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Inhibitory effect of TGF-β gene modified human amniotic mesenchymal stem cells on rejection after xenotransplantation of peripheral nerves

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Abstract. – OBJECTIVE: To explore the inhibitory effect of transforming growth factor-beta (TGF-β) gene modified human amniotic mesenchymal stem cells on rejection after xenotransplantation of peripheral nerves.

MATERIALS AND METHODS: In this study, 6 placentas collected in our hospital were selected as the source of human amniotic mesenchymal stem cells. A total of 60 C57BL/6 experimental mice (mouse sciatic nerves were removed before the experiment) were taken as research objects. Mice were randomly divided into experimental group 1, experimental group 2 and experimental group 3 (xenogenous peripheral nerves were introduced to all experimental groups), and a control group (autologous peripheral nerves were introduced). Among them, TGF-β gene modified (overexpression) human amniotic mesenchymal stem cells were introduced to experimental group 1; TGF-β gene modified (inhibition) human amniotic mesenchymal stem cells were introduced to experimental group 2; normal human amniotic mesenchymal stem cells were introduced to experimental group 3; and autologous sciatic nerves were introduced to control group. The messenger ribonucleic acid (mRNA) and protein expressions of the TGF-β in different human amniotic mesenchymal stem cells were detected by quantitative polymerase chain reaction (qPCR) and Western blotting, respectively. Mouse sciatic nerve function in each group after 2 weeks of procedures was detected *via* the CatWalk system. Expression level of interleukin-22 (IL-22) in the peripheral tissues of transplanted nerves and blood was detected using immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). Its mRNA level was examined via fluorescence quantitative PCR.

RESULTS: TGF-β1 was highly expressed in mice of experimental group 1, but lowly expressed in experimental group 2 relative to that of experimental group 3 ($p<0.05$). CatWalk test results revealed that the main indexes in experimental group 1 were superior to those in other groups, while the main indexes in experimental group 2 were inferior to those in other groups. According to immunohistochemistry and ELISA results, there were significant differences in the expression level of IL-22 in mice of different treatment groups ($p<0.05$). IL-22 level was the lowest in control group [(5.05±0.15) pg/mL], followed by that in experimental group 1 [(6.52±0.24) pg/mL], and it was the highest in experimental group 2 [(9.47±0.31) pg/mL].

CONCLUSIONS: Human amniotic mesenchymal stem cells overexpressing TGF-β can inhibit rejection after xenotransplantation of peripheral nerves.

Key Words
TGF-β, Human amniotic mesenchymal stem cells, Xenotransplantation of peripheral nerve, Sciatic nerve, Immunological rejection.

Introduction

In recent years, the incidence rates of neurological diseases related to the phenomena, such as Parkinson's syndrome, Alzheimer's disease, malignant nerve sheath tumor and sciatic nerve diseases, have increased due to aging and environmental pollution[1,2]. Most of these diseases are related to the peripheral nerve injury. At present, there is no effective treatment for the above-mentioned diseases.

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Neural stem cell (NSC) is lowly differentiated and has the potential to differentiate into neurons and related nervous systems. It has important significance on the treatment of the above-mentioned peripheral nervous system injury diseases and can be used as the seeds that differentiate into corresponding nerve cells[3]. However, due to the extremely low level of NSCs in the human body, it is difficult to culture and amplify[4]. Human amnion-derived stem cells have gradually attracted the attention of researchers in recent years due to their low degree of differentiation and differentiation potential to nerve cells. Human amniotic mesenchymal stem cells belong to human amnion-derived stem cells. Konala et al[5] have shown that human amniotic mesenchymal stem cells can not only express nestin, a specific protein of neural stem cell markers, but also efficiently express octamer-binding transcription factor-4 (OCT-4). Hence, human amniotic mesenchymal stem cells may have therapeutic effects on peripheral nerve injury diseases[6]. However, rejection after xenotransplantation of nerve cells is a common complication during transplantation of many organs and cells. Prevention of such rejection has become a research focus in this field. In recent years, it has been found[7] that transforming growth factor-beta 1 (TGF-β1), as an important member of the immune system family, plays an important regulatory role in cell proliferation and differentiation as well as in the development of the nervous system. For example, it has been found[8] that TGF-β1 can promote the synthesis of the extracellular matrix and regulate the transformation of bone marrow mesenchymal stem cells into osteoblasts and chondrocytes. However, there are few reports on the study of the regulatory effect of TGF-β1 modified human amniotic mesenchymal stem cells on rejection after xenotransplantation of peripheral nerves. Therefore, in this work, sciatic nerves in peripheral nerves were taken as the research objects to preliminarily explore the inhibitory effect of TGF-β modified human amniotic mesenchymal stem cells on rejection after xenotransplantation of peripheral nerves. We aim to provide a certain theoretical and experimental basis for prevention of rejection after xenotransplantation of peripheral nerves.

Materials and Methods

General Data

In this study, pregnancies' placentas (full-term) obtained in our hospital from 2016 to 2017 were selected as the research objects, and were approved by the pregnant women and their family members in writing and reviewed by the Academic Ethics Committee of our hospital. At the same time, 60 C57BL/6 experimental mice (removal of sciatic nerve in advance) were randomly divided into experimental group 1, experimental group 2 and experimental group 3, with 20 mice in each group, and a control group was set up. Mice in experimental groups underwent xenotransplantation of sciatic nerves. TGF-β gene modified (overexpression) human amniotic mesenchymal stem cells were introduced to experimental group 1, TGF-β gene modified (inhibition) human amniotic mesenchymal stem cells were introduced to experimental group 2, normal human amniotic mesenchymal stem cells were introduced to experimental group 3, and autologous sciatic nerves were introduced to control group.

Experimental Instruments

The inverted phase difference microscope was purchased from Nikon (Tokyo, Japan), the fluorescence quantitative polymerase chain reaction (PCR) instrument from ABI (Foster City, CA, USA), the carbon dioxide incubator from Thermo Fisher Scientific (Waltham, MA, USA), and the protein Western blotting imager from Tiangen (Beijjng, China).

Main Reagents

Roswell Park Memorial Institute-1640 (RPMI-1640) medium and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA), the fluorescence quantitative PCR kit and RNA extraction kit from TaKaRa (Dalian, China), the immunohistochemical kit and animal total protein extraction kit from Thermo Fisher Scientific (Waltham, MA, USA), the interleukin-22 (IL-22) primary antibody (anti-mouse IL-22 monoclonal antibody) and secondary antibody (horseradish peroxidase (HRP)-labeled goat ant-mouse antibody) from PLlabs (Vancouver, Canada), and other reagents from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China).

Intracellular Ribonucleic Acid (RNA) Extraction

RNA extraction was conducted according to the instructions of the TaKaRa kit (Dalian, China)[9], and the specific procedures were as follows:

(1) About 0.1 g frozen tissue sample was taken out from liquid nitrogen, melt on ice and added with 0.45 mL RNA plus. Then, the tissue was ground in a pre-cooled mortar and moved into a 1.5 mL Eppendorf (EP) tube. After another 0.45 mL RNA

Plus was added to the mortar, the tissue was transferred into a centrifuge tube after washing. (2) 200 μL chloroform was added to the centrifuge tube, shaken violently for 15 s and let stand on ice for 15 min. (3) Centrifugation was carried out at 12000 rpm, 4°C for 15 min. (4) The supernatant was transferred into the RNase-free EP tube. After that, the same amount of isopropyl alcohol was added, gently mixed, and let stand on ice for 10 min. (5) Centrifugation was carried at 12000 rpm, 4°C for 10 min. (6) After the supernatant was discarded, the tube was added with 750 μL 75 % ethanol, gently mixed and centrifuged at 12000 rpm, 4°C for 10 min. (7) The supernatant was discarded, and the residual ethanol was removed as much as possible. (8) A proper amount of RNase-free water was added. The quality of the extracted RNA was measured, and the rest was used for reverse transcription (RT)[10].

Fluorescence Quantitative PCR

In this study, the kit used for fluorescence quantitative PCR was purchased from TaKaRa (Dalian, China) and the experiment was carried out using a three-step method. The primers used were shown in Table I.

Enzyme-Linked Immunosorbent Assay (ELISA)

In this study, the double antibody sandwich method was applied to detect the gene expression levels of TGF-β1 and IL-22[11], the specific procedures were as follows:

(1) Coating: in this study, the antibody protein was appropriately diluted with phosphate-buffered saline (PBS) buffer at pH 9.0, with a concentration of about 1-10 μg/mL. 0.1 mL protein was added to a 96-well plate and treated at 4°C overnight. At the next day, the liquid in the 96-well plate was discarded, followed by washing with washing liquid for 5 times, with 2 min each time.
(2) Sample addition: 0.1 mL serum sample was added, treated in the above 96-well plate and let stand at 37°C for 1 h, followed by washing with washing buffer for 5 times, with 2 min each time.

Table I. Primers used in fluorescence quantitative PCR.

Gene	Primer sequence
TGF-β1	F: 5'-TAGCTGAGGATCGCTAGCTA-3' R: 5'-CGATCGGGCATGCTACGATC-3'
IL-22	F: 5'-CGATCGGCGCATCGTCAGTAC-3' R: 5'-CTAGGCGCATTAAGCTCGATC-3'
GAPDH	F: 5'-CGATCGGGCATGAGGACTCGCATC-3' R: 5'-CGAGAGGCTAGCACGCTAGCATC-3'

(3) Addition of the secondary antibody: after washing, 0.1 mL newly prepared secondary antibody to the 96-well plate was added for incubation at 37°C for 0.5-1.2 h. Next, the protein was dyed red, followed by washing with washing buffer for 5 times, with 2 min each time.
(4) Addition of the chromogenic substrate: after washing, 0.1 mL newly configured substrate solution tetramethylbenzidine (TMB) (Solarbio, Beijing, China) was added to the 96-well plate for incubation at 37°C for 30 min.
(5) Addition of termination solution: at the end of the experiment, 0.005 mL 0.2 M sulfuric acid termination solution was added to the above 96-well plate.
(6) Qualitative detection: the above 96-well plate was placed on the plain paper for qualitative observation through the color depth, i.e., the darker the color was, and the higher the TGF-β1 protein content would be, with a colorless negative control well. Quantitative detection: the 96-well plate was placed on a microplate reader for quantitative detection, with a wavelength of 450 nm and zeroed through a blank well. If the optical density (OD) value was larger than 1.2 times that of the negative control value in the experimental results, the results would be determined as positive[10].

Extraction of the Total Intracellular Protein and Western Blotting Assay

In this study, the animal cell protein extraction kit (Roche, Basel, Switzerland) was used to extract the total protein in samples (the specific operation was carried out according to the instructions)[11]. Subsequently, according to the product instructions provided by Roche (Basel, Switzerland), the antibody dilution was carried out, with the final dilution multiple of 1:5000, and the related operations were carried out according to the Molecular Cloning Manual.

TGF-β1 Inhibition and Cell Line Construction

The overexpression of human amniotic mesenchymal stem cells and the construction of TGF-β1 cell lines were described in the article of Sha et al[12]. TGF-β1 primers used were synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China), and primer sequences were shown in Table II. Experiments such as cell transfection and liposome construction were performed according to the experiments of others.

Table II. Primers used for TGF-β1 gene knockout and overexpression.

Name	Sequence
TGF-β1 overexpression	F: 5'-AGCTGCGCTAGCTCGCATCGAT-3' R: 5'-CGTAGCGGCATGCTCAGCTAC-3'
TGF-β1 knockout	F: 5'-CGGCGCATCGATCGATCAGCT-3' R: 5'-CGGCGCTACGTCGCGCGATCGCT-3'

CatWalk

In order to explore the inhibitory effect of TGF-β1 modified human amniotic mesenchymal stem cells on rejection after xenotransplantation of peripheral nerves, the behavior of experimental mice under different treatment conditions was detected by the CatWalk experiment. The experimental procedures were carried out according to that of Prasad et al[13], and the recovery effect of mice after transplantation was evaluated by measuring the three indexes, namely, the minimum pressure, maximum contact area and paw print length.

Statistical Analysis

All experimental results were statistically processed using Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA). The data were expressed as ($\bar{x}\pm s$). The univariate analysis of variance was conducted for multi-sample mean comparison, the *t*-test was adopted for the comparison of the difference between two groups, and the Q test was used for the pairwise comparison of the difference. $p<0.05$ represented that the difference was significant.

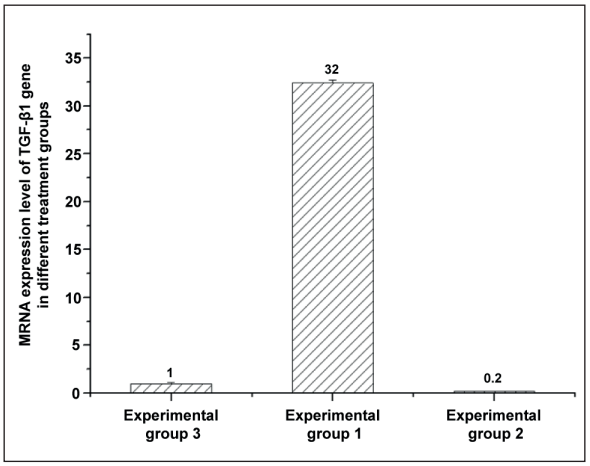

Figure 1. MRNA expression level of TGF-β1 in human amniotic mesenchymal stem cells in different treatment groups detected *via* fluorescence quantitative PCR. The mRNA level of TGF-β1 is markedly different in the experimental groups ($p<0.05$).

Results

MRNA Expression Level of TGF-β1 in Human Amniotic Mesenchymal Stem Cells in Different Treatment Groups Detected Via Fluorescence Quantitative PCR

In this study, the mRNA expression level of TGF-β1 in human amniotic mesenchymal stem cells in different experimental groups and control group was detected. As shown in Figure 1, the results manifested that the mRNA expression level of TGF-β1 in human amniotic mesenchymal stem cells in experimental group 1 significantly increased compared with that in experimental group 3 ($p<0.05$). At the same time, the mRNA expression of TGF-β1 in experimental group 1 was notably higher than that in experimental group 2, with a significant difference ($p<0.05$). However, the mRNA expression of TGF-β1 in experimental group 3 was evidently higher than that in experimental group 2, displaying a significant difference ($p<0.05$).

Protein Expression Level of TGF-β1 gene in Human Amniotic Mesenchymal Stem Cells in Different Treatment Groups Detected via Western Blotting

Western blotting was adopted to determine the protein expression level of TGF-β1 in different treatment samples in experimental groups. The results (Figure 2) revealed that compared with that in ex-

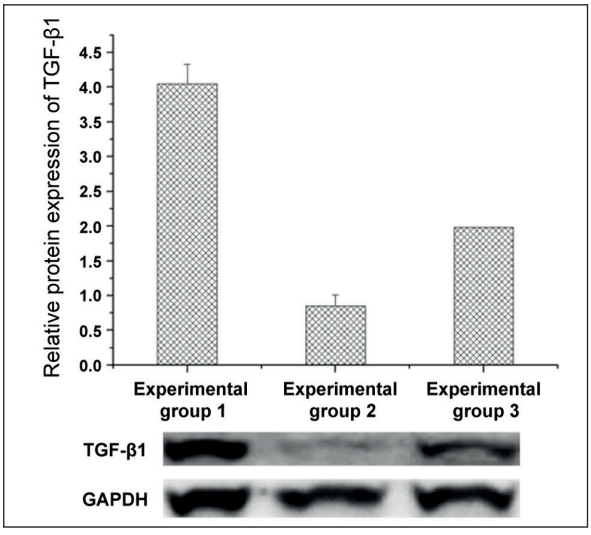

Figure 2. Protein expression level of TGF-β1 in human amniotic mesenchymal stem cells in different treatment groups detected *via* Western blotting. The protein expression level of TGF-β1 is markedly different in the experimental groups ($p<0.05$).

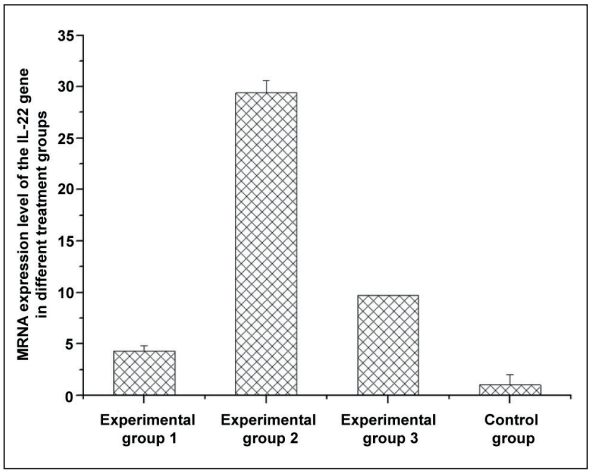

Figure 3. MRNA expression level of IL-22 in experimental mouse samples in different treatment groups detected *via* fluorescence quantitative PCR. The relative mRNA expression level of IL-22 in control group is significantly different from those in experimental groups ($p<0.05$).

perimental group 1 (TGF-β1 overexpression), the protein expression level of TGF-β1 in experimental group 2 notably decreased, showing a significant difference ($p<0.05$). Compared with that in experimental group 3 (normal human amniotic mesenchymal stem cells) the expression level of TGF-β1 in human amniotic mesenchymal stem cells in experimental group 2 significantly decreased, which was markedly upregulated in experimental group 1 displaying significant differences ($p<0.05$).

The mRNA Expression Level of IL-22 Gene in Experimental Mouse Samples in Different Treatment Groups Detected via Fluorescence Quantitative PCR

Interleukin, as a kind of cytokine produced by leukocytes, is mainly involved in information transmission, activation and regulation of immune cells in the human body in response to external stimuli, mediating the differentiation of T and B cells and other processes. Ball et al[14] has indicated that in the process of allogenic organ and tissue transplantation, leukocytes are the main factors causing rejection. To explore the relationship between TGF-β1 and rejection, IL-22 level in mice in different experimental groups was detected. It was found from Figure 3 that compared with that in control group, the mRNA level of TGF-β1 in experimental groups markedly increased, displaying significant differences ($p<0.05$). Meanwhile, IL-22 level in mice receiving different treatment methods in experimental groups was compared. IL-22 level in mice introduced with human amniotic mesenchymal stem cells in experimental group 1 (TGF-β1 overexpression) was obviously lower than that in experimental group 3, showing a significant difference ($p<0.05$). Besides, IL-22 level in experimental group 3 was markedly lower than that in experimental group 2 ($p<0.05$), indicating that TGF-β1 overexpression can significantly reduce IL-22 level in the blood.

Protein Expression Level of IL-22 in Peripheral Nerve Tissues in Different Treatment Groups Detected via Immunohistochemistry

Protein expression level of IL-22 in experimental samples in different treatment groups was detected by immunohistochemistry. According to the results (Figure 4), the number of IL-22-positive cells in experimental group 1 was lower than that in control group, and the difference was significant ($p<0.05$). At the same time, among experimental groups, the number of IL-22-positive cells was the highest in experimental group 2, successively followed by the experimental group 3 and experimental group 1. It is suggested that

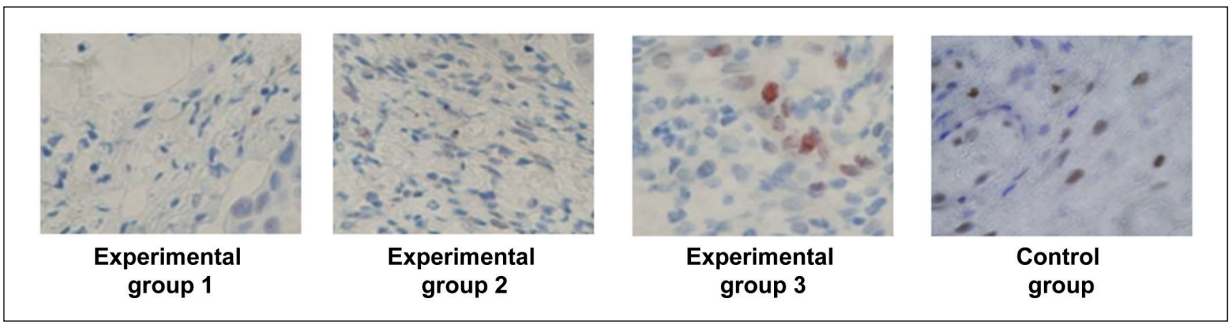

Figure 4. Protein expression level of IL-22 in peripheral nerve tissues in different treatment groups detected *via* immunohistochemistry. The number of IL-22-positive cells (purple) in control group is significantly different from those in experimental groups ($p<0.05$).

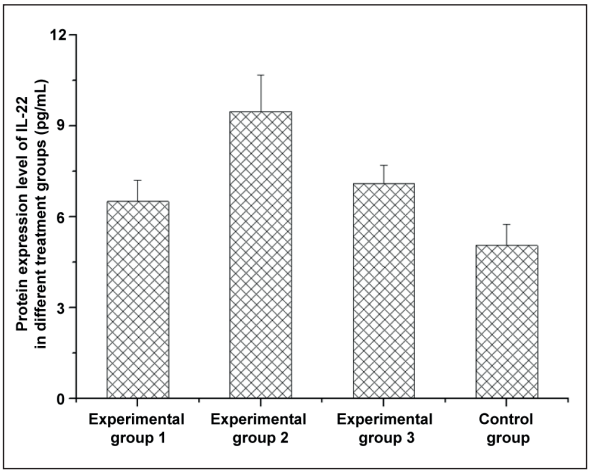

Figure 5. Protein expression level of IL-22 in blood in different treatment groups detected via ELISA. The relative protein expression level of IL-22 in control group is significantly different from those in experimental groups (p<0.05).

overexpression of TGF-β1 can reduce the intracellular level of IL-22.

Protein Expression Level of IL-22 in blood in Different Treatment Groups Detected via ELISA

The protein expression level of IL-22 in the blood samples of mice in different treatment groups was determined by ELISA. The results (Figure 5) demonstrated that compared with the protein expression level of IL-22 in the blood of mice receiving autotransplantation [(5.05±0.15) pg/mL], it notably increased in experimental groups, displaying significant differences (p<0.05). At the same time, compared with the protein expression level of IL-22 in experimental group 3 [(7.08±0.31) pg/mL], TGF-β1 overexpression [(6.52±0.24) pg/mL] could reduce the protein level of IL-22 in the blood, while TGF-β1 inhibition [(9.47±0.31) pg/mL] could increase this level in the blood.

Behaviors of Experimental Mice in Different Treatment Groups Detected via CatWalk

Based on the evaluation of the behavior of mice after xenotransplantation of peripheral nerves, it could be found that the indexes of mice in experimental group 1 with TGF-β1 overexpression were similar to those of mice undergoing autotransplantation in control group, showing no significant difference (p>0.05). However, there was a significant difference between experimental group 2 (TGF-β1 inhibition) and control group (autotransplantation without rejection) (p<0.05). It is indicated that TGF-β1 modified human amniotic mesenchymal stem cells can significantly reduce rejection after xenotransplantation of peripheral nerves (Table III).

Discussion

At present, with the continuous progress of the medical technology, the treatment methods for nervous system diseases and organ failure have been continuously improved. Stem cells in related tissues and organs have been extensively used in autologous organ and tissue culture in the later stage due to their low differentiation degree in this process. However, as the number of stem cells in the human body decreases greatly with aging, finding alternative stem cells and xenogenous organs has become an important treatment method for organ failure and other diseases[15,16]. Prevention of immune rejection after xenotransplantation of organs and tissues in this process is an important issue that must be solved in xenotransplantation of organs and tissues[17]. Human amniotic mesenchymal stem cells are considered as a kind of excellent stem cells because of their low degree of differentiation and extensive sources[18,19]. In this study, the role of TGF-β1 gene modification in rejection of human amniotic mesen-

Table III. CatWalk results of different treatment groups.

Group	Minimum pressure (Pa)	Maximum contact area (cm²)	Claw print length (cm)
Control group	55.37±3.71	0.28±0.11	2.04±0.24
Experimental group 1	61.51±2.34	0.23±0.15	1.83±0.31
Experimental group 2	12.18±1.79	0.13±0.05	0.83±0.27
Experimental group 3	21.29±1.53	0.11±0.07	1.13±0.46
t	5.793	3.208	5.489
p	0.001	0.018	0.012

chymal stem cells after xenotransplantation was firstly explored. The results showed that TGF-β1 overexpression in human amniotic mesenchymal stem cells could remarkably reduce immunological rejection in mice after xenotransplantation of peripheral nerves (sciatic nerves). IL-22 level in human amniotic mesenchymal stem cells of mice with TGF-β1 overexpression [(6.52±0.24) pg/mL] was lower than that of controls [(7.08±0.31) pg/mL], displaying a significant difference ($p<0.05$). Protein level of IL-22 in all experimental groups was higher than that in control group after autotransplantation [(5.05±0.15) pg/mL]. The above results indicated that although TGF-β1 modified human amniotic mesenchymal stem cells can markedly decrease the level of interleukins leading to immunological rejection, it did not fundamentally eliminate the occurrence of immunological rejection after xenotransplantation of peripheral nerves. We believed that TGF-β1 may not be involved in the process of immunological rejection alone, but may be involved by cooperating with other genes. This is an important direction for the follow-up research.

Conclusions

We demonstrated that human amniotic mesenchymal stem cells overexpressing TGF-β1 can inhibit rejection after xenotransplantation of peripheral nerves.

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Conflict of Interests

The authors declared no conflict of interest.

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