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Implantation of adipose-derived stem cells cures the optic nerve injury on rats through inhibiting the expression of inflammation factors in the TLR4 signaling pathway

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Abstract. – OBJECTIVE: The use of adipose-derived stem cells (ADSCs) to cure the optic nerve injury was never shown previously. Here, we implanted purified ADSCs into optic nerve injury of rats.

MATERIALS AND METHODS: Male Sprague Dawley (SD) rats were used in this study. The vision degeneration was detected by Flash-visual evoked potential (F-VEP) assay. The expression of Macrophage-1 (Mac-1), myeloid differentiation factor 88 (MyD88), and nuclear transcription factor-κB (NF-κB) were studied by Western blot. The expression of interleukin (IL)-6 and tumor necrosis factor (TNF)-α in the optical nerve lysates were assessed by enzyme-linked immunosorbent assay (ELISA).

RESULTS: We found out that ADSC implantation inhibits the amplitude decrease and latency increase of the P1 wave caused by the optic nerve injury. The expression of the inflammation associated proteins of the toll-like receptor 4 (TLR4) signaling pathway, including Mac-1, MyD88, NF-κB, IL-6, and TNF-α, were inhibited in the ADSC therapy group compared to the control group.

CONCLUSIONS: Our results indicated that ADSC implantation can inhibit the inflammation after the optic nerve injury and improve the functional vision impairment. These findings suggested ADSC implantation as a translational therapy method for optic nerve injury in clinics.

Key Words:
Adipose-derived stem cells, Optic nerve injury, TLR4 signaling pathway.

Abbreviations

ADSCs = adipose-derived stem cells; F-VEP = flash-visual evoked potential; TLR4 = toll-like receptor 4; Mac-1 = macrophage-1; MyD88 = myeloid differentiation factor 88; NF-κB = nuclear transcription factor-κB; IL = interleukin; TNF = tumor necrosis factor; POH = progressive osseous heteroplasia; PBS = phosphate-buffered saline; DMEM = Dulbecco's modified Eagle's medium; FBS = fetal bovine serum; PFA = paraformaldehyde; HRP = horseradish peroxidase; ISCEV = International Society for Clinical Electrophysiology of Vision; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA = enzyme-linked immunosorbent assay; SD = standard deviation; LSD = least significant difference.

Introduction

Human pathologies indicate that there are multipotent progenitor cells contained in adipose tissue, called adipose-derived stem cells (ADSCs). Histological analysis of the progressive osseous heteroplasia (POH) patients demonstrates the presence of osteoblasts and chondrocytes in addition to adipocytes[1]. POH is caused by the mutation of GNAS1 gene, which is responsible for the coupling of transmembrane hormone receptors to adenylate cyclase[2,3]. This inborn metabolic error suggested that adipose tissue-derived stem cells are tri-potential, with the capability of adipogenic, chondrogenic, and osteogenic differentiation potential.

Obesity presents further evidence supporting the existence of stem cells within adipose depots. *In vivo* models of adipogenesis suggest that the mature adipocyte is a terminally differentiated cell, with limited capacity for proliferation and replication[4,5]. But the radioactive tracer studies have found that the turnover rate for cells within

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adipose depots ranges between 6 to 15 months in humans and rodents. These indicated that a stem cell population within adipose tissue is responsible for replacing mature adipocytes through the lifetime of the individual[6].

The initial methods to isolate cells from adipose tissue were pioneered in 1966[7,8]. The isolation method was improved by multiple investigators through the half-century[9,10]. Identification of the ADSC surface immunophenotype has provided a mechanism to enrich or purify the stem cell population directly from the heterogeneous stromal vascular fraction cells obtained from adipose tissue[11].

ADSCs can be differentiated to multiple kinds of terminal cells, including cardiomyocyte, chondrocyte, endothelial, myocyte, neuronal-like cells, and osteoblast[12-17]. ADSCs have been widely used in regenerative medicine. A previous study[18] showed that ADSCs had regeneration effects on sciatic nerve injury.

Here, we firstly investigated the regeneration effects of ADSCs in optic nerve injury. Functional improvement of vision responses and relief of inflammation were observed in our study. The results indicated that ADSCs has the potential for the therapy of optic nerve injury in clinics.

Materials and Methods

Reagents

Chloral hydrate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human basic fibroblast growth (FGF-basic) was purchased from R&D system. Phosphate-buffered saline (PBS) and 3% hydrogen peroxide was purchased from Gibco (Rockville, MD, USA). Mouse anti-rat Nestin monoclonal antibody and biotinylated rabbit anti-mouse polyclonal antibody were purchased from Abcam (Cambridge, MA, USA).

Experimental Animals

Male Sprague Dawley (SD) rats were bought from Guandong Medical Experiment Animal Resource Center (No. 44007200021628) and were used at the age of 22 months. Rats were bred and housed in specific pathogen-free conditions in groups of three to four rats per cage with free access to food and water. Animal breeding and experiments were conducted according to Yantaishan Hospital Animal Welfare guidelines and were approved by the Animal Ethics Committee of the Yantaishan Hospital.

Isolation, Expansion, and Identification of ADSCs

Rats were euthanized by over-dose injection of chloral hydrate and cleaned with 70% ethanol. The isolation procedure is operated under restrict sterile condition. The adipose tissue under the skin of groin was isolated, washed with phosphate-buffered saline (PBS) containing 100 μg/mL streptomycin, minced into small pieces and digested with 0.1% clostridiopeptidase A for one hour at 37°C. After the digestion, the minced tissue was diluted in PBS and filtered with 50 μm strainers. After 10 min centrifugation under 1200 g, the cells were resuspended and cultured in the low glucose Dulbecco's modified Eagle medium (DMEM-low) with 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C with 5% CO_2.

The cell was identified by nestin expression using streptavidin-biotin complex based immunohistochemistry. Briefly, cells seeded on coverslips were fixed with 4% paraformaldehyde (PFA) on ice for 30 minutes and permeabilized with 0.5% TritonX-100 solution twice for 5 minutes each at room temperature. The coverslips were treated with 3% hydrogen peroxide for 15 minutes at room temperature and washed with PBS. Then, the cells were incubated with mouse anti-rat Nestin antibody (1:500, 4°C overnight) and biotinylated rabbit anti-mouse polyclonal antibody (1:1000, room temperature for one hour) sequentially. Horseradish peroxidase (HRP) labeled streptavidin and 3,3'-diaminobenzidine tetrahydrochloride (DAB) were added for coloration.

Optic Nerve Injury Model

The rat was anesthetized with 10% chloral hydrate (3 ml/kg), and the surrounding area of the right eye was sterilized by 70% ethanol. The Tenon's capsule was opened and the lateral rectus was separated. Then, the tissues along the lateral temporal sclera surface were separated to expose the optic nerve. The optic nerve was clamped 2-3 mm away from the bulbus oculi for 15 seconds. In some groups, 800 Au recombinant human FGF-basic or 106 ADSCs was injected into the tunica vaginalis of the injured area. After closed the incision, the retinal blood supply was assessed by an ophthalmoscope and the surgical area was treated with an antibiotic.

F-VEP Assay

The electrophysiological examination follows the principles of International Society for Clinical Electrophysiology of Vision (ISCEV). After

Table I. The dynamic changes of the P1 wave amplitude (μV) after the optic nerve injury.

Group	Time					
	Before	Day 3	Day 7	Day 14	Day 21	Day 28
control	6.79±1.20	5.83±1.31	5.01±1.1	4.56±0.87	3.86±0.89	3.12±0.91
FGF	6.81±1.57	6.51±0.98	6.34±0.88**	6.09±0.92**	5.89±1.13**	5.32±0.75**
ADSC	6.82±1.19	6.31±1.21	6.11±0.76*	5.86±0.87**	5.45±1.05**	4.76±0.938**

Mean ± SD, *$p < 0.05$, **$p < 0.01$ when comparing with control group.

anesthetized with 10% chloral hydrate (3 ml/kg), mydriasis was induced by dropped tropicamide. After adopted in dark chamber, 3193 CD/m^2, 119 Hz flash stimulation was applied. The amplitude and latency of the P1 wave were recorded at day 3, week 1, 2, 3, and 4.

Western Blot

Eyeballs and optical nerves were harvested immediately after euthanasia at day 3, week 1, 2, 3, and 4. Optical nerves were isolated and stored in liquid nitrogen. Nerve lysates were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblotting using goat polyclonal antibodies react with human Mac-1, MyD88 and NF-κB receptor (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and horseradish peroxidase (HRP)-conjugated donkey anti-goat antibody (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sample loading amounts in the assay were normalized by β-actin (goat polyclonal antibody react with human β-actin, 1:2000, Santa Cruz Biotechnology (Santa Cruz, CA, USA) blotting. The blots were visualized by G:BOX Gel and Blot Imaging Systems (Syngene, Cambridge, UK).

Enzyme-Linked Immunosorbent Assay (ELISA)

Expression of IL-6 and TNF-α in the optical nerve lysates were assessed by using ELISA kits following manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). The quantification was performed by a microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Statistical analysis was performed with SPSS19.0 (IBM Corp., IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY, USA). Data were presented as mean ± standard deviation (SD). Single data points were presented in some graphs. The means for the data sets were compared, each separately, using Least Significant Difference (LSD) test. $p < 0.05$ were considered statistically significant.

Results

ADSC Implantation Improved the Vision Degeneration Caused by Optical Nerve Injury

Assessed by F-VEP assay, we observed a continuous decrease (from 6.79 ± 1.20 to 3.12 ± 0.91 μV) of the P1 wave amplitude after the optical nerve injury (Table I). In the group treated with recombined human FGF-basic, the decrease of the P1 wave amplitude is slower (from 6.81 ± 1.57 to 5.32 ± 0.75 μV) compared to the untreated control. The significant difference between FGF-basic treated and control group was showed starting at week one after the optical nerve injury ($p = 0.008$). Notably, the ADSC implantation group also showed a slower decrease of the P1 wave amplitude (from 6.82 ± 1.19 to 4.76 ± 0.94 μV) compared to the untreated control group. The significant difference between ADSC implantation and control group was also showed one week after the optical nerve injury ($p = 0.011$).

Meanwhile, a continuous increase of the P1 wave latency was observed (from 91.9 ± 2.2 to 118.9 ± 2.1 ms) after the optical nerve injury (Table II). In the group treated with recombined human FGF-basic, this increase was inhibited (from 90.8 ± 1.6 to 105.1 ± 1.5 ms). The significant difference between FGF-basic treated and control group was observed starting at day three after the optical nerve injury ($p < 0.01$). The ADSC implantation showed the inhibitory effect on the latency increase (from 90.2 ± 1.9 to 112.3 ± 3.8 ms) compared to the untreated control. The significant difference between ADSC implantation and control group was reached starting from day three after the optical nerve injury ($p < 0.01$).

Overall, these results indicated that ADSC implantation can improve the vision degeneration caused by optical nerve injury.

Table II. The dynamic changes of the P1 wave latency (ms) after the optic nerve injury.

Group	Time					
	Before	Day 3	Day 7	Day 14	Day 21	Day 28
control	91.9±2.2	105.9±3.4	109.6±2.1	118.3±1.7	119.6±1.9	118.9±2.1
FGF	90.8±1.6	95.9±3.1**	99.3±1.6**	103.3±2.2**	104.1±1.1**	105.1±1.5**
ADSC	90.2±1.9	99.5±2.1**	103.1±1.6**	109.8±2.7**	110.4±1.5**	112.3±3.8**

Mean ± SD, *$p < 0.05$, **$p < 0.01$ when comparing with control group.

ADSC Implantation Inhibit the Up-Regulations of Proteins Involved in the TLR4 Pathway Caused by Optical Nerve Injury

In our Western blot assay, we found that Mac-1 expression was upregulated in the optic nerve tissue, which indicated the microglia was activated (Figure 1a). The group treated with recombined human FGF-basic and implanted with ADSCs showed lower expression level than the control group (Figure 1b, $p < 0.05$). We also observed similar result for MyD88, which is another important inflammation regulator. MyD88 was upregulated after the optical nerve injury (Figure 2a) and reached the peak value at week two. This upregulation was limited by both FGF-basic treatments and ADSC implantations. Significant difference was reached (Figure 2b, $p < 0.05$). NF-κB, another important regulator of inflammation, was also shown upregulated in the optic nerve tissue by the optical nerve injury (Figure 3a) and reached the peak value at week 2. The trend is similar to the expression of MyD88. Both the FGF-basic treatments and ADSC implantations inhibited this upregulation and reached the significant differences (Figure 3b, $p < 0.05$). Using ELISA, we quantitatively assessed the expression of both IL-6 and TNF-α, which are two important cytokines involving in TLR4 mediated inflammation. Our results showed that both IL-6 (Table III) and TNF-α (Table IV) were continuously increased after the optic nerve injury, and reached the peak value at week three to four. Both the FGF-basic treatments and ADSC implantations can inhibit these increases. The significant difference between FGF-basic treatments and control groups was reached starting at week one ($p < 0.01$) for both IL-6 and TNF-α. The significant inhibition of IL-6 and TNF-α upregulation in the ADSC implantations group compared to the control group was observed at week one ($p < 0.05$) and week two ($p < 0.01$), respectively. Overall, these results indicated that ADSC implantation can effectively suppress the inflammations. The suppression of optic nerve tissue inflammation might be the reason for improving the vision degeneration caused by the optical nerve injury.

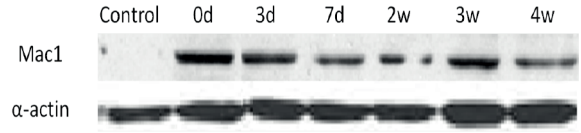

Figure 1. The expression of Mac-1 in the optic nerve tissue. ***(a)*** Western blot of Mac-1 in the optic nerve tissue harvested from different days. ***(b)*** The expression of Mac-1 quantified from three independent experiments. Mean ± SD, *$p < 0.05$, **$p < 0.01$.

Discussion

The previous study[19] showed that bone marrow-derived cells can be used to treat optic nerve diseases. Although it can be translated to clinics, the pain of bone marrow aspiration becomes an obstacle. In our work, we showed that ADSCs has similar curative effect on optic nerve injury. ADSCs are easier to be acquired if compared to bone marrow cells. Meanwhile, the ADSC implantation is an autologous (self) transplantation method. The autologous transplantation avoids the immunological rejection of the graphs, which is the main problem for transplantation and regenerative medicine[20]. The most common example of the autologous transplantation in clinics is en-

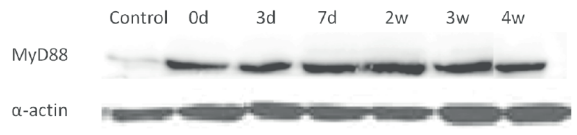

Figure 2. The expression of MyD88 in the optic nerve tissue. ***(a)*** Western blot of MyD88 in the optic nerve tissue harvested from different days. ***(b)*** The expression of MyD88 quantified from three independent experiments. Mean ± SD, *$p < 0.05$, **$p < 0.01$.

Table III. The dynamic changes of the IL-6 expression (ng/mg) after the optic nerve injury.

Group	Time					
	Before	Day 3	Day 7	Day 14	Day 21	Day 28
control	31.1±2.3	41.9±1.9	47.7±1.8	53.5±2.0	52.9±2.2	51.4±2.2
FGF	30.8±2.5	40.7±2.0	43.5±2.0**	41.0±2.3**	38.4±1.8**	34.1±2.0**
ADSC	31.9±2.0	41.2±2.3	45.7±1.9*	44.7±2.1**	40.5±2.1**	35.7±2.4**

Mean ± SD, *$p < 0.05$, **$p < 0.01$ when comparing with control group.

Table IV. The dynamic changes of the TNF-α expression (ng/mg) after the optic nerve injury.

Group	Time					
	Before	Day 3	Day 7	Day 14	Day 21	Day 28
control	92.1±1.9	111.9±2.2	127.7±2.1	133.5±2.4	192.9±2.1	181.4±2.3
FGF	90.8±2.2	110.7±2.0	123.5±2.0**	121.0±2.7**	128.4±2.0**	124.1±2.1**
ADSC	91.9±2.3	111.2±2.5	129.7±1.9*	124.7±2.3**	155.5±2.4**	145.7±2.2**

Mean ± SD, *$p < 0.05$, **$p < 0.01$ when comparing with control group.

grafting the stem cells from cord blood[21]. Autologous transplantation of bone marrow stem cells is also developed to fight against cancer[22], limb ischemia, and heart disease. Autologous transplantation of ADSCs was shown to have a therapeutic effect on skin diseases, cardiovascular diseases, ischemic limb diseases, urinary system diseases, and neural system diseases[23-28]. The optic nerve, also known as cranial nerve II, is a paired nerve that transmits visual information from the retina to the brain. The optic nerve injury, also known as traumatic optic neuropathy, refers to an acute injury of the optic nerve secondary to trauma. The optic nerve axons may be damaged either directly or indirectly and the visual loss may be partial or complete. An indirect injury to the optic nerve typically occurs from the transmission of forces to the optic canal from blunt head trauma. This is in contrast to direct injury, which results from an anatomical disruption of the optic nerve fibers from penetrating orbital trauma, bone fragments within the optic canal, or nerve sheath hematomas. Optic nerve injury used to happen in patients having a head trauma, usually causing to visual decrease or incurable blind[29]. The regenerative therapy is one of the best strategies to treat the optic nerve injury. And our results present an important method of using ADSCs to improve the optic nerve injury. The secondary injury of the optic nerve caused by surrounded inflammation after trauma is a nonnegligible factor in treating the optic nerve injury. Toll-like receptor (TLR) signaling, an important pathway of inflammation, is critical for the functional recovery after peripheral nerve injury. It is reported that TLR4 in the central nervous system is involved in inflammation and autoimmune diseases. The critical role of the TLR4 response in neuroinflammation, brain injury, and potentially neurodegeneration induced by chronic ethanol intake was discovered[30]. Microglial TLR4 expression on microglia was demonstrated to be vital in the regulation of neuropathic pain[31], neuronal precursor cell migration, and differentiation[32]. It was demonstrated that TLR4-mediated signaling pathway is activated following optic nerve injury in mice. The inhibition of this pathway will prevent neural cell death following optic nerve injury.

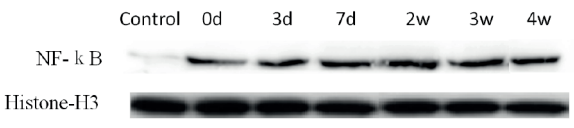

Figure 3. The expression of NF-κB in the optic nerve tissue. ***(a)*** Western blot of NF-κB in the optic nerve tissue harvested from different days. ***(b)*** The expression of NF-κB quantified from three independent experiments. Mean ± SD, *$p < 0.05$, **$p < 0.01$.

Consistent with the previous study, we observed the up-regulation of the inflammation factors in the TLR4-mediated signaling pathway, including Mac-1, MyD88, NF-κB, IL-6, and TNF-α. The up-regulation caused by the optic nerve injury was inhibited by the implantation of ADSCs. This indicated that ADSCs may not only provide regenerative therapy on the optic nerve injury, but

also modulate the microenvironment homeostasis and inhibit inflammation, which further improves the prognosis after optic nerve injury.

Conclusions

This study demonstrates that the implantation of ADSCs inhibits the vision functional degradation and the TLR4 signaling pathway mediated inflammation. Our finding suggests ADSC implantation as a translational therapy method for optic nerve injury in clinics.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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