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Original Article

A study on how bone marrow-derived MSCs inhibit rejection reaction of alkali burn-induced corneal neovascularization

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Abstract: Objective: The study was to explore the mechanism of action of bone marrow-derived mesenchymal stem cells (MSCs) inhibition of corneal neovascularization (CNV) of rabbits and to test the impact of MSCs on endogenous vascular endothelial growth factor (VEGF) and interferon-γ (IFN-γ) in the CNV model. Methods: The bone marrow of rabbits was taken under aseptic conditions and MSCs were separated thereafter. MSCs after 3 generations of culture were digested and made into a cell suspension and inoculated on amniotic membrane graft for routine culture. The alkali burn-induced CNV model of rabbits was established. Thirty New Zealand rabbits 8 to 12 weeks old were equally divided into three groups: Empty Group (Group A), MSCs Transplant Group (Group B) and Conventional Transportation Model Group (Group C) two weeks later. With penetrating keratoplasty, the amniotic membrane graft with MSCs of Group B and Group C was respectively fastened to the shallow sclera through interrupted suture. Group A was subjected to no treatment whatsoever. The eye surface of rabbits was examined before and after the transplant, and the CNV area tested, and rejection reaction and turbidity graded. Expression of VEGF and IFN-γ mRNA in corneal tissues was measured through real-time quantitative polymerase chain reaction (PCR). Results: The screened MSCs of rabbits had a uniformity of 97.54%, and after the transplant, the comprehensive score of eye surface of Group B was remarkably reduced ($P<0.05$). Group C had rejection reaction and a statistical difference from Group B ($P<0.05$). Group A featured remarkably improved expression of IFN-γ mRNA and was statistically different from Group B and Group C ($P<0.05$). Group A offered noticeably reduced expression of VEGF mRNA in comparison to Group B and Group C ($P<0.05$). Group B boasted notably lowered expression of IFN-γ mRNA and VEGF mRNA in comparison to Group C ($P<0.05$). Conclusion: Bone marrow-derived MSCs could remarkably inhibit the rejection reaction of alkali burn-induced corneal neovascularization.

Keywords: Mesenchymal stem cells, corneal neovascularization, cell apoptosis, alkali burn, rabbit model

Introduction

Transplant rejection refers to the immunological reaction of destroying, eliminating, and attacking the transplant organ after the receptor's immune system identifies it following allotransplantation [1]. Surveys indicate that patients with corneal chemical injury induced corneal neovascularization (CNV) have rejection reaction rates as high as 75% after the transplant [2]. This exerts a huge impact on the recovery of patients after the transplant. Patients experiencing rejection reaction must undergo multiple transplants. Together with the shortage of cornea donors, mental and economical burdens of the patients have been considerably enhanced. Ocular chemical injury is one of the frequently seen diseases in the Ophthalmic Emergency Department. Severe corneal ocular chemical injury will lead to serious damage of limbal stem cells (LSCs) and corneal tissues and subsequently give rise to perforated ulcer, CNV total corneal opacity and symblepharon, and eventually result in blindness of the patients [3-6]. As adult stem cells, mesenchymal stem cells (MSCs) are capable of transdermal differentiation [7]. According to current researches, MSCs play a significant role in immunosuppression, and transplant of cultured amniotic membrane with human MSCs to

alkali burn animals can reduce the incidence rate of inflammatory reaction [8].

However, research on the immunological rejection of MSCs in keratoplasty has not been reported. Through the expression impact on endogenous vascular endothelial growth factor (VEGF) and interferon-γ (IFN-γ) in a CNV model, this paper explores what role MSCs play in the rejection reaction of alkali burn-induced CNV.

Materials and methods

Animals of study

Thirty healthy New Zealand rabbits 8 to 12 weeks old weighing 2,000 to 2,500 g (15 female rabbits and 15 male rabbits) were purchased from Jiangsu Zhenlin Biotechnology Co., Ltd. The rabbits were fed in separate cages under light. A 3-week-old SPF grade New Zealand white rabbit was further purchased and cultivated for donor of MSCs.

Reagents and instruments

The reagents, 0.25% trypsin, low sugar culture medium DMEM, and PBS solution were purchased from Shanghai Beinuo Biotechnology Co., Ltd. RNA extracting solution TRIzol and the RT-PCR kit were purchased from Invitrogen. The 7500 qRT-PCR instrument was from ABI, the reverse transcription kit and the TaqMan miRNA kit were purchased from Applied Biosystems. The rat anti-rabbit CD29 antibody and the anti-CD45 antibody were purchased from Epigentek, Beckman DxFLEX Flow Cytometry purchased from Beckman.

Methods

Culture of rabbit bone marrow-derived MSCs: The iliac marrow of the SPF grade New Zealand white rabbit was taken under aseptic conditions, mixed evenly with 5 mL of culture medium and resuspended with low sugar culture medium DMEM. When the first generation MSCs grew to 85% to 90% (in terms of area of Petri dish), 0.25% trypsin-EDTA solution (250 μg trypsin + 50 μg EDTA dissolved into 100 mL PBS solution) was used to digest and the MSCs were observed under a microscope. After completely digested, the MSCs were transferred to the sterilized centrifuge tube with a gun pipette and centrifuged for 5 min at 1,000 rpm. The supernatant was then removed. After flushing with PBS for 3 times, the MSCs were transferred to the low sugar culture medium DMEM for culture (in a 5% CO_2 incubator at 37°C). The third generation MSCs were collected and the growth curve drawn [9].

Verification of rabbit bone marrow-derived MSCs: To verify the third generation MSCs collected, 0.25% μg/mL trypsin was used for digested counting and preparation of suspension liquid. The MSCs were respectively inoculated to the 24-well plates at a density of $1.0*10^5$/L and 0.25% μg/mL trypsin-EDTA solution (250 μg trypsin + 50 μg EDTA dissolved into 1,00 mL PBS solution) was added to the plate every 24 hours. Finally, 0.25% μg/mL trypsin was adopted for digested counting. In addition, the third generation MSCs collected should be digestively counted and made into suspension liquid with 0.25% μg/mL trypsin, and washed for 3 times for 30 to 60 s per time with PBS solution. Subsequently rat anti-rabbit CD29 antibody and anti-CD45 antibody were added and incubated without light for 30 min. The MSCs were washed with PBS and tested with flow cytometry for 3 times [9].

Preparation of human amniotic membrane and plantation of MSCs: The human amniotic membrane provided by the Ophthalmology Department of General Hospital of Guangzhou Military Command of PLA (at a temperature of -20°C in glycerinum) was first repeatedly washed with normal saline, soaked in gentamicin in a proportion of 1:2000 for 30 min, and washed in water bath of 0.25% trypsin-EDTA solution (250 μg trypsin + 50 μg EDTA dissolved into 1.0 mL PBS solution) for 60 min at a temperature of 37°C. Afterwards, the human amniotic membrane was placed upside down under the microscope. The epithelial cell layer was blown down, and dried for later use. The third generation MSCs collected were digestively counted and made into suspension liquid with 0.25% μg/mL trypsin. After adjustment to a density of $1.0*10^5$/L, the MSCs were planted on the amniotic membrane and cultured with regular culture solution (low sugar DMEM) in a 5% CO_2 incubator at 37°C, and transplanted 5 days later [9].

Alkali burn animal model building: A model was established to inspect the appendage and anterior segment of both eyes of the rabbits with surgical microscope, and binocular corne-

Table 1. Primer sequence

Gene	Upstream primer	Downstream primer
VEGF	5'-AACTTTCTGCTGTCTTGGGTG-3'	5'-ACAAATGCTTCTCCGCTCT-3'
IFN-γ	5'-AGAGCCAGATTATCTCTTTCTACCTCAG-3'	3'-CTTTTTTCGCCTTGCTGCTG-5'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCT-TCACGAATTTGCGT-3'

Note: VEGF, vascular endothelial growth factor; IFN-γ, interferon-γ.

al sodium fluorescein used to avoid interference from other diseases. Phenobarbitone (Guangdong Bangmin Pharmaceutical Co., Ltd.) was injected into the vein on the edge of rabbit ear at 30 mg/kg, and 50 uL 1% tetracaine hydrochloride eye drop (General Hospital of Guangzhou Military Command of PLA under the batch number of 130808) injected for local anesthesia. Dry cotton balls were used to wipe off excessive water. Tweezers were used to fully soak the single-layer filter paper with a diameter of 10 mm in 1 mol/L NaOH. After excess liquid was absorbed with absorbent paper, the filter paper was placed on the center of the New Zealand white rabbit's cornea for 60 s and then discarded. The PBS solution (100 mL) was then used to wash the burned area for 10 min. The animal models were examined every day. This study had been approved by the Laboratory Animal Welfare & Ethics Committee of the Institute in General Hospital of Guangzhou Military Command of PLA [9].

Transplant and grading of MSCs human amniotic membrane: Two weeks after the model was established, thirty New Zealand rabbits were equally divided into three groups--Empty Group (Group A), MSCs Transplant Group (Group B) and Control Group (Group C), with Group B and Group C subject to PKP [10]. Empty Group (Group A), MSCs Transplant Group (Group B) and Conventional Transportation Model Group (Group C) two weeks later. MSCs human amniotic membrane was used for transplant in respect of Group B and conventional human amniotic membrane was used for transplant in respect of Group C. Group A had no treatment whatsoever. The rabbits were fed in separate cages after the transplant with no light interference. The immunological rejection index (RI) was evaluated from slit-lamp examination. One observation was conducted every 2 days within 2 weeks after the transplant and every 3 days after 2 weeks of the transplant, and observations lasted for 12 weeks. The turbidity, swelling, and neovascularization of the implant were graded as per the scoring method of clinical observations established by Holland, et al. (The sum of the three is RI of corneal implant, and rejection reaction is identified when RI ≥6) [11].

Examination of rabbit corneal repair under slit-lamp microscope: A slit-lamp observation was conducted every week to evaluate the transparency of cornea and growth of neovascularization. The transparency of the cornea was evaluated in four grades: Grade 0, representative of completely transparent; Grade 1, indicative of lightly turbid and iris texture perceivable; Grade 2, representative of intermediately turbid, iris texture unperceivable and pupil perceivable; Grade 3, indicative of highly turbid and iris texture unperceivable. The length of CNV growing from the corneosclera limbus was measured from corneal photographs and recorded to calculate the growth area of CNV (calculation formula: $S=C/12*3.1416(r^2-(r-L)^2)$, wherein C represents cumulative hours of corneal vascular network; L represents the growth length of CNV; r represents the radius of cornea) [12].

Detection of expression of VEGF and IFN-γ mRNA in corneal tissues via qRT-PCR: The animals were suffocated in the CO_2 anesthesia chamber three weeks after the transplant. The corneal tissues were treated with Trizol reagent and total RNA extracted in strict accordance with the manual of the kit. UV spectrophotometer was used to measure the concentration and purity of RNA. Sample quality requirements: The A_{260}/A_{280} of total RNA solution ranged from 1.8 to 2.1, and extraction was reconducted in case of noncompliance. The extracted total RNA were reverse transcribed as instructed in the reverse transcription kit. The cDNA sample extracted was stored at -20°C. VEGF and IFN-γ primers were designed as per Paydas S and Silva for experiments as shown in **Table 1** [13, 14]. The PCR system was configured as provided in the manual. PCR conditions: 94°C 10 min; 94°C 30 s; 60°C 45 s; 72°C 30 s, 45 cycles in total, U6 used as reference gene, 3 repeated experiments. See **Table 1**.

Statistical analysis

Statistical analysis was conducted on the data collected from this study through SPSS22.0

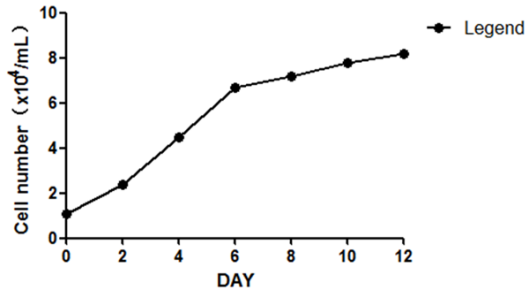

Figure 1. Growth curve of MSCs. Cells began to increase from day 1 to day 2, grew exponentially from day 5 to day 7, and entered into a plateau of slow growth afterwards. MSCs, mesenchymal stem cells.

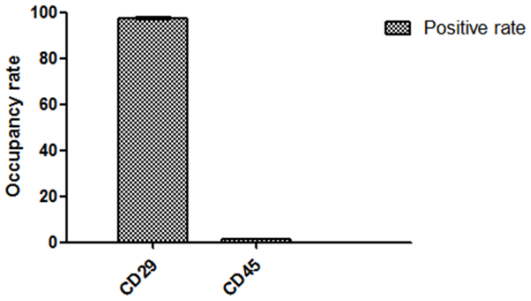

Figure 2. Cell verification. It was found through flow cytometry that the bone marrow cells cultured were mostly rabbit MSCs. MSCs, mesenchymal stem cells.

software package. Mean ± standard deviation ($\bar{x} \pm sd$) were adopted to represent the enumeration data, t test used for comparison between groups and ANOVA for analysis over two groups. The data were considered statistically significant when $P<0.05$.

Results

Growth curve of MSCs

Observations on inoculation of primary rabbit bone marrow-derived MSCs revealed that anchorage-dependent cells started to grow after 3 to 4 hours of inoculation and cell adhesion and cell proliferation perceivable after 24 hours of growth. Certain colonies were formed from Day 2 and certain cells started to have extruded cytoplasm. Cell colonies increased rapidly from Day 5 to Day 7, primarily spindle cell colonies. It can be seen from the growth curve that the cells began to increase from Day 1 to Day 2, grew exponentially from Day 5 to Day 7, and entered into a plateau of slow growth afterwards as shown in **Figure 1**.

Verification of MSCs

Flow cytometry of the cultured bone marrow cells were rabbit MSCs and no hematopoietic cell were detected ($P=0.001$) as shown in **Figure 2**.

Therapeutic evaluation

An evaluation on the corneal transparency of the animal models indicates that the grades of Group B and Group were remarkably higher than Group A before the transplant and statistically significant ($P<0.05$). The grades of Group B after the transplant gradually reduced over time in comparison to the grades before the transplant, and had statistical significance ($P<0.05$). The grades of Group B gradually lowered in comparison to Group C after the transplant and had statistical significance ($P<0.05$). Group B and Group C had no statistical significance ($P>0.05$) before the transplant. See **Table 2**.

Rejection reaction

A comparison of RI values of different groups after the transplant reveals that the RI values of Group B and Group C 1 week after the transplant were statistically insignificant ($P>0.05$); the RI grades of Group C were noticeably higher than those of Group B from 2 weeks to 2 months after the transplant ($P<0.05$), and no immunological rejection was detected in Group B and immunological rejection was detected in Group C 1 month after the transplant (RI value >6). Group A was subject to no treatment whatsoever. See **Table 3**.

Rabbit alkali burn-induced CNV area comparison

Observations on the animal models after alkali burn reveal that the CNV area of Group A 1 week to 2 months after alkali burn increased remarkably in comparison to that of Group B and Group C and had statistical significance ($P<0.05$). The CNV area of Group B and Group C gradually decreased over time 1 week to 2 months after alkali burn, and was statistically significant. See **Table 4**.

Tissue mRNA expression inspection through qRT-PCR

Expression of VEGF and IFN-γ mRNA in the tissues of animal models was inspected 2 months

Table 2. Therapeutic evaluation of animal models in different groups

Group	Group A (n=10)	Group B (n=10)	Group C (n=10)	F	P
Before the operation	2.34±0.42	7.60±1.40	7.90±1.65	60.375	0.001
One week postoperatively	2.58±0.32	4.65±1.65[a,b]	7.55±1.50	36.844	0.001
Two weeks after the operation	2.77±0.63	3.45±1.50[a,b]	6.60±1.25	29.759	0.001
One month after the operation	2.81±0.44	3.00±1.00[a,b]	6.00±1.45	29.144	0.001
Two months after the operation	3.04±0.65	2.65±0.75[a,b]	5.54±1.20	30.421	0.001

Note: [a]P<0.05 in comparison to grades before the transplant; [b]P<0.05 in comparison to Group C after the transplant. Comparison of Group B 1 week after the transplant and before the transplant (t=4.311, P=0.001); comparison of Group B 2 weeks after the transplant and before the transplant (t=6.396, P=0.001); comparison of Group B 1 month after the transplant and before the transplant (t=8.455, P=0.001); comparison of Group B 2 months after the transplant and before the transplant (t=9.856, P=0.001). Comparison of Group C 1 week after the transplant and before the transplant (t=4.113, P=0.001); comparison of Group C 2 weeks after the transplant and before the transplant (t=5.102, P=0.001); comparison of Group C 1 month after the transplant and before the transplant (t=5.386, P=0.001); comparison of Group B 2 months after the transplant and before the transplant (t=6.458, P=0.001). Group A, Empty Group; Group B, MSCs Transplant Group; Group C, Conventional Transportation Model Group.

Table 3. Rejection reaction grading

Group	Group B (n=10)	Group C (n=10)	t	P
One week postoperatively	1.35±0.34	1.47±0.51	0.619	0.543
Two weeks after the operation	1.38±0.44	4.54±0.78	11.158	0.001
One month after the operation	1.78±0.58	6.89±0.85	15.703	0.001
Two months after the operation	2.62±0.76	9.58±1.02	17.302	0.001

Note: Group B, MSCs Transplant Group; Group C, Conventional Transportation Model Group.

Table 4. CNV area comparison

Group	One week	Two weeks	One month	Two months
Group A (n=10)	31.58±1.88	48.64±2.45	43.28±1.57	40.54±1.66
Group B (n=10)	23.58±1.74[a]	18.62±1.68[a]	12.55±1.58[a]	9.37±1.68[a]
Group C (n=10)	27.68±1.84[a,b]	38.81±2.14[a,b]	36.29±1.74[a,b]	32.84±2.34[a,b]
F	48.26	524.30	866.70	715.50
P	0.001	0.001	0.001	0.001

Note: CNV, corneal neovascularization. Differences of different groups are detected through ANOVA analysis. Group B and Group C have statistical difference in comparison to Group A ([a]P<0.05); Group C has statistical difference in comparison to Group B ([b]P<0.05). Comparison of Group A and Group B & Group C 1 week after the transplant (t=9.876, P=0.001, t=4.688, P=0.001); comparison of Group A and Group B & Group C 2 weeks after the transplant (t=31.956, P=0.001, t=9.556, P=0.001); comparison of Group A and Group B & Group C 1 month after the transplant (t=44.605, P=0.001, t=9.432, P=0.001); comparison of Group A and Group B & Group C 2 months after the transplant (t=41.735, P=0.001, t=8.487, P=0.002). Comparison of Group B and Group C 1 week after the transplant (t=5.120, P=0.001); comparison of Group B and Group C 2 weeks after the transplant (t=23.467, P=0.001); comparison of Group B and Group C 1 month after the transplant (t=31.941, P=0.001); comparison of Group B and Group C 2 months after the transplant and before the transplant (t=25.765, P=0.001). Group A, Empty Group; Group B, MSCs Transplant Group; Group C, Conventional Transportation Model Group.

after the transplant through qRT-PCR and it was found that the expression of IFN-γ mRNA remarkably increased and had statistical difference in comparison to that of Group B and Group C (t=7.567, P=0.001, t=2.825, P=0.011). Expression of VEGF mRNA of Group A was noticeably reduced in comparison to that of Group B and Group C (t=7.820, P=0.001, t=2.283, P=0.035). The expression of IFN-γ mRNA of Group B lowered notedly in comparison to Group C (t=4.914, P=0.001) and the expression of VEGF mRNA of Group also decreased significantly in comparison to that of Group C (t=6.041, P=0.001). See **Table 5**.

Discussion

Statistics in recent years indicate that ocular injury and keratitis are the primary cause for blindness in the country. Keratoplasty was the best treatment of keratopathy and the only treatment for vision restoration today [15]. There were mainly three approaches for vision restoration: transplant of LSCs cultured *in vitro*, transplant of autologous LSCs and transplant of bone marrow-derived MSCs. The currently widely adopted transplant of LSCs

Table 5. Expression of IFN-γ mRNA

Group	IFN-γ mRNA	VEGF mRNA
Group A (n=10)	3.24±0.57	4.57±0.88
Group B (n=10)	1.76±0.24[a]	1.82±0.68[a]
Group C (n=10)	2.58±0.47[a,b]	3.74±0.74[a,b]
P	0.001	0.001
F	26.82	33.45

Notes: VEGF, vascular endothelial growth factor; IFN-γ, interferon-γ. [a]P<0.05 in comparison to that of Group A; [b]P<0.05 in comparison to that of Group B. Comparison of IFN-γ mRNA of Group A and Group B & Group C (t=7.567, P=0.001, t=2.825, P=0.011). Comparison of VEGF mRNA of Group A and Group B & Group C (t=7.820, P=0.001, t=2.283, P=0.035). Group A, Empty Group; Group B, MSCs Transplant Group; Group C, Conventional Transportation Model Group.

cultured *in vitro* and autologous LSCs offered a relatively high success rate [16]. However, behind the high success rate are issues like the uncontrollability of volume of healthy corneal limbus tissues, requirements of repeated transplants in case of rejection reaction, and shortage of cornea donors [17]. A research study revealed that MSCs in the adult bone marrow with strong proliferation ability can be divided into different cells of the human body (myocardial cells, epithelial cells, adipose cells and osteoblasts through varying inductions, and have clinical benefit response in the treatment of alkali burned animal models after in vitro culture with corneal stroma [18]. Due to the extremely complicated and low content of stem cells and difficulty in sampling of bone marrow-derived MSCs, however, it's difficult for clinical promotion.

With special biological and anatomical features, amniotic membrane has a thin and transparent basilar membrane, no antigenicity and degradability in the body and easy accessibility, and multiple factors (IV collagen, fibronectin and laminin) it contains can accelerate cell adhesion, growth and proliferation [19]. Inhibition of growth of alkali burn-induced CNV through amniotic membrane transplant experimented by domestic and international scholars have delivered favorable results [20].

Through the treatment of alkali burned animals with bone marrow-derived MSCs amniotic membrane as a carrier, we examined the growth of CNV and expression of VEGF and IFN-γ mRNA. Studies show that the multiple differentiation abilities of MSCs are related to the environment and pluripotency [21]. The former is a reflection of pluripotency, determined by the features of the body and regulated by pluripotency genes; the latter is a manifestation of different signal molecules in the environment induced into various cells.

In this study, the iliac marrow of animal models was collected for *in vitro* culture and counted through flow cytometry and it was found that MSCs of rabbits had a uniformity of 97.54%, proving that the cells cultured in this study were MSCs. Subsequent inspections and grading on the corneal turbidity of animal models revealed that the untreated Group A had a noticeably lower turbidity grade than Group B and Group. The grades of Group B subject to transplant of MSCs amniotic membrane reduced markedly over time, and had significant difference from Group C subject to transplant of conventional amniotic membrane. It proved that the MSCs of Group B had successfully differentiated into corneal stem cells and had the features of epithelial cells after transplant of MSCs amniotic membrane. It further attested that MSCs could be induced into various directions as per varying signal molecules in different environments.

It's assumed from the above findings that the probability of rejection reaction through transplant of MSCs amniotic membrane may reduce. To prove this, the rejection reaction on Group B and Group C after the transplant was graded. It was found that the RI values of Group B and Group C had no differences 1 week after the transplant, but the RI values of Group C rose noticeably 2 weeks after the transplant and exceeded 6 one month after the transplant, indicating rejection reaction of Group C. In contrast, Group B experienced no rejection reaction (RI<6) despite of slightly increase of RI values after the transplant and had statistical significance in comparison to Group C. A comparison of CNV area of different groups after the transplant indicated that the CNV area of Group B subject to transplant of MSCs amniotic membrane gradually decreased, remarkably reduced in comparison to and had differences from untreated Group A and Group C subject to transplant of conventional amniotic membrane. The above tests illustrate that transplant of MSCs amniotic membrane has a lower probability of rejection reaction and a remarkably better postoperative recovery than

transplant of conventional amniotic membrane.

Research indicates that MSC treatment on chemical burn promotes the repair of corneal tissues and anti-neovascularization through anti-inflammatory transplant [22]. As a primary excreted factor of Th1 cells, IFN-γ primarily activates macrophages, and thus leads overexpression and antigen presentation of MHC molecules and subsequently results in the inhabitation of Th2 cells [23]. As unique mitogen in vascular endothelial cells, VEGF can induce the growth of blood vessels [24]. Through qRT-PCR, the expression of IFN-γ and VEGF mRNA of different groups is inspected 2 months after the transplant. It has been found that expression of IFN-γ mRNA and VEGF mRNA of Group B remarkably reduced and had differences in comparison to the other two groups. According to the experiments of treating alkali burned animal models with Dexamethasone conducted by Gong et al., expression of IFN-γ mRNA of the untreated control group significantly increased and that of the experimental group weakened [25]. Weakened expression of VEGF demonstrated that transplant of MSC amniotic membrane reduced the expression of VEGF and greatly narrowed the CNV area, and might further inhibit rejection reaction.

However, this experiment is not without flaws. The mechanism of action is yet to be further explored and MSCs rebuilding eye surface structure yet to be further studied. The samples are in a small quantity. It is also difficult to collect human bone marrow. Therefore, the samples are expected to be increased and studies in MSCs rebuilding surface structure to be unfolded in future experiments. It is our hope that the accuracy and feasibility of this study can be further verified with clinical experiments.

In conclusion, bone marrow-derived MSCs play an inhibitory role in the rejection reaction of alkali burn-induced corneal neovascularization and are expected to be promoted clinically.

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Disclosure of conflict of interest

None.

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