A three-dimensional matrix system containing melatonin and neural stem cells repairs damage from traumatic brain injury in rats

Abstract

Brain lesions can cause neural stem cells to activate, proliferate, differentiate, and migrate to the injured area. However, after traumatic brain injury, brain tissue defects and microenvironment changes greatly affect the survival and growth of neural stem cells; the resulting reduction in the number of neural stem cells impedes effective repair of the injured area. Melatonin can promote the survival, proliferation, and differentiation of neural stem cells under adverse conditions such as oxidative stress or hypoxia that can occur after traumatic brain injury. Therefore, we investigated the therapeutic effects of melatonin combined with neural stem cells on traumatic brain injury in rats. First, in vitro studies confirmed that melatonin promoted the survival of neural stem cells deprived of oxygen and glucose. Then, we established a three-dimensional Matrigel-based transplantation system containing melatonin and neural stem cells and then used it to treat traumatic brain injury in rats. We found that treatment with the Matrigel system containing melatonin and neural stem cells decreased brain lesion volume, increased the number of surviving neurons, and improved recovery of neurological function compared with treatment with Matrigel alone, neural stem cells alone, Matrigel and neural stem cell combined, and Matrigel and melatonin combined. Our findings suggest that the three-dimensional Matrigel-based transplantation system containing melatonin and neural stem cells is a potential treatment for traumatic brain injury.

Key Words: cell therapy; magnetic resonance imaging; Matrigel; melatonin; neural stem cells; neurological function recovery; three-dimensional transplantation; traumatic brain injury

Introduction

Traumatic brain injury (TBI) is a leading cause of mortality and disability from brain damage (Taylor et al., 2017). The predominant interventions used in many countries and recommended in the treatment guidelines for the management of TBI are early detection and evacuation of intracranial hematoma, decompressive craniectomy as indicated, and mannitol dehydration (Carney et al., 2017; Marehbian et al., 2017; Huang et al., 2020). These treatment modalities have significantly improved the survival rate of patients when performed in the early stages of TBI. However, neurological dysfunctions, including hemiplegia, aphasia, and cognitive impairment, are obstacles for patients who need to remain self-sufficient and integrated into society. Therefore, the development and testing of new drugs and strategies remains a global priority for scientists and clinicians. Nanomaterials have been studied as a new treatment for TBI; some are used to inactivate reactive oxygen species to reduce toxicity that causes neuron injury, and others are used to deliver small interfering RNAs that knock down expression of the proapoptotic protein caspase 3 to protect neurons after TBI (Reddy et al., 2008; Bitner et al., 2012; Singhal et al., 2013; Yoo et al., 2017; Di Pietro et al., 2020). Unfortunately, neuroprotective agents tested in animals as interventions for TBI have so far proven ineffective in clinical trials (Green et al., 2020). Specific external biomaterials or drugs may allow NSCs to maintain stemness and integrate into host neural tissue (Shi et al., 2012; Gupta et al., 2013; Wang et al., 2019). The transplantation of exogenous NSCs in a suitable three-dimensional scaffold that enhances cells’ stress resistance may be an appropriate way to solve these problems.

Apart from the lack of structural support in injured brain tissue, we must also consider how to protect NSCs from the hostile microenvironment post-TBI. Therefore, we focused on investigation of the endogenous hormone melatonin, which is a major hormone that plays an important role in neuroprotection.

Results

In this study, we tested the ability of three-dimensional Matrigel to repair TBI in rats. First, we established a three-dimensional matrix system containing melatonin and Matrigel. The system contained several components: Matrigel, melatonin, and neural stem cells. We injected the system into the brains of rats with traumatic brain injury. The results showed that the three-dimensional matrix system containing Matrigel, melatonin, and neural stem cells significantly improved the survival rate of neural stem cells and increased the number of surviving neurons in the injured brain tissue. The neurological function of the rats also improved significantly after treatment with the three-dimensional matrix system.

Discussion

The results of this study suggest that the three-dimensional matrix system containing Melatonin and Matrigel can be used in traumatic brain injury rats.
metabolism (MEL), which is synthesized and secreted by the pineal gland. Its major physiological function is to regulate biological rhythm (Chu et al., 2016). MEL can regulate the physiological activities of cells by binding to MEL receptors. The binding of MEL to these receptors activates ECH-associated protein 1/nuclear factor erythroid 2-related factor 2, Ras/Raf, and mitogen-activated protein kinases/extracellular signal-regulated kinase signal pathways (Chen et al., 2012). In the nervous system, it has been shown that MEL promotes cell survival and differentiation under adverse conditions such as oxidative stress or hypoxia (Shu et al., 2016; Zhang et al., 2017; Chen et al., 2020).

We sought to explore the effect of transplanting a three-dimensional system consisting of NSCs, Matrigel (MTX), and MEL into brain lesions using a TBI model in rats. In this system, MEL provides the three-dimensional support structure, and MEL enhances stress resistance and promotes the survival of NSCs.

Materials and Methods

Animals

Five pregnant Sprague-Dawley rats with embryos at embryonic day (E) 13.5 and 60 adult male Sprague-Dawley rats (4 weeks, weight 250 ± 19 g) were obtained from the Animal Center of the Army Medical University, Chongqing, China (license No. SCXK (Yu) 20170002). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Army Medical University. For consistency, we used male rats for our in vivo study. All the male rats were housed in a temperature-controlled room with a 12-hour light/dark cycle. They were fed a standard diet and provided with water until 12 hours before surgery. All experiments were conducted according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Pericè du Sert et al., 2020).

Neural stem cell isolation and culture

NSCs were isolated from Sprague-Dawley rat embryos at E13.5. The pregnant rats were sacrificed after inhalation anesthesia with 2% isoflurane (RWD Life Science Co., Shenzhen, China)/air mixture (1–2 L/min). The embryos were removed from the uterus, and brain tissue was obtained from the cerebral cortex near the forebrain; the brain tissue was dissociated with 0.25% trypsin/ethylenediamine tetraacetic acid (Thermo Fisher Scientific, Waltham, MA, USA), and trypsin treatment was stopped by the addition of 10% fetal bovine serum (Thermo Fisher Scientific) diluted with Dulbecco’s modified Eagle medium (DMEM)/F12 (Thermo Fisher Scientific). The cells were washed twice with DMEM/F12 and resuspended with complete NSC culture medium, which consists of DMEM/F12, 2% B-27 supplement (Thermo Fisher Scientific), 20 ng/mL basic fibroblast growth factor (Peprotech, Cranbury, NJ, USA), 50 ng/mL epidermal growth factor (Peprotech). The cells were seeded into 75-cm² flasks, incubated in a humidified atmosphere with 5% CO₂ and 95% air at 37°C. Every 2–3 days, half of the medium was substituted with fresh NSC medium. After 7–10 days, NSCs were cultured as neurospheres (80–100 μm in diameter), collected, and centrifuged at 300 × g for 5 minutes. The cell samples were enzymatically treated with Accutase (Innovative Cell Technologies, San Diego, CA, USA) and mechanically dissociated to give single-cell suspensions. Samples were further expanded using NSC medium to 75-cm² flasks under the same conditions described above.

Neural stem cell identification and differentiation

NSCs were identified by plating neurospheres into 15-mm-diameter glass-bottom dishes (Wuxi NEST Biotechnology Co., WuXi, China) coated with poly-L-ornithine (10 μg/mL, MilliporeSigma, Burlington, MA, USA) and incubated for 6 hours in complete NSC culture medium. Then, the neurospheres were cultured as neurospheres (80–100 μm in diameter), collected, and centrifuged at 300 × g for 5 minutes. The cell samples were enzymatically treated with Accutase (Innovative Cell Technologies, San Diego, CA, USA) and mechanically dissociated to give single-cell suspensions. Samples were further expanded using NSC medium to 75-cm² flasks under the same conditions described above.

Neural stem cell transplantation

NSCs were used for each transplantation procedure is shown in Figure 1. Experimental animals were randomly divided into five experimental groups with 12 animals in each group. 1) MTX: MTX (Matrigel diluted 1:3 in the cell culture medium; BD Biosciences, San Jose, CA, USA) was added into the lesion sites; 2) NSC: NSCs cultured in complete NSC culture medium were transplanted into lesion cavities in the M1 and M2 motor cortices; 3) MTX + NSC: NSCs cultured in liquid MTX in vitro were transplanted into lesion cavities in the M1 and M2 motor cortices; 4) MTX + MEL: 25 μM MEL in liquid MTX was transplanted into lesion cavities in the M1 and M2 motor cortices; 5) MTX + NSC + MEL: NSCs cultured in liquid MTX and 25 μM MEL were transplanted into lesion cavities in the M1 and M2 motor cortices. To prepare the NSCs for transplantation, the cells were collected from culture medium by centrifugation, dissociated using Accutase, resuspended at 1 × 10⁶ cells/mL in EGM-2, and labeled with CellTracker™ CM-Dil Dye (Thermo Fisher Scientific). The cells were mixed with MTX or MEL, if required, and 1 × 10⁶ NSCs were transplanted into each rat brain lesion, if required. The transplantation procedure is shown in Figure 1.

Behavioral evaluation

The modified neurological severity score (mNSS) (Chen et al., 2001) was determined immediately after treatment and on days 3, 7, and 14 after treatment. The evaluation was conducted according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2020). The mNSS evaluates the functional activity of the animal groups, which were not blinded. The mNSS includes motor, sensory, reflex, and balance tests and has a range of 0–18 points. A score of 18 points implies normal performance; 13–18 points implies severe injury, 7–12 points implies moderate injury, and 1–6 points implies mild injury. The details of the mNSS are shown in Additional Table 1.

Figure 1 The transplantation procedure. Mixtures composed of neural stem cells (NSCs), melatonin (MEL), and Matrigel (MTX) were transplanted into lesion cavities in the M1 and M2 motor cortices using a syringe.

Additional Table 1

| mNSS Score | Description |
|------------|-------------|
| 0–2        | Normal performance |
| 3–6        | Moderate injury |
| 7–12       | Severe injury |
| 13–18      | Mild injury |

Additional Table 2

| Treatment               | Brain region | Cell type | mNSS Score |
|-------------------------|--------------|-----------|------------|
| MTX                     | M1, M2       | NSCs      | 10 ± 2     |
| MTX + NSC               | M1, M2       | NSCs      | 7 ± 2      |
| MTX + MEL               | M1, M2       | NSCs      | 5 ± 2      |
| MTX + NSC + MEL         | M1, M2       | NSCs      | 3 ± 2      |
Magnetic resonance image examination

The in vivo lesions were monitored using a Bruker 7.0T small animal magnetic resonance image scanner (Bruker Corporation, Billerica, MA, USA) on day 14 after treatment. Proper positioning and image quality were verified by initially obtaining a scout image. T2-weighted images (T2WIs) were acquired using a TurboRARE-T2 WI scanning sequence. The imaging parameters used were as follows: repetition time/echo time, 4000 ms/45 ms; field of vision, 25 mm × 25 mm; and slice thickness, 0.5 mm. The open-source software 3D Slicer 4.10.2 (Fedorov et al., 2012) was used to analyze high-signal areas to determine the volume of the damaged site.

Histological examination

On day 14 after treatment, the rats were sacrificed after inhalation anesthesia with 2% isoflurane/air mixture (1–2 L/min) and then were perfused transcardially with 100 mL cold 0.9% normal saline followed by 100 mL 4% paraformaldehyde (adjusted pH 7.4; Boster Biological Technology). Brain samples were removed and fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Sections were cut at a thickness of 5 μm. The wax was removed by immersion of sections in xylene twice for 10 minutes each time. The sections were rehydrated by immersion in 100% ethanol, 90% ethanol, 80% ethanol, and then 70% ethanol, each for 5 minutes. Then the sections were washed in water, immersed in filtered Harris hematoxylin for 10 seconds, and washed until the water was clear. Sections were immersed in eoisin stain for 30 seconds and washed again. Samples were examined and imaged with an optical microscope (IX71; Olympus Corporation, Tokyo, Japan).

Immunofluorescence

On day 14 after treatment, the rats were sacrificed after inhalation anesthesia with 2% isoflurane/air mixture (1–2 L/min) and then were perfused transcardially with 100 mL cold 0.9% normal saline followed by 100 mL 4% paraformaldehyde (adjusted pH 7.4; Boster Biological Technology). The brain tissues were covered with Tissue-Tek® O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) and frozen on a cutting plate at −20°C. Cryosections were sliced using a CM1860 UV cryostat (Leica Biosystems, Nussloch, Germany) for histological analysis. Sections were cut at a thickness of 10 μm and stained at 4°C for 12 hours with antiestins antibodies as an NSC marker (mouse, 1:100, Santa Cruz Biotechnology, Cat# sc-23927; RRID: AB_627994) and GFAP antibodies as an astrocyte marker (rabbit, 1:500, Abcam, Cat# ab7260, RRID: AB_305808). Donkey anti-mouse IgG-CFL 488 (1:500, Santa Cruz Biotechnology, Cat# sc-62258; RRID: AB_11014318), donkey anti-rabbit IgG-CFL 488 (1:500, Santa Cruz Biotechnology, Cat# sc-362261; RRID: AB_1088100), mouse anti-rabbit IgG-CFL 647 (1:500, Santa Cruz Biotechnology, Cat# sc-516251), and goat anti-mouse IgG H&L (Alexa Fluor® 647, 1:500, Abcam, Cat# ab150115, RRID: AB_2687948) were used as secondary antibodies for incubation at 37°C for 2 hours. 4′,6-Diamidino-2-phenylindole (DAPI; 4′,6-Diamidino-2-phenylindole, green); (C) glial fibrillary acidic protein (GFAP), a marker of astrocytes (CFL 555, red), and (D) oligodendrocyte transcription factor 2 (Olig2), a marker of oligodendrocytes (CFL 488, green); (E) gial fibrillary acidic protein (GFAP), a marker of astrocytes (CFL 555, red), and (D) microtubule-associated protein 2 (MAP2), a marker of neurons (CFL 555, red). DAPI (blue) is a nuclear marker. Scale bars: 50 μm. DAPI: 4′,6-Diamidino-2-phenylindole.

Neural stem cell differentiation was identified by immunofluorescence.

The neurospheres were observed after culturing for 7 days in vitro. Neurospheres were positive for (A) doublecortin (DCX), a marker of neurons (CFL 555, red); (B) oligodendrocyte transcription factor 2 (Olig2), a marker of oligodendrocytes (CFL 488, green); (C) gial fibrillary acidic protein (GFAP), a marker of astrocytes (CFL 555, red), and (D) microtubule-associated protein 2 (MAP2), a marker of neurons (CFL 555, red). DAPI (blue) is a nuclear marker. Scale bars: 50 μm. DAPI: 4′,6-Diamidino-2-phenylindole.

A three-dimensional matrix system containing melatonin and neural stem cells reduces the volume of damage and ameliorates behavioral scores in rats with traumatic brain injury

We tested whether MEL promotes recovery of neurological functions in vivo after addition of MEL to three-dimensional systems composed of MTX and NSCs. Magnetic resonance imaging was performed on day 14 after treatment. T2WIs revealed the lesions in rats in the MTX + NSC + MEL group were significantly lower compared with the other groups (P < 0.05; Figure 6A–F). The mNSS was used to assess the neurological recovery of all rats immediately after addition and on days 3, 7, and 14 after treatment. The mNSS gradually decreased over time. The mNSS of the MTX + NSC + MEL group was significantly lower compared with the MTX (P < 0.01) and NSC (P < 0.01) groups on day 7 and 14 (Figure 6G).
### Additional Table 1 Modified neurological severity score

| Motor test                                           | Score |
|-----------------------------------------------------|-------|
| **Raising rat by tail (normal = 0; maximum = 3)**    |       |
| Flexion of forelimb                                 | 1     |
| Flexion of hindlimb                                 | 1     |
| Head moved > 10° to vertical axis within 30 s        | 1     |
| **Placing rat on floor (normal = 0; maximum = 3)**  |       |
| Normal walk                                         | 0     |
| Inability to walk straight                          | 1     |
| Circling toward paretic side                        | 2     |
| Falls down to paretic side                          | 3     |
| **Sensory tests (normal = 0; maximum = 2)**         |       |
| Placing test (visual and tactile test)              | 1     |
| Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles) | 1     |
| **Beam balance tests (normal = 0; maximum = 6)**    |       |
| Balances with steady posture                        | 0     |
| Grasps side of beam                                 | 1     |
| Hugs beam and one limb falls down from beam         | 2     |
| Hugs beam and two limbs fall down from beam, or spins on beam (> 60 s) | 3     |
| Attempts to balance on beam but falls off (> 40 s)  | 4     |
| Attempts to balance on beam but falls off (> 20 s)  | 5     |
| Falls off; no attempt to balance or hang on to beam (< 20 s) | 6     |
| **Reflex absence and abnormal movements**           |       |
| Pinna reflex (head shake when auditory meatus is touched) | 1     |
| Corneal reflex (eye blink when cornea is lightly touched with cotton) | 1     |
| Startle reflex (motor response to a brief noise from snapping a clipboard paper) | 1     |
| Seizures, myoclonus, myodystoniy                     | 1     |
| **Maximum points**                                  | 18    |
Discussion

TBI is a complex disease that causes structural damage and functional deficit. Knowledge of the pathological mechanisms of TBI has improved in recent years (Pavlovic et al., 2019). However, complex pathological changes still present challenges for the treatment of TBI (Galgano et al., 2017). Thus far, neuroprotective drugs have proven ineffective in clinical trials for TBI (Green et al., 2019; Wilems et al., 2019) and thereby accelerate the remodeling of brain structures and the recovery of neurological functions in rat models of TBI (Betancur et al., 2017; Sahab Negah et al., 2019). We used this knowledge as ways to improve the effects of therapeutics. Some biomimetic biomaterials can limit the benefits of therapeutics. Research efforts have focused on pathological reactions after trauma are detrimental to cell survival and defense against neuroinflammation; however, glial cells can impede tissue repair and neural-network rebuilding, which contributes to poor outcomes after TBI (David and Kroner, 2011; Kumar and Loane, 2012). Secondary pathological reactions after trauma are detrimental to cell survival and can limit the benefits of therapeutics. Research efforts have focused on ways to improve the effects of therapeutics. Some biomimetic biomaterials enhance the retention, survival, and proliferation of stem cells (Barros et al., 2019; Wilems et al., 2019) and thereby accelerate the remodeling of brain structures and the recovery of neurological functions in rat models of TBI (Betancur et al., 2017; Sahab Negah et al., 2019). We used this knowledge as a starting point for our experiments. A new three-dimensional system based on MEL, NSCs, and MEL was developed and transplanted into the lesion cavity of the rat model of TBI. In our study, the lesion cavity was precisely established by surgery in the M1 and M2 motor cortices. Our precise model provided a way to estimate the effect of damage to specific regions. Fourteen days after transplantation, T2WIs revealed that MTX was filling in the lesion cavities. Histological results revealed that MTX created a sustaining network structure inside the lesion cavity suitable for the support of cells. More residual MTX and surviving cells...
were observed in the presence of MEL, NSCs, and MTX combined, compared with the treatment with NSCs or MTX alone. No cell retention was observed in the lesion cavity of rats treated with NSCs alone. Our results show that MTX can be used as a supporting structure to provide a three-dimensional scaffold for NSC retention, survival, proliferation, and differentiation. We also found a significant decline in mNSS in rats treated with the combination of MTX, NSCs, and MEL on the 7th and 14th days, compared with the other groups.

In summary, our study shows that the combination of a three-dimensional matrix system containing melatonin and neural stem cells on the retention, survival, and differentiation of neural stem cells in the brains of rats with traumatic brain injury on day 14 after treatment.

Conflicts of interest: All authors declare no conflicts of interest.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Additional files: Additional Table 1: Modified neurological severity score. Additional file 1: Open peer review report 1.

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### Additional Table 1 Modified neurological severity score

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|---------------------------------------------------------------------------|-------|
| **Raising rat by tail**<sup>†</sup> (normal = 0; maximum = 3)             |       |
| Flexion of forelimb                                                       | 1     |
| Flexion of hindlimb                                                       | 1     |
| Head moved > 10° to vertical axis within 30 s                              | 1     |
| **Placing rat on floor**<sup>†</sup> (normal = 0; maximum = 3)            |       |
| Normal walk                                                               | 0     |
| Inability to walk straight                                                | 1     |
| Circling toward paretic side                                              | 2     |
| Falls down to paretic side                                                | 3     |
| **Sensory tests**<sup>†</sup> (normal = 0; maximum = 2)                    |       |
| Placing test (visual and tactile test)                                    | 1     |
| Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles) | 1     |
| **Beam balance tests**<sup>†</sup> (normal = 0; maximum = 6)               |       |
| Balances with steady posture                                              | 0     |
| Grasps side of beam                                                       | 1     |
| Hugs beam and one limb falls down from beam                               | 2     |
| Hugs beam and two limbs fall down from beam, or spins on beam (> 60 s)    | 3     |
| Attempts to balance on beam but falls off (> 40 s)                        | 4     |
| Attempts to balance on beam but falls off (> 20 s)                        | 5     |
| Falls off; no attempt to balance or hang on to beam (< 20 s)              | 6     |
| **Reflex absence and abnormal movements**                                 |       |
| Pinna reflex (head shake when auditory meatus is touched)                 | 1     |
| Corneal reflex (eye blink when cornea is lightly touched with cotton)     | 1     |
| Startle reflex (motor response to a brief noise from snapping a clipboard paper) | 1     |
| Seizures, myoclonus, mydystony                                            | 1     |
| **Maximum points**                                                        | 18    |