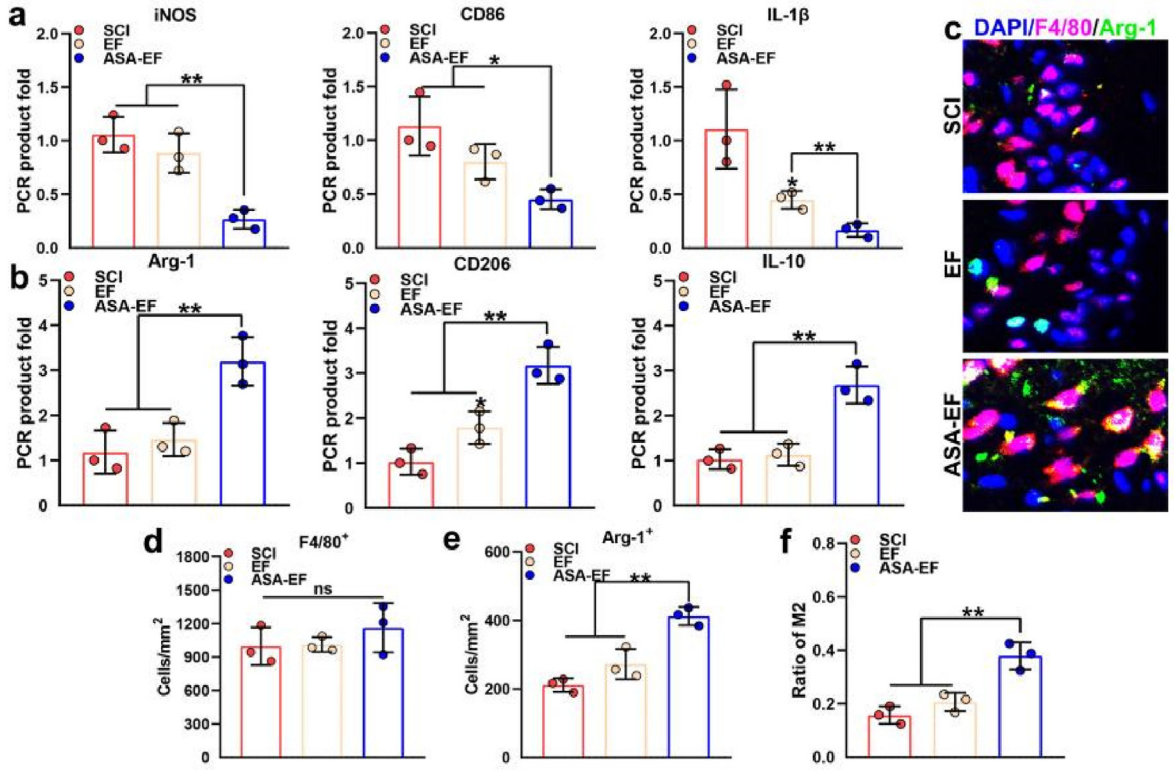

Fig. 4. In vivo macrophages polarization of ASA-EF. (a) Representative quantification of mRNA levels of iNOS, CD86 and IL-1β. (b) Representative quantification of mRNA levels of Arg-1, CD206 and IL-10. (c–f) Representative images and quantification of colocalization in vivo. *P < 0.05; **P < 0.01; ***P < 0.001.

luminal axon regeneration, which damages the survival and integration of recruited nerve cells and ultimately hinders SCI repair. Therefore, electrospinning may provide a favorable ECM microenvironment for the inward growth of cells. In fact, as shown in Fig. 3d, the number of cells infiltrating into the lesions increased from about 60 cells per field of SCI to about 300 cells per field of EF, and about 620 cells per field of ASA-EF. In addition, ASA-EF treated mice were beneficial to the recovery of hind limb motor function. ASA-EF has the ability to enhance cavity clearance and cell infiltration, which may provide an improved niche to attract endogenous cells to promote axon regeneration and SCI repair.

2.4. Macrophage polarization induced by ASA-EF at the injured lesion

Under the predominant state of proinflammatory microglia/macrophages (M/Ms), the microenvironment of the injured spinal cord is detrimental to axon regeneration and tissue repair [14–17]. The phenotype of M/Ms, as M1 and M2 polarization, has a different capability to exert functions in SCI. M1 M/Ms, highly labeled inducible nitric oxide synthase (iNOS), CD86 and interleukin-1β (IL-1β), have cytotoxic effect on SCI. As highly expressed arginase-1 (Arg-1), CD206, interleukin-10 (IL-10), M2 M/Ms can promote regeneration and inhibit inflammation after SCI [18]. The analysis of markers for M1 and M2 macrophages in the injured spinal cord showed that the mRNA levels of iNOS, CD86 and IL-1β in ASA-EF group were significantly lower than those in SCI and EF groups (Fig. 4a). As key markers of M1 phenotype, these genes are related to inflammatory response and the release of proinflammatory cytokines, and contribute to the hyperinflammation microenvironment in the lesion. In contrast, the mRNA levels of Arg-1, CD206 and IL-10 in ASA-EF group were significantly increased as compare to SCI and EF groups (Fig. 4b). Subsequently, these data were tested to quantify the total number of CD206+cells (Hoechst+/Arg-1+), macrophages (Hoechst+/F4/80+) and M2 macrophages (Hoechst+/F4/80+/Arg-1+) on the 7th day after SCI (Fig. 4c). It was no statistical difference that the total number of infiltrated macrophages in these groups (Fig. 4d). Moreover, compared with other groups, the number of Arg-1+cells in ASA-EF significantly increased (Fig. 4e). In addition, in the ASA-EF group, the ratio of M2 phenotype to the total number of macrophages was significantly increased 2-fold (Fig. 4f). Therefore, the synergistic effect of ASA and EF could not only polarize M1 macrophages toward M2, but also enhance the expression of proteins related to anti-inflammatory function, which may provide the possibility of rebuilding the microenvironment. Therefore, ASA-EF should be a suitable choice for nerve injury repair.

2.5. Remyelination enhanced by ASA-EF in injured lesion

Next, we evaluated the pro-regenerative effect of ASA-EF administration at the beginning of chronic phase of SCI (the 28th day post-injury) (Fig. 5). Spinal cord tissues were labeled with neurofilament 200 (NF200, red), myelin basic protein (MBP, blue) and myelin protein 0 (P0, green). Compared with SCI and EF groups, ASA-EF administration significantly increased the number of NF200+ cells (Fig. 5b) and the number of myelinated axons (NF200+/MBP+, Fig. 5c). Moreover, the significantly greater number of oligodendrocyte- (NF200+/MBP+/P0−) and Schwann cell-derived myelinated (NF200+/MBP+/P0+) axons were observed for ASA-EF administration as compared to PBS group, which is similar to the axons on the uninjured opposite side (Fig. 5b–d). Collectively, these results showed that ASA-EF administration promoted remyelination and functional recovery after SCI.

3. Conclusion

In summary, we developed ASA-EF to investigate their therapeutic effects as targeting nanomedicines for SCI. ASA-EF was efficiently polarized toward M2 macrophages. Furthermore, in vivo studies showed that ASA-EF had significantly improved therapeutic efficacy. This study

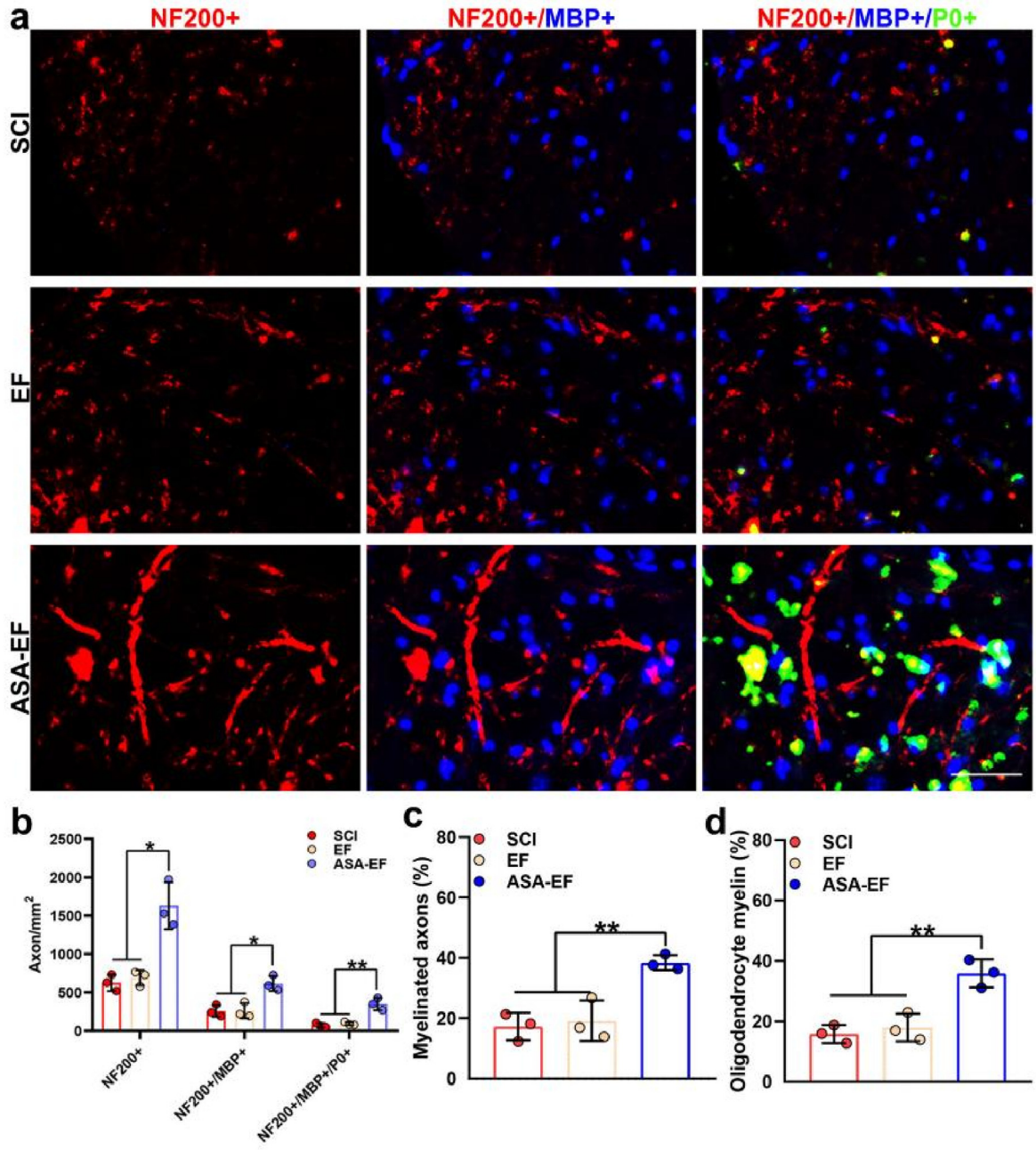

Fig. 5. In vivo remyelination of ASA-EF. (a–b) Representative images and quantification of axons in vivo. (c) Representative quantification of myelinated axons in vivo. (d) Representative quantification of oligodendrocyte axons in vivo.*P < 0.05; **P < 0.01.

demonstrated the great potential of ASA-EF as a nanodrug via reprogramming glial cells for SCI.

Authors' contributions

S.Lin and Y. Guo designed the idea of this work and wrote the manuscript. D. Wang and H. Zhao conducted the material preparation, supervised the project. C. Xu performed in vitro experiment.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

All animal procedures were approved by the Committee on Animals of the Jinzhou Medical University. All animal procedures were performed in accordance with the Ethical Committee of Care and Use of Laboratory Animals at Jinzhou Medical University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2022.100529.

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