Tissue-Engineered Regeneration of Hemisected Spinal Cord Using Human Endometrial Stem Cells, Poly ε-Caprolactone Scaffolds, and Crocin as a Neuroprotective Agent

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Abstract Loss of motor and sensory function as a result of neuronal cell death and axonal degeneration are the hallmarks of spinal cord injury. To overcome the hurdles and achieve improved functional recovery multiple aspects, it must be taken into account. Tissue engineering approaches by coalescing biomaterials and stem cells offer a promising future for treating spinal cord injury. Here we investigated human endometrial stem cells (hEnSCs) as our cell source. Electrospun poly ε-caprolactone (PCL) scaffolds were used for hEnSC adhesion and growth. Scanning electron microscopy (SEM) confirmed the attachment and survival of stem cells on the PCL scaffolds. The scaffold-stem cell construct was transplanted into the hemisected spinal cords of adult male rats. Crocin, an ethanol-extractable component of Crocus sativus L., was administered to rats for 15 consecutive days post injury. Neurite outgrowth and axonal regeneration were investigated using immunohistochemical staining for neurofilament marker NF-H and luxol-fast blue (LFB) staining, respectively. TNF-α staining was performed to determine the inflammatory response in each group. Functional recovery was assessed via the Basso-Beattie-Bresnahan (BBB) scale. Results showed that PCL scaffolds seeded with hEnSCs restored the continuity of the damaged spinal cord and decreased cavity formation. Additionally, hEnSC-seeded scaffolds contributed to the functional recovery of the spinal cord. Hence, hEnSC-seeded PCL scaffolds may serve as promising transplants for spinal cord tissue engineering purposes. Furthermore, crocin had an augmenting effect on spinal cord regeneration and proved to exert neuroprotective effects on damaged neurons and may be further studied as a promising drug for spinal cord injury.

Keywords Spinal cord injury · Human endometrial stem cells · Poly ε-caprolactone scaffolds · Crocin · TNF-α

Introduction

Spinal cord injury (SCI) as a result of traumatic injury or disease has for many years been a challenging problem to overcome [1, 2]. Primary injury as an irreversible process destructs the spinal cord tissue causing the loss of neurons, axon breakage, and hemorrhagic necrosis. Limited regeneration capacity of the spinal cord in contrast to the peripheral nervous system (PNS) is a result of the post-traumatic environment such as ischemia, inflammation, immune response, and glial scar formation [3–5]. Although recent findings show a weak ability of the spinal cord to regenerate after injury, glial scars hinder the regeneration of nerve fibers towards their synaptic targets [6–8]. Management of this secondary injury cascade has been the focus of SCI research including, stem cell transplantation, drug treatment, and neural tissue engineering approaches [9–11]. A bridging biomaterial scaffold can help prevent glial scar formation, guide axon growth through the lesion site into the distal tissue, and act as a vehicle to deliver stem cells [12–15]. Choosing an appropriate biomaterial for this purpose is critical to treat SCI [16]. Poly ε-caprolactone (PCL), a synthetic,
biocompatible, and biodegradable semi-crystalline polymer, has been commonly used for neural repair [17, 18].

Transplanted stem cells can provide new neurons as well as secrete cytokines to form new functional neural circuits and promote axonal regeneration [16, 19, 20]. Stem cell-based therapy promotes lost neuron replacement, apoptosis inhibition, axonal regeneration, and myelination [21]. Recent studies have deployed neural stem cells (NSCs) and

Fig. 1 Flow cytometric analysis of isolated hEnSCs for mesenchymal stem cell markers (CD90 and CD105), hematopoietic marker (CD34), endothelial marker (CD31), and endometrial stem cell marker (CD146). Results are positive for CD90, CD105, and CD146 and negative for CD31 and CD34.
mesenchymal stem cells (MSCs) for cell replacement therapy of the injured spinal cord. Nevertheless, NSCs demonstrate low neuronal differentiation ability and favor glial differentiation which may result in enhanced glial scar formation and poor functional recovery [12, 22, 23]. To this extent and because MSCs have shown the ability to downregulate pro-apoptotic factors, upregulate anti-apoptotic molecules, hinder axon demyelination and degeneration, have been advocated as a promising source for cell therapy in SCI [24]. However, host immune response, lack of neural differentiation, and low survival of grafted cells are some of the limitations of using MSCs [25]. Endometrial-derived stem cells (EnSCs), a recently characterized type of MSCs engaged in the monthly regeneration of the endometrium, are a new promising source of dynamic stem cells suitable for use in SCI treatment [26–28]. These cells are non-invasively isolated, rapidly expandable, demonstrate high clonogenic potential while maintaining karyotypic normality, and have an ability to differentiate into neurogenic cell types [29, 30].

The post-trauma inflammatory response through active secondary processes results in the loss of structure and function of the spinal cord. Reactive inflammatory cells produce cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) which mediate the recruitment of immune cells to the site of injury. TNF-α has an important role in the cytokine cascade and as a regulator of the inflammatory response [31]. Previous studies have demonstrated successful results in preclinical models of SCI through blocking TNF-α with biological agents [32, 33]. Crocin is an ethanol-extractable component of *Crocus sativus* L. that has demonstrated suppressive effects on pro-inflammatory mediators such as TNF-α, IL-1β, and reactive oxygen species. Crocin has shown to modulate the mRNA expression of Bcl-2 which results in caspase-3 activation and consequently suppress TNF-α induced cell death in neuronally differentiated PC-12 cells [34]. These results and results from other studies suggest that Crocin has a neuroprotective effect on damaged neurons [34–36].

In this study, hEnSCs were seeded onto electrospun PCL nanofibrous scaffolds and transplanted into dorsally hemisected spinal cords of adult male rats. Spinal cord regeneration, axon remyelination, neurite outgrowth, and motor function were analyzed after implantation. Furthermore, the concurrent effect of Crocin was assessed in this process.

**Material and Method**

**Isolation and Culture of hEnSCs**

Human endometrial stem cells (hEnSCs) were isolated and purified. In brief, endometrial biopsies were obtained from the fundus of the uterus of women with an average age of 32 years in accordance with the Tehran University of Medical Sciences ethic committee. Specimens were
dissociated in Hank’s balanced salt solution (HBSS; Sigma, USA, H-6136) supplemented with HEPES (25 mM) and collagenase type 1 (1 mg/mL; Sigma, USA, C0130) with agitation at 37 °C for approximately 45 min. After nullifying the cell suspension with Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12; Sigma, USA, D6421), it was passed through a 70 μM filter and centrifuged. Mononuclear cells were isolated via Ficoll 400 (Sigma, USA, F2637), washed in PBS, and cultured in DMEM/F12 comprising of 10 % fetal bovine serum (FBS; Gibco, USA, 16000-044) and 1 % Penicillin/Streptomycin (50 U/mL). Cells were propagated at 37 °C and 5 % CO2.

Electrospinning and Fabrication of PCL Scaffolds

Poly ε-caprolactone (MW: 80,000 w, Sigma-Aldrich) was dissolved in chloroform/methanol 4:1 (v/v) and a 10 % (wt) PCL solution was obtained. The polymer solution was loaded into a 5-mL plastic syringe. A grounded drum collector was placed at a 12-cm distance from the cannula tip. A high voltage generator producing 20 kV was attached to the syringe, and the PCL mats were woven on aluminum foil with an ejecting rate of 1 mL/h at 30 °C.

Cell Seeding of hEnSCs on PCL Scaffolds

PCL scaffolds were cut into 3 mm pieces and sterilized overnight with ultraviolet light. Human EnSCs were digested with 2× trypsin for 5 min at 37 °C and resuspended in DMEM/F12 media. The cells were then seeded onto the prepared PCL scaffolds with a density of 1 × 10⁶ cells/scaffold and incubated in DMEM/F12 at 37 °C and 5 % CO2 for 2 h before being transplanted.

SEM

Scanning electron microscopy (SEM) was performed on PCL scaffolds seeded with hEnSCs. The scaffolds were fixed by incubation with 2.5 % glutaraldehyde for 1 h followed by a series of dehydration with an incremental concentration of ethanol at 37 °C. The cell-containing scaffolds were then coated with gold using sputter coater. Images were obtained using a scanning electron microscope (Philips XL-30, Netherland) operated at 25 kV.

Surgical Procedure

Twenty-seven male Sprague-Dawley (SD) rats weighing an average of 250 g were used for surgical procedures. All rats were individually caged, received food, and water ad libitum and were kept under 12-h light-dark cycles. Animal care, handling, and surgical procedures were performed in accordance with the institutional guidelines of Tehran University of Medical Sciences for animal care. Rats were anesthetized by intraperitoneal (IP) injection of Xylazine (5 mg/kg) and Ketamine (100 mg/kg). After dorsal skin was cleaned with betadine solution and shaved, a midline incision was made and a T9 laminectomy was performed. The exposed dura was incised and pulled laterally, followed by a T9 dorsal hemisection. The PCL scaffolds with or without cells were

**Fig. 4** Hindlimb movements were evaluated using the 21-point BBB rating scale for 8 weeks. Best results were obtained in rats treated with PCL + hEnSC + Crocin. All results were expressed as means ± standard deviation (SD). (*, P < 0.05, one-way ANOVA, n = 3)
implanted into the lesion sites and muscle, and skins were sutured individually using absorbable vicryl suture. All rats were randomly divided into 7 groups: (i) sham-operated group: laminectomy without SCI (referred as sham), (ii) control group: SCI without treatment (referred as SCI), (iii) SCI with PCL without cell (referred as PCL), (iv) SCI with PCL without cell that received IP injection of 20 mg/kg Crocin for 15 consecutive days [37] (referred as PCL/Crocin), (v) SCI without PCL that received IP injection of 20 mg/kg Crocin for 15 consecutive days (referred as Crocin), (vi) SCI with PCL seeded with hEnSC (referred as PCL/hEnSC), and (vii) SCI with PCL seeded with hEnSC that received IP injection of 20 mg/kg Crocin for 15 consecutive days (referred as PCL/hEnSC/Crocin). Post-operative care for all animals included, IP injection of Cefazoline (1 mg/d) for 7 days and manual bladder expression twice daily until the animal regained control of bladder function.

**Hind Limb Motor Function Assessment**

Basso-Beattie-Bresnahan (BBB) open-field-gait assessment is a 21-point scale developed to assess hindlimb locomotor function of rats after spinal cord injury [38].

![Fig. 5 Immunohistochemical staining of neurofilaments (NF-H) for spinal cords at 8 weeks post-surgery.](image-url)
The tests were performed by two observers blinded to the groups at weekly time points for 8 weeks. BBB scores were averaged from both hindlimbs and final scores were averaged across the observers.

**Tissue Fixation and Histological Preparation**

For histopathological analysis of the tissue damage and inflammation progression, all rats were euthanized via deep anesthesia (pentobarbital 50 mg/kg IP) at the completion of the 8-week study. The animals were transcardially perfused with 100 mL ice cold PBS followed by 100 mL 4 % paraformaldehyde (pH 7.4). Dissected spinal column and cord were post-fixed in 4 % paraformaldehyde for 2 days at 4 °C. The spinal cords were then separated from the spinal column and post-fixed for another 2 days in 4 % paraformaldehyde. Sections encompassing the lesion were cut and after tissue processing were embedded in paraffin. Serial transversal and longitudinal sections were cut using a microtome and mounted on glass slides.

**Luxol-Fast Blue Staining**

Myelin was assessed in the tissue sections via luxol-fast blue (LFB) staining. Sections 200 μm caudal from the largest lesion area were selected and used for analysis. Sections were de-waxed and cleared in ethanol solutions (100 and 95 %) followed by staining with LFB staining solution at 60 °C. The excess dye was rinsed with 95 % ethanol followed by differentiation of the tissue in lithium carbonate and final rinsing with 70 % ethanol. Integrity of the dorsal funiculi was assessed with intact tissue determined as myelinated and without scar.

**Immunohistochemistry**

Immunohistochemical analysis of the spinal cord tissue was performed for neuron marker NF-H and pro-inflammatory marker TNF-α. Sections were permeabilized with 0.2 % Triton X-100 (Sigma, USA, T8787) in TBS for 30 min at room temperature followed by 30 min incubation with 5 % BSA in TBS to minimize non-specific adsorption. Slides were then treated with 5 % BSA in TBS with primary antibodies against NF-H (Anti-300 kD neurofilament heavy antibody-neuronal marker; ab8135, Abcam, USA, 1/200) and TNF-α (Anti-TNF alpha antibody; ab9635, Abcam, USA, 1/200) overnight at 4 °C. Sections were washed twice with TBS. Endogenous peroxidase activity was blocked by incubating in 0.3 % hydrogen peroxidase in TBS for 15 min. Then, sections were incubated with secondary antibody goat anti-mouse peroxidase IgG (ab6823, Abcam, USA, 1/500) for 1 h at room temperature. Sections were imaged with a Zeiss LSM 510 Meta microscope.

<table>
<thead>
<tr>
<th>Image</th>
<th>Volume of NF-H+ tissue (μm³)</th>
<th>Percentage of NF-H+ tissue</th>
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<tr>
<td>a</td>
<td>10⁵</td>
<td>3.41 %</td>
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<td>b</td>
<td>3.75 x 10⁵</td>
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<td>c</td>
<td>6.35 x 10⁵</td>
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<td>d</td>
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<td>e</td>
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<td>13.87 x 10⁵</td>
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Fig. 6  a Data obtained from stereological analysis of spinal cord sections for volume estimation of NF-H positive tissue according to the principle of Cavalieri using a test-point counting grid. All results are expressed as mean volume (n = 3). b Formula used for volume estimation. V is the volume of NF-H positive tissue; t is the section thickness; a/p is the interpoint area; P is the number of points touching the NF-H positive tissue

Fig. 7 Luxol fast blue staining of spinal cord sections at 2-weeks post-surgery. a Section from rats in sham group b Section from rats in SCI group. Representative images are shown
Statistical Analysis

To evaluate functional outcome, the BBB test was used to compare groups using one-way ANOVA followed by unpaired student’s t test. P values less than 0.05 were considered significant. All results in this study were expressed as means ± standard deviation (SD) from at least three independent experiments.

Results

Characterization of Isolated hEnSCs

After three passages, flow cytometry results confirmed that isolated cells were indeed hEnSCs. Results showed that isolated cells expressed mesenchymal stem cell markers CD90 and CD105 and endometrial stem cell marker CD146.

Fig. 8 Luxol fast blue staining of spinal cord sections at 8-weeks post-surgery. a SCI. b Crocin. c PCL. d PCL + Crocin. e PCL + EnSC. f PCL + EnSC + Crocin. g Sham. Images confirm that hEnSCs have a significant impact on remyelination of damaged neurons. Representative images for all groups are shown.

Fig. 9 Immunohistochemical staining of TNF-α at 2 weeks post-surgery. a, b Section from rats in SCI group. c, d Sections from rats in Crocin group. Images show that receiving 2 weeks of Crocin resulted in significantly lower expression of TNF-α. Representative images are shown.
However, they were negative for hematopoietic stem cell marker CD34 and endothelial marker CD31 (Fig. 1).

**Morphology of hEnSC-Derived Neuron-like Cells Cultured on PCL Scaffolds**

PCL scaffolds were randomly entangled to form a strong, flexible, and porous three-dimensional matrix Fig. 2. Scanning electron micrographs demonstrated the strong attachment of hEnSC to the PCL nanofibrous scaffold. The cells efficiently adhered, spread, and grew on the scaffolds. The attained results confirmed the biocompatibility of the PCL scaffolds as a bridging structure for neural tissue repair Fig. 3a, b.

**Basso-Beattie-Bresnahan Hindlimb Analysis**

Behavioral analysis was performed every week using the 21-point BBB scale to assess hindlimb function. Day 1 post hemisection all rats had a score of 0. Locomotor function showed incremental progress however, the first 4 weeks demonstrated the most increase suggesting the importance of therapeutic intervention in the initial weeks post injury. The rats in groups that had received PCL

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![Immunohistochemical staining of TNF-α at 8 weeks post-surgery. a SCI. b PCL. c PCL + EnSC. d PCL + Crocin. e Crocin. f PCL + EnSC + Crocin. (g) Sham. Groups that received Crocin demonstrated a lower degree of inflammation. Red arrows indicate cavity formation and apoptosis. Representative images for all groups are shown.](image-url)
transplants showed a significantly higher progress in hindlimb function compared to rats in Crocin and control group. Furthermore, the BBB scores of rats who had received PCL transplants seeded with hEnSCs treated with Crocin showed the highest recovery rate. Treatment with Crocin alone resulted in the rapid improvement of motor function throughout the first 5 weeks. Thereafter BBB scores reached a plateau Fig. 4.

Morphological and Immunohistochemical Study

Immunohistochemical staining of specimens in 8 weeks post-operation revealed that PCL grafts had successfully integrated into the lesion site. Neuronal regeneration was assessed via immunohistochemical staining for the neuron marker NF-H. Results at 8 weeks revealed that linear fibrous tissue had increased in the PCL/hEnSC/Crocin-treated and PCL/hEnSC groups compared to other groups. PCL as a bridging structure demonstrated a positive impact on the density of NF-positive neural fibers Fig. 5. Moreover, results show that NF-positive-fibers had extended into the PCL structure further increasing the assimilation of the scaffold into the spinal cord tissue Fig. 5h. Stereological analysis of the images for volume estimation of NF-H positive tissue according to the principle of Cavalieri using a test-point counting grid quantitatively confirmed these findings Fig. 6. To further evaluate the neural fibers, LFB staining was performed Fig. 7. Qualitative analysis of images was as expected demonstrating that the extent of remyelination was significantly higher in groups treated with hEnSCs. The density of myelinated fibers was greater in the PCL/hEnSC/Crocin group compared to PCL/hEnSC which could be related to the attenuating effect of Crocin on secondary response Fig. 8. Results from NF-H staining and LFB were in alignment with the results attained from behavioral tests and confirmed that rats treated with PCL/hEnSC/Crocin demonstrated the highest improvement. Post-trauma inflammation was qualitatively assessed by immunohistochemical staining for TNF-α as a regulator of inflammatory response Fig. 9. All rats that had received 15 days of Crocin treatment demonstrated less expression of TNF-α. Figure 10d–f. Specimens obtained from rats that were subjected to PCL/hEnSC/Crocin manifested the least inflammatory cells followed by lower degree of apoptosis and necrosis with more efficient regeneration Fig. 10f. The results confirmed the neuroprotective effect of Crocin on damaged neurons.

Discussion

Spinal cord injury as an outcome of physical trauma and the ensuing secondary injury cascade results in the death of neurons, axonal breakage, and demyelination [3, 4]. The limited regenerative capacity of impaired neurons is hindered by hemorrhagic necrosis, immune response, inflammation, and subsequent cavity formation [6–8]. Managing this secondary injury cascade is a crucial target of SCI research. Recent development of regenerative medicine research has set the foundation for deploying stem cells as a therapeutic approach in spinal cord injury. Stem cells can promote neuronal regeneration by differentiating into neurons and secreting neurotrophic cytokines [16, 19]. Different types of stem cells that have been used in spinal cord repair include neural stem cells (NSCs) and mesenchymal stem cells (MSCs). Previous studies have confirmed the effect of MSCs on inhibiting necrosis, promoting axonal regeneration, and remyelination [24]. However, host immune response and lack of sufficient neural differentiation have hindered the use of these stem cells [25].

Eight weeks post-surgery neuronal regeneration characterized by NF-H expression was mostly apparent in spinal cord of subjects treated with PCL/hEnSC/Crocin and PCL/hEnSC. PCL as a bridging structure exerted impact on guiding axon growth through the lesion into the distal tissue. Furthermore, SEM images revealed how hEnSCs efficiently adhered and grew on the PCL nanofibrous scaffolds. NF positive fibers efficaciously infiltrated the PCL structure further increasing the assimilation of the scaffold into the spinal cord tissue. Choosing an efficient biomaterial as the bridging construct is crucial for tissue engineering approaches. Our results confirmed that PCL is a synthetic biocompatible polymer that is suitable for spinal cord tissue engineering.

LFB staining was performed to further assess the fibers. The extent of remyelination was higher in groups treated with hEnSCs. Moreover, rats that were concurrently treated with hEnSCs and Crocin exhibited the most density of myelinated fibers. In high-stress SCI conditions, endoplasmic reticulum (ER) stress contributes to the inflammatory demyelination and neurodegeneration of damaged neurons [39]. Crocin can co-regulate neuro-inflammation and ER stress to prevent demyelination and neurodegeneration [36]. Observations from this study provide evidence for the augmenting effect of Crocin on myelination. The ability to prevent demyelination may be the reason why rats treated with PCL/hEnSC/Crocin exhibit higher amounts of myelinated axons compared to those treated with PCL/hEnSC.

BBB scores revealed that locomotor function attained the highest improvement in the rats treated with PCL/hEnSC/Crocin. The acute phase of secondary injury can be temporally divided to the early acute (2–48 h) and subacute (2 days–2 weeks) phase. Subacute phase is the time period in which
cell-based therapeutic strategies may be successfully applied [40]. Obtained data from the BBB locomotor analysis demonstrated and confirmed the importance of intervention in the first 2–4 weeks post-injury. Results from NF-H staining and LFB were in alignment with the results attained from behavioral tests and confirmed that rats treated with PCL/hEnSC/Crocin demonstrated the best results with higher recovery rate in the first 5 weeks.

Crocin has the ability to attenuate post-trauma inflammatory response by suppressing pro-inflammatory mediators such as TNF-α which is strongly upregulated at the lesion site and the subsequent glutamate-mediated neuronal cell death [31, 34]. Observations from immunohistochemical staining of spinal cords for TNF-α after 2 weeks of crocin administration indicated a significant decrease in TNF-α expression designating an anti-inflammatory role for crocin. Concurrent treatment with PCL/hEnSC/Crocin exerted the greatest effect resulting in decreased inflammation and necrosis and increased regeneration efficiency. The results suggest a neuroprotective role for crocin in the injured spinal cord.

In conclusion, the present study shows that implantation of hEnSCs seeded on PCL nanofibrous mats into a dorsally hemisected SCI rat model can attenuate secondary response and promote neuronal regeneration, axonal remyelination, and motor function recovery. Furthermore, concurrent administration of crocin as an anti-inflammatory and neuroprotective mediator may suppress the neurodegenerative process and help improve neuronal regeneration. These results may have a promising impact on future therapeutic approaches towards spinal cord injury.

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