Research Article

Shengyu Cui, Xinhui Zhu, Dawei Xu, Wei Liu, Hong Yi, and Jun Yan*

Novel design and combination strategy of minocycline and OECs-loaded CeO₂ nanoparticles with SF for the treatment of spinal cord injury: In vitro and in vivo evaluations

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Abstract: Generally, several mechanisms influenced the secondary injury chutes following acute spinal cord injury (SCI). Though current SCI therapeutic approaches mostly target single elements in the injury chutes, they have been mostly ineffective in clinical trials. The aim of this study was to design and develop a novel cerium oxide/silk fibroin (CeO₂/SF) hydrogel material loaded with minocycline (MCN) and transplantation of olfactory ensheathing cells (OEC) for SCI treatment. The prepared CeO₂/SF hydrogel has an advantageous porous morphological structure and CeO₂ NPs were greatly encapsulated on the surface, which was confirmed by microscopic observations. The results of in vitro analyses established favourable biocompatibility of 94.65% and 89.45%, sustained drug release rate of 89% and 58%, and significant reduction in pro-inflammatory factors for the treatments using cerium oxide loaded silk fibroin (CSF) and CeO₂ NPs, respectively. Meanwhile, the administration of MCN@OEC greatly provides an efficient improvement in BBB score, decreased bladder weight, and histological improvement after SCI when compared to the control. Therefore, the combined MCN and OEC-loaded CSF hydrogel sample could be proved as a low cost, safe, and potential material for the treatment of SCI.

Keywords: spinal cord injury, silk fibroin, hydrogel, CeO₂ nanoparticles, OECs

1 Introduction

Spinal cord injury (SCI) is an enervating problem for patients, which leads to reduce the life span, owing to the health issues such as respiratory pain, partial or complete paralysis, and bladder dysfunction [1,2]. Most of the young people or even those below 40 years affected by SCI mainly suffer from sensory, motor, and autonomic dysfunction all through their life. Generally, SCI is classified into two categories, namely, traumatic and non-traumatic aetiologies. Traumatic SCI occurs due to accidents, sports injury, and falling from heights which acutely damages the spinal cord; however, non-traumatic SCI arises when an acute or chronic disease like tumour, degenerative disc disease, or injury creates primary injury [3]. Mainly, traumatic SCIs occur due to loss of neuronal tissues, which are categorized by fractional or complete dysfunction in the nervous system. In the treatment of SCI functional recovery, effective regeneration of injured axons across the injury site is mandatory. The mechanisms of SCI recovery function are more complicated process when compared to the other healing processes because it has been disturbed by extremely complex inhibitory factors. Importantly, cellular fibroblasts, endothelial cells, and neuroglia would produce an inhibitory atmosphere for neurons by persuading the formation of glial scars. Therefore, the development of clinical treatments for SCI recovery has been designed with combinations of anti-inflammatory drug molecules and transplantation of various kinds of cells including peripheral nerves, Schwann cells, embryonic stem cells, neural stem cells, and stromal cells which provide promising functional recovery with pain relief and the
prevention of secondary injuries [4–6]. The acute phase of SCI occurs at the initial stage and thereafter, the primary mechanical tissue will cause a secondary injury cascade, the outcomes of which are oxidative stress, apoptosis, axon degeneration, and inflammation. Besides, the oxidative stress and electrophilic stress are the crucial factors to induce SCI owing to the presence of high level of oxidative metabolic activity, intense production of reactive oxygen species (ROS), such as hydroxyl radicals, superoxide radicals, lipid radicals, etc., and lower production of antioxidant. ROS can easily interact with nucleic acids and proteins which results in the molecular and cellular damage as a consequence of neurological dysfunction. Therefore, the scavenging of ROS is an important process in the treatment of SCI [7–9].

Natural polymers such as wool, amber, silk, rubber, collagen, and keratin are explored as a potential material for various applications. Among these polymers, silk is an important natural polymer obtained from Bombyx Mori silkworm cocoons family. Silk mainly consists of two types of proteins, silk fibroin (SF) and sercin, that are made of amino acids such as glycine, serine, and alanine. The SF biopolymer has a special attention for tissue engineering applications as well as drug delivery applications due to the potential properties of biocompatibility, bio-degradability, self-assembly into β-sheet networks, and mechanical stability [10]. Furthermore, different forms of SF-based biomaterials are processed by various methods from regenerated SF solutions such as films [11], hydrogels [12], microspheres [13], scaffolds [14], and NPs [15]. Besides, SF hydrogels are cross linked 3D hydrophilic networks which swell, nonetheless insoluble, when contacting water. SF hydrogel was formed through sol–gel transition and leads to the aggregation of fibroin molecule into β-sheet structure to create an interconnected network in an aqueous medium [16]. Gelation of SF was stimulated by factors like concentration, change in pH, heat and cold treatment, and incorporation of cross linkers. Owing to their excellent properties, SF hydrogels are considered as an efficient polymeric material for biomedical applications such as bone tissue engineering, drug delivery, and inhibitors for cancer in therapeutics [17,18]. For instance, a few examples are described here, Kaplan and co-workers described a synthesis of doxorubicin-loaded SF hydrogel for the treatment of primary breast cancer [19]. Van et al. reported a study on the biomimetic SF hydrogel for the inhibition of tumour growth [20]. Singh et al. investigated a potential of agarose/SF blended hydrogel for in vitro cartilage tissue engineering application [21]. Further, Chouhan et al. investigated the in situ preparation of an injectable SF hydrogel used to promote skin regeneration of burn wounds [22]. Chung et al. reported a study on bi-layer SF scaffolds and small intestinal scaffolds submucosa matrices to support bladder tissue regeneration of SCI in a rat model [23]. Zhang and co-workers described an efficient approach for transplantation of neutral scaffolds consisting of dermal fibroblast reprogrammed neurons and 3D SF materials promoting the repair of SCI [24]. Bone mesenchymal stem cells seeded in porous SF scaffolds promote myelination, the nerve regeneration, and functional recovery of SCI in rat models [25].

Cerium oxide nanoparticles (CeO$_2$ NPs) have got a great attention in research community, due to their interesting properties like ultra violet absorbance, oxygen transfer ability, high refractive index, etc., and find applications in various fields [26,27]. CeO$_2$ have a unique behaviour to scavenge ROS, namely, superoxide anions, hydrogen peroxide, and peroxynitrite. Interestingly, CeO$_2$ have dual oxidation states +3/+4, thus capable to act as oxidation and reduction catalyst and also closely resemble properties of the antioxidant enzyme superoxide dismutase [28]. Further, CeO$_2$ NPs have an exclusive electronic structure which creates oxygen defects or reactive sites on the surface of NPs. These sites act as free radical scavengers, and consequently these NPs have been investigated as therapeutic interventions in biological applications [29]. Herein we quoted some of the reports regarding CeO$_2$ NPs: Xia et al. investigated a comparative study of the mechanism of toxicity of ZnO and CeO$_2$ NPs based on dissolution and oxidative stress properties [30]. Hickman and co-workers reported the properties of an auto-catalytic anti-oxidant behaviour and biocompatibility of CeO$_2$ NPs for SC repair and other central nervous system [31]. As previous reports described shape-dependent CeO$_2$ NPs for enhancement of enzyme (super oxide dismutase) mimetic activity in therapeutic applications [32–35].

Minocycline (MCN) is a tetracycline antibiotic as well as anti-inflammatory drug used to treat various bacterial infections such as pneumonia, acne, and urinary tract infections [36]. Moreover, this drug was explored as a potential drug for SCI due to its anti-inflammatory, anti-oxidant, and anti-apoptotic properties [37]. Besides, MCN mainly targets to destroy the following problems: free radicals, oxidative stress, glutamate excitotoxicity, and ischemia. Therefore, MCN was considered as a potential neuroprotective drug for SCI in a recent methodical review of pre-clinical data [38]. In this present investigation, we studied facile combination of MCN and transplantations of olfactory ensheathing cells (OEC) by the carrier of CeO$_2$/SF hydrogel materials for the functional...
recovery of SCI. The combination effect of MCN and OEC was found to have favourable action of enhanced cell compatibility and suppressed pro-inflammatory expressions with the assistance of sustained releasing ability of hydrogel sample. The therapeutic potential of the prepared materials has provided improved BBB scale and bladder functions, which was confirmed by in vivo animal experiment.

2 Experimental details

2.1 Chemicals used

The chemicals (NH₄)₂Ce(NO₃)₆, ethylenediamine, hydrazine monohydrate, ethanol, Na₂CO₃, LiBr, and PVP were purchased from Jiangsu Zhongshan Chemical Co., Ltd. Silk worm cocoons were obtained from Jiangsu Academy of Agricultural Science, PR China. Biological mediums (BSA, DMEM, FBS, and EDTA) were obtained from Tansoole Co., Ltd., Shanghai, China. The purchased chemicals were used as analytical grade (AR) reagents.

2.2 Synthesis of CeO₂ NPs by hydrothermal method

For the synthesis of CeO₂ NPs, 0.25 g of (NH₄)₂Ce(NO₃)₆ was added into 30 mL of distilled water and stirred for 20 min to get a completely dissolved solution. Thereafter, 0.15 mL of ethylenediamine was dissolved in 25 mL of deionised water and was added into the above solution in a drop-wise manner under constant stirring. Then, 1 mL of hydrazine monohydrate was added, continuously stirring for 15 min at room temperature. Finally, the solution was transferred into an autoclave and heated at 100°C for 24 h. After the completion of reaction, the autoclave was completely cooled to room temperature. The final product mixture was centrifuged at 5,000 rpm for 20 min to get white precipitate which was washed with water (3 × 10) and ethanol (3 × 10) thrice, filtered, and dried at 60°C for 2 h. Finally, the white powder was calcined at 500°C for 2 h to get CeO₂ NPs.

2.3 Synthesis of SF hydrogel

SF solution was prepared from a natural B. mori silkworm cocoons through a previously reported literature [39,40]. First, the cocoons were cut into tiny pieces and boiled in Na₂CO₃ (0.02 M) for 30 min. The degumming process was repeated thrice using deionized water in order to remove sericin and wax from the silk. Then, the silk fibres were dried at room temperature and dissolved in LiBr (9.3 M) solution at 60°C for 2 h. The solution was dialysed in distilled water for 3 days using dialysis membrane at ambient condition to remove LiBr salt. Thereafter, the SF solution was centrifuged at 8,000 rpm for 30 min. Then, the final SF solution was stored at 4°C for further studies.

2.4 Preparation of CeO₂ NPs embedded SF hydrogel

In this preparation, 100 mg of synthesized CeO₂ NPs (white powder) was dispersed in 10 mL of ethanol. Meanwhile, 5 wt% of PVP was prepared in ethanol and was added to the CeO₂ NPs' solution at constant stirring. Thereafter, the prepared SF solution was poured into the above mixture under ultrasonication for 3 h to get CeO₂ loaded SF hydrogel. Then, the SF hydrogel nanocomposite was freeze dried at −40°C and preserved at 4°C for further studies.

2.5 Characterisation

Functional groups and other important moieties present in the synthesized SF hydrogel and CeO₂ incorporated hydrogel were studied by Infrared Spectrophotometer (Shimadzu −8400, Japan) using KBr pellet method. SF hydrogel and CeO₂/SF were investigated by Ultra-High-Resolution Schottky Scanning Electron Microscope SU7000. The size and morphology of CeO₂ NPs were analysed by TEM, JEOL JEM-2100 with an accelerating voltage of 200 kV. Panalytical X-ray diffractometer with a Cu-κα X-ray source operated at 40 kV with a current of 30 mA (λ = 0.15406 nm) was used to investigate the crystalline behaviour of NPs.

2.6 The OECs culture method

The Nash method was used to attain OEC from the sources of adult rats' nerve fibres and olfactory bulbs as per previous reports [41]. Chiefly, the adult rats were
successfully anesthetized using an appropriate dose of chloral hydrate. Thereafter, the olfactory nerves were separated and bulbs were placed into the magnesium- and calcium-free HBSS solution obtained from Sigma-Aldrich, USA. The isolated blood vessels- and meninges-removed tissues were pulverized and incubated using DMEM medium supplemented with trypsin (0.1%) at biological conditions (37°C; 5% of CO2) for 30 min. After that, the suspension was centrifuged with FBS solution after inactivation of trypsinitization. Then, the suspension was placed into a culture flask with DMEM medium added with FBS (10%), standard antibiotics, and L-glutamine (2 mM). After 48 h of incubation, OECs were attached onto the precoated poly L-lysine plates.

2.7 The spinal cord in vivo animal model

The adult female rat models (230–260 g) were purchased from Slac Laboratory Animal Co. Ltd., PR china for the animal testing of SCI treatments. All animal experiments and protocols were in agreement with the policies of the Institutional Animal Care and Use Committee, Nantong University, Jiangsu, China. The adult rat animal models were anesthetized using chloral hydrate (450 mg per 1 kg) by intraperitoneal injection. The spinal cord of rats was exposed under laminectomy at vertebral level T11 and spinal injury was created, which was followed by animal experiments. The spinal cord of rats were anesthetized using an appropriate dose of chloral hydrate. Thereafter, the olfactory nerves were separated and bulbs were placed into the magnesium- and calcium-free HBSS solution obtained from Sigma-Aldrich, USA. The isolated blood vessels- and meninges-removed tissues were pulverized and incubated using DMEM medium supplemented with trypsin (0.1%) at biological conditions (37°C; 5% of CO2) for 30 min. After that, the suspension was centrifuged with FBS solution after inactivation of trypsinitization. Then, the suspension was placed into a culture flask with DMEM medium added with FBS (10%), standard antibiotics, and L-glutamine (2 mM). After 48 h of incubation, OECs were attached onto the precoated poly L-lysine plates.

2.8 Administration of MCN

The MCN with dissolving sterile PBS solution was administered intraperitoneally to the injury created animal models. The SCI created animal models proximately received 80 mg/kg of MCN after SCI injury and then administered after one day as established by previous reports. The animal injected with bare PBS solution with absence of MCN was considered as control sample. At the same time, the rat without injury with non-therapeutic approaches was sacrificed and was considered as sham.

2.9 Cell transplantation

The cell transplantation was done 7 days after initial SCI surgery with treatment of MCN. In brief, the adult rats were re-exposed through laminectomy after being anesthetized. The cultured OECs (6 µL/450,000) cells were transplanted to the defected SCI site by using syringe, and the injected place was marked. The OEC culture was injected at the places of lesion (0.8 mm), rostral (1 mm), and caudal (1 mm) epicentres. The animal injected with DMEM biological medium was considered as control sample.

2.10 The measurement of BBB score

The BBB scores were evaluated to measure the recovery function of MCN and transplanted cells followed by BBB scale technique, which measured the hind limb function in the ranges between 0 and 21. The augmented score indicated the improvement in the recovery functions including joint movements, limb movements, weight behaviours, and other recovery functions. The scores were obtained and calculated following SCI locomotion recovery progression in different time intervals (1, 7, 14, 21, 28, and 35 days).

2.11 Histological analysis

The treated animal models were sacrificed feasibly with an overdose of anaesthetic cocktail (ketamine and xylazine) at the end of the experiment day. After that, the treated spinal cords of rats were carefully detached and stored in sucrose solution (30%) dissolved in PBS medium (pH 7.4) for 12 h. The spinal cords with injury site of 3 cm in size were separated sensibly. Then, the samples were cut into small square shape sections (10 mm) using the cryostat device and stained by haematoxylin and eosin (H and E) dye. The presence of connective and nervous tissues on the treated spinal cords were observed at 10× and 40× magnifications and visualized using the light microscopic technique.

2.12 Statistical analysis

The statistical significance value of the presented results was examined using the one-way analysis of variance
technique. All the values are mentioned as the mean value ± SD (standard deviations).

3 Results and discussion

3.1 Characterisation of CeO$_2$ NPs

Herein we described a fabrication of drug-loaded novel therapeutic agent containing SF/CeO$_2$ for SCI recovery (Figure 1). CeO$_2$ NPs were synthesized by well-established hydrothermal method and the formation of NPs were confirmed by the combination of x-ray diffraction studies (XRD) and Transmission electron microscopic studies (TEM. Figure 2a shows the XRD pattern for CeO$_2$ NPs and the sharp peaks present in between the angles 20° and 80° clearly indicates the formation of NPs. Moreover, the spectrum shows intense sharp peaks appearing at 28.4, 33.2, 47.5, 56.4, 59.6, 70.3, and 76.7 having the miller indices (111), (200), (220), (311), (222), (154), and (331), respectively. These peaks vividly indicate the crystalline nature of CeO$_2$ NPs. Further, the size and structural morphology of CeO$_2$ NPs were examined under TEM and the TEM images showed that the NPs are spherical structure having ~20–25 nm (Figure 2b). Along with spherical

Figure 1: Schematic representation of the present investigation including synthetic and structural schemes.

Figure 2: (a) XRD pattern for CeO$_2$ NPs and (b) TEM images of CeO$_2$ NPs.
particles, some thread like morphologies were also observed in the TEM micrograph.

### 3.2 Characterisation of CeO$_2$-loaded SF hydrogel

The functional groups present in the SF were confirmed by FTIR spectroscopy. An overlapped FTIR spectrum for SF hydrogel and CeO$_2$ NPs-loaded SF hydrogel is given in Figure 3a. The spectrum (a) displays a band at 1,632 cm$^{-1}$ for N–H stretching which confirmed the presence of (amide groups) amino acids such as glycine, alanine, and so on. These characteristic bands were considered as the absorption peak of the peptide backbone. The important peaks at 1,632, 1,519, and 1,231 were assigned to amide I, amide II, and amide III bands for β-sheet conformation. The characteristic band appeared at 1,519 cm$^{-1}$ due to N–H bending vibration and additional peaks were observed at 1,231 and 1,336 cm$^{-1}$ due to C–N stretching vibrations. Moreover, these characteristic absorbance peaks are clearly the evidence for the hydrogen bonding in N–H groups. We observed that a small peak at 3,289 cm$^{-1}$ is ascribed to O–H hydroxyl groups in SF hydrogel material. In addition, two peaks appeared at wavenumber 2,983 and 2,895 cm$^{-1}$ which are attributed to C–H symmetric and C–H asymmetric vibrations, respectively. As seen in spectrum (b), in addition to all the peaks obtained for SF hydrogel, a small sharp peak appeared at around 600 cm$^{-1}$ for Ce–O metal to oxide linkage revealing the incorporation of CeO$_2$ NPs in SF hydrogel.

Further, CeO$_2$-loaded SF hydrogel was studied by X-ray diffraction studies. The amorphous structure of freeze-dried SF hydrogel exhibiting a broad peak at around 20° is shown in Figure 3b (pattern a), and the obtained spectrum is closely analogous to the XRD pattern for β-sheet SF structure. The lyophilized SF hydrogel displays only typical broad peak at around 20°, obviously no other peaks were observed in the spectrum which specify the possibility of more α-helical structure. As seen in Figure 3b (pattern b), the XRD pattern for CeO$_2$-loaded SF hydrogel showing broad peak at 20° reveals the amorphous nature of SF material, and along these wide peaks at small angle region, seven more additional peaks appeared owing to the existence of CeO$_2$ NPs in hydrogel.

Besides, the structural morphology of SF hydrogel and CeO$_2$ NPs-loaded SF hydrogel was investigated through scanning electron microscopic (SEM) technique. Figure 4a shows that the SEM image of SF hydrogel are porous in nature and have exposed interconnected heterogeneous porous architecture. Moreover, the freeze-dried process also enhances the porosity of the hydrogel. The surface morphology of CeO$_2$ NPs-loaded SF hydrogel was examined under SEM and Figure 4b shows that the microstructure of hydrogel composite have porous architecture and CeO$_2$ NPs were embedded in the surface of hydrogel matrix. Figure 4c displays vividly that the CeO$_2$ NPs were properly blended to the SF solution, and the NPs’ spherical as well as elongated thread like morphologies are well matched to the TEM images of CeO$_2$ NPs. These results strongly support the existence of NPs in the hydrogel matrices. Additionally, we carried out TEM analysis (Figure 5) of CeO$_2$/SF hydrogel which confirmed the
incorporation of NPs in hydrogel. TEM images obtained for CeO$_2$/SF hydrogel displayed a dark portion indicating CeO$_2$ NPs entrenched in NPs in the porous architecture of hydrogel matrix. Moreover, Figure 5c indicates a zoom version of image b and displays an intense image of CeO$_2$ NPs.

After the cell cultures in appropriate time, we can observe the successful forms of OECs through the optical and fluorescence microscopic images as shown in Figure 6. The fluorescence microscopic observation of OECs exhibits the findings of NGFRp75 (Figure 6). The production of repressive glial scars and spinal cord neurons have been significantly damaged by the secondary injury after SCI. The current single strategic therapies of SCI have targeted its own complications that could limit the functions of post-injury recovery, which provides inadequate developments in the recovery functions of SCI. Hence, we have designed and developed combined therapies for SCI recovery using
OECs' transplantation and distribution of MCN to overwhelm the treatment of SCI that enhances therapeutic potential. The present investigation results established that combination potential of OECs' transplantation and MCN have great effects than treating separately with cells' transplantation and MCN. The cell compatibility of the prepared nanomaterials was evaluated on cultured OECs at different concentrations using CCK-8 assay. The results of cell viability exhibited that the prepared nanomaterials displayed non-toxicity on the OECs at appropriate concentrations, which demonstrated greater biological compatibility with normal cells as exhibited in Figure 6a. Specifically, the prepared CeO₂ NPs and CeO₂/SF nanovesicles displayed cell viability percentages of 89.45% and 94.65%, respectively, at higher concentration levels, which revealed that molecular structures and concentrations of prepared nanoformulated materials have not disturbed the normal cell lines. These observed results of in vitro drug release and cell viability demonstrated that therapeutic dose of nanomaterials could provide effective treatment for SCI recovery, signifying the combination therapy of MCN and OECs. The analysis result of in vitro drug release was evaluated using simulated body fluid (PBS; pH 7.4 supplemented with 2% of DMSO) with treatment of MCN-loaded CeO₂ NPs and CeO₂/SF composite vesicles. The drug molecules’ (MCN) release from CeO₂ NPs were significantly faster when compared to the CeO₂/SF nanovesicles, which confirm that the developed CeO₂/SF nanoformulation provides controlled release platform. The drug release rate of MCN is 75% and 100% from CeO₂/SF nanocomposite and CeO₂ NPs, respectively, at 5 days, which revealed that CeO₂/SF composite have effective and sustained release platform as shown in Figure 6b. The anti-inflammatory efficiency of MCN-loaded CeO₂ NPs and CeO₂/SF composite was investigated by quantification-based measurements using ELISA method. Both nanomaterials distributed with MCN have significantly reduced expressions of IL-1β, TNF-α, and IL-10 when compared with control (LPS) group as exhibited in Figure 7. The prepared MCN-loaded

Figure 6: Optical and Immuno-fluorescence microscopic images of cultured OECs: (a) in vitro cell compatibility evaluations of prepared materials on OECs and (b) in vitro drug release ability of the nanomaterials with MCN.
CeO$_2$/SF composite displayed that IL-1$\beta$ and TNF-\(\alpha\) expressions significantly assuaged, due to the effective anti-inflammatory action of MCN and CeO$_2$ nanoparticulate system.

The improvement of SCI recovery function was evaluated by the investigation of locomotor behaviour of hind limbs such as BBB score. The results of locomotor function demonstrated that animal models of SCI have gradual enhancements in the hind limb movements at increasing time intervals at about 35 days. Though the hind limb movement and motor functions were progressively developed, which was treated separately with bare OECs and MCN on different days (14, 21, 28, and 35 days) when compared with SCI group, the combination results of OECs and MCN exhibited augmented BBB scores at increasing days post-treatment, which confirms the amplified functional recovery as displayed in Figure 8. The results established that the combination of MCN and OECs’ transplantation have significant progress in BBB scores and tissue sparing than SCI models treated with bare MCN and OECs alone. High recovery rate after SCI was observed and recorded after 2–5 weeks with the treatment of MCN + OECs group, which revealed that the transplantation of OECs has significantly influenced the functional recovery improvement. Meanwhile, the grafting of OECs provides favourable biological setting for the SCI recovery mechanisms with decreasing actions of pro-inflammatory factors and reducing formation of glial scars. At the same time, the transplantation of OECs may regulate the expressions of glial fibrillary acidic protein (GFAP), which avert the glial scar formation and inhibitory molecules secretion. Previous reports recorded that OECs’ graftings have greatly

Figure 7: Evaluations of pro-inflammatory expressions on the TNF-\(\alpha\) (a), IL-10 (b), and IL-1$\beta$ (c) in treated groups of SCI, MCN, MCN@CSF, and OEC + MCN@CSF.
influenced the GFAP expressions after SCI. The treatment of MCN to SCI therapy has providing modulation of apoptosis, microglia, and caspases, which proved the auspicious therapeutic potential for regeneration of SCI. In agreement with the previous reports, we have investigated and confirmed that MCN combined with OECs have importantly enhanced the functional recovery and regeneration effects after SCI.

The histopathological observation was performed to demonstrate the visualization of SCI recovery after administration of MCN and MCN + OECs-loaded CeO$_2$/SF nanomaterial as displayed in Figure 9. The H and E staining observation of combination therapeutic approach exhibited significant histological enhancement when compared to the nanomaterial with MCN (alone), OECs (alone), and SCI control group. The SCI control group without any bio-pharmaceutical treatment exhibited more pseudocysts with very less progressed development. In treatment group of MCN + OECs-loaded CeO$_2$/SF nanomaterial, the recovery of spinal injury was significantly improved, and successfully re-established SCI section as exhibited in Figure 9. These results of histopathological

![Figure 8: Evaluation of BBB behaviour motor function: (a) on hind limb of rat models and bladder weight (b) treated with different samples including MCN, OEC, and combined OEC + MCN materials loaded on CeO$_2$/SF nanomaterials.](image)

![Figure 9: The analysis of histopathological study of the SCI (untreated sample), MCN, OEC, and MCN + OEC loaded on CSF nanomaterial; H and E and MTS staining images were observed after 8 weeks of post-injury under microscopic technique.](image)
4 Discussion

The previous reports presented that the hydrogel-based materials have significant tissue repair and regeneration abilities and high suitability for SCI treatments. In the present investigation, silk fibroin hydrogel material was prepared with incorporation of CeO$_2$ nanoparticles. The prepared CeO$_2$/SF hydrogel material exhibited superior biocompatibility with high cell density, which confirmed the suitability of the material for the in vivo treatment. Precisely, in vivo histological observations showed significant ability to influence regeneration after SCI due to its biocompatibility, functional recovery, and anti-oxidant ability of the encapsulated ceria nanoparticles as reported in many previous reports [31,43]. Generally, high porosity of the hydrogel materials has excessive attention and noteworthy consideration for cell attachment, infiltration, and differentiation in the applications of tissue engineering and wound healing application, which provides auspicious advantageous for cellular mechanisms and supply of needed nutrients to the cell growth. The designed high-porous SF hydrogel material in the current study would be favourable for the cell compatibility and growth [5,44,45]. Generally, acute SCI injuries induced by ROS factors such as NO, peroxynitrite, H$_2$O$_2$, and hydroxyl radical are suggestively concerned to the formation of exacerbate pathological environments, which make more complications like locomotor dysfunctions. Hence, some anti-oxidative materials have been involved in the SCI therapies to prevent the ROS agents’ influences after SCI. To reduce high dosage of antioxidant pharmacological agents, we have used biocompatible ceria nanoparticles to improve the neuronal regeneration and locomotor function after SCI. Consequently, ceria nanoparticles could have favourable action against SCI, at the same time, ceria NPs could be considered as a promising material for sustained delivery of therapeutic drug molecules to attain efficient SCI recovery function as reported in the previous reports [46,47].

The secondary injury of spinal cord after treatment leads to the complicated issues of loss of neurons and inhibitory glial scar formation. Many of the current therapies have embattled and provided one-way treatments of single complications that have limitations to post-injury repair, which offer limited progresses in SCI functional recovery. The previous observations and research reports presented that effective axonal regeneration is highly possible in the SCI therapies, while preventing inhibitory glial scar to permit favourable CNS axons [48]. In the present investigation, we have designed and developed promising SCI therapies with combinations of MCN and OECs’ transplantations into the SCI targeted site to overcome the complications by single therapies and to achieve efficient recovery functions. The observations of the investigated results demonstrated the combined and promising actions of MCN and OECs into the biochemical activity and apoptosis when compared to the bare actions of both components independently. In addition, the combined action of MCN and OECs have provided noteworthy improvement in BBS score compared to these components’ (OECs and MCN) transplantation alone. The higher recovery rate and favourable bladder weight were achieved with the treatment of combined MCN and OECs, which established the development in the functional recovery function after SCI [49,50].

The results of anti-inflammatory factors described that implantation of OECs and MCN through CeO$_2$/SF biomaterials offers a suitable biological environment for cell grafting by improving anti-inflammatory molecules and reducing formation of glial scars. The previous studies reported by researchers exhibited that the late implantations of OECs had transitional actions on pro-inflammatory expressions after SCI [49,51,52]. Hence, the combination potential of MCN and OECs has immediate protective effect and boosted the locomotor function, which may facilitate the inhibition of the posttraumatic astrogliosis. Also, MCN has greatly contributed to the neuroprotection and axonal regeneration by prevention of RGMa, which was reported using rat reperfusion model. Similar to those studies, we have confirmed that MCN provides significant improvement in functional recovery and effective restorative effect with combination of OECs’ transplantation. The histological observation of MCN with OECs transplanted groups exhibited significantly reduced cavity size and tissue sparing when compared to components alone, which are in well-agreement with the previous studies [8,50,53]. Specifically, the treatment using MCN has significantly prevented neuronal death and cyst cavity and also greatly enhanced locomotor function after SCI in rat models. At the same time, tissue protection, axonal regeneration and neurotrophic support was greatly influenced by OECs due to the biological factor’s excretion OECs, which favours the existence of neural cells after damage and nerve growth...
factor. As same as previous reports, we established that combination of OECs and MCN treatment have promisingly reduced cytokines of pro-inflammatory factors (IL-1β, TNF-α, and IL-10) after SCI. Overall, many studies have investigated and reported that treatment using MCN on SCI regeneration significantly contributed to the secondary injury mechanisms and provide protection of neural tissues by its strong anti-inflammatory, anti-apoptotic, and anti-oxidant properties with addition of lipid peroxidation and oligodendrocyte apoptosis inhibitions. It was clearly established that the SCI therapies with combinations of MCN and OECs have greater improvement in the neuronal regeneration and functional recovery after SCI [51,53,54].

5 Conclusion

Overall, the presented results summarized that MCN combined with OEC administered to animal models could have significant improvement in neuronal regeneration and locomotor function as demonstrated by the results of BBB and ladder scores. The developed MCN@OEC encapsulated CSF hydrogel has favourable morphological behaviour including higher porous structure, which provides greater cell compatibility and controlled MCN release for the SCI therapy. The expressions of pro-inflammatory factors (IL-1β, TNF-α, and IL-10) were significantly reduced by the MCN@OEC-loaded CSF hydrogel, which lead to the effective functional recovery after SCI in vivo model. In addition, the results of BBB and ladder scores established the effective recovery of neuronal injury and reactive astocytes and inflammation inhibition when treated with MCN@OEC-loaded CSF hydrogel. Therefore, the combined MCN and OEC-loaded CSF hydrogel sample could be proved as a low cost, safe, and potential material for the treatment of SCI. To conclude, our observations confirm that MCN and OEC could be considered as a promising therapeutic agent to treat acute SCI regeneration therapy.

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