Transplantation with Cultured Stem Cells Derived from the Human Amniotic Membrane for Corneal Alkali Burns: an Experimental Study

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Abstract. Amniotic membranes (AM) have been used in a wide range of clinical applications. We successfully extracted mesenchymal stem cells (MSCs) from human AM, but little is known about the use and efficacy of human amniotic membrane-derived mesenchymal stem cells (hAM-dMSCs) for the treatment of alkali burns. We utilized hAM-dMSCs transplantation, AM grafting, and their combined use in the treatment of alkali burns. An experimental model in rabbits was devised to analyze the use of these techniques with immunocytochemistry and ELISA. The survival and migration of hAM-dMSCs labeled by SPION in the host were assessed with Prussian blue staining. Compared with the control group, the treated groups demonstrated faster reconstruction of the corneal epithelium, and lower levels of corneal opacification and neovascularization within corneal alkali burns. Furthermore, dark blue-stained particles were detected in the limbus corneae at day 28. These results demonstrated the ability of hAM-dMSCs to enhance epithelial healing and reduce corneal opacification and neovascularization in corneal alkali wounds.

Key Words: Amniotic membrane, mesenchymal stem cells, alkali burns.

Introduction

Alkaline substances permeate the surface of the eye when the cornea is burned by alkali, where they induce a severe inflammatory reaction through cytotoxic infiltration and secretion of enzymes and cytokines [1-6]. The amniotic membrane (AM) has potent epithelialization and anti-inflammatory effects on the ocular surface [7-11]. Following AM transplantation, rejection does not occur as the AM does not express antigens such as HLA-A/-B and DR. Thus, the AM is a biological material that is suitable for oculo-surface reconstitution following an alkali burn, and AM transplantation has been successfully undertaken in this context [12]. In addition, AM suspensions have an active effect on reconstitution of the corneal epithelium, suggesting it plays an important role in repair [13].

Mesenchymal stem cells (MSCs) are generally used in the regeneration and repair of damaged tissue. To date, studies have demonstrated that bone marrow mesenchymal stem cells (BM-MSCs) and umbilical cord blood mesenchymal stem cells (UC-MSCs) can be transplanted for the treatment of alkali burns [14,15]. The bone marrow is the main cell source of MSC, although it is a limited resource. As donor age increases, the differentiation potency of BM-MSCs decreases [16]. Umbilical cord blood has no anti-inflammatory or anti-scarring roles, but the AM contains human amniotic membrane-derived mesenchymal stem cells (hAM-dMSCs), which differentiate into neurons in specific chemical and cytokine microenvironments [17-20].
The corneal epithelium rapidly secretes cytokines, such as IL-1 and IL-6, after alkali burns, with IL-6 being produced by IL-1 [5]. Sekine-Okano et al. reported that the corneal epithelium secretes TNF-α when inflamed, which then causes cytokine secretion [21].

The aim of this study was to define the curative effect of AM transplantation alone and combined with hAM-dMSCs transplantation, and the value of hAM-dMSCs for the treatment of alkali burns. This was achieved through an assessment of morphology and pathology, using immunocytochemistry and ELISA.

Materials and Methods

Alkali burn animal model. Forty-six adult albino rabbits (both sexes; weight range, approximately 2.5–3.5 kg) were used in this study. All animals were treated in accordance with the guidelines laid out in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and the study was approved by the Committee for Animal Research at the Catholic University of Medicine. The rabbits were deeply anesthetized with intravenous 2% pentobarbital sodium (1 ml/kg). Alkali injuries to the eyes were randomly induced with a 60 second exposure of the central cornea to an 8-mm diameter disc of filter paper soaked in 1 N NaOH, followed by rinsing with sterile saline (40 ml).
Amniotic membrane and cell preparation. The study was approved by the medical ethics board of the First Affiliated Hospital of Zhengzhou University, China. Once informed consent had been obtained, placental samples were obtained from full-term pregnant women during elective caesarean section.

Cleansed AMs were tiled on nitrocellulose filter (NC filter), with the epithelium away from the filter, and cut into 20 mm×20 mm pieces. The pieces were then maintained at 4ºC in Dulbecco's modified eagle's medium-low glucose (LG-DMEM) containing 1×10^5 U/L penicillin and streptomycin, and were used within 12 hours.

Cells were prepared according to the previously described methods of Yang et al. [22]. A commercial SPION suspension (diluted at 1:10; Resovist; Schering, Germany) was used in accordance with the method described by Zeng et al. [20].

hAM-dMSCs and AM transplantation. Once the model had been created, animals were randomly allocated into four treatment groups. The control group (n=10) was treated topically with PBS immediately after the alkali injury, four times per day for two days. The first study group (hAM-dMSCs group, n=12) was treated with a subconjunctival injection in the injured eye using a Hamilton syringe, and into the temporal quadrant of the bulbar conjunctiva. One hundred microliters of hAM-dMSCs suspension, containing 1×10^6 hAM-dMSCs, which had been labeled by SPION, was used. The second study group (AM group, n=12) was treated with AM grafting, and the third group (association group, n=12) was treated with labeled hAM-dMSCs in combination with AM grafting.

Scoring for corneal opacity and neovascularization. A previously described scoring system was used to measure the degree of corneal opacification from 0 to 5+: 0=a clear and compact cornea; 1+= minimal superficial opacity; 2+= mild deep (stromal) opacity with pupil margin and iris vessels visible; 3+= moderate stromal opacity with only pupil margin visible; 4+= intense stromal opacity with anterior chamber visible; 5+= maximal corneal opacity with total obscuration of the anterior chamber [23].

All corneas were photographed using a digital camera (Nikon Coolpix 995, Tokyo, Japan) connected to a surgical microscope (Carl Zeiss, OPMI-1, Berlin, Germany). Areas containing blood vessels were traced on the computer monitor, calculated using image analysis software, and reported in square millimeters. Dilated limbal vessels not penetrating the corneal stroma were considered not representative of corneal neovascularization (NV). Areas of corneal neovascularization were outlined using the following formula: C/12×3.1416[r^2-(r-L)^2], where C refers to the hours taken for neovascularization to occur around the corneal circumference, r refers to the radius of the cornea, and L refers to the length of neovascularization. All gradings were conducted in a masked fashion. The results are expressed in square millimeters.

Histological documentation of inflammatory cell infiltration. Two albino rabbits in each group were killed following clinical examination on days 1,3,7,14, and 28, and 0.3 ml of aqueous humor was withdrawn from the limbus corneae using a Hamilton syringe and stored at -80ºC until use. For histological evaluation, eyes in each group were enucleated and fixated in 4% formaldehyde. Corneas were sectioned centrally, routinely dehydrated and embedded in paraffin, and 4 μm sections were stained with hematoxylin and eosin. The corneas were then stained with Prussian blue, using the same isolation method as on day 28.

Enzyme-linked immunoassay (ELISA). The expression of TNF-α in aqueous humor was determined using ELISA systems (Innogenetics; Ghent, Belgium). The test sample was obtained as described above.

Statistical analysis. Chi-square tests were used to evaluate cell activity after they were labeled with SPIONs, or the therapeutic effect of the different methods on the corneal burn. Student’s t-tests were used to compare the corneal neovascularization and TNF-α scores between the four groups. The Kruskal-Wallis test was used to determine whether there were significant differences in the opacity grading, and the Mann-Whitney U test was used to assess the presence of significant differences between the groups. P-values <0.05 were considered statistically significant. All analyses were performed using SPSS statistical software, version 19.0 (SPSS Inc., Chicago, IL, USA).

Results

Growth characterization and surface antigens of hAM-dMSCs in primary culture. The growth characteristics of hAM-dMSCs that were isolated by digestion from the amnion amniotic membranes (identity proven by flow cytometry) in primary culture are shown in Figure 1A. Cells were simple fusiform fibers by the third passage (Figure 1B). The hAM-dMSCs that were cultured in cytokine induction medium (CIM), which contained Dulbecco's
modified Eagle’s medium–low glucose (DMEM-LG; Gibco BRL, USA), presented with neuron-like differentiation.

**SPION-labeled methods affect hAM-dMSCs.** Dark blue-stained particles were detected in the cytoplasm of SPION-labeled hAM-dMSCs using Prussian blue staining and confirmed that the SPIONs labeled ratio was 100% (Figure 1C).

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to assess cytotoxicity. Cultured in cytokine growth medium (CGM) at 24 hours, samples that were not labeled with SPIONs had cytoactivity greater than 80%, which was approximately equal to samples labeled with SPIONs at concentrations of 3.5, 7, and 14 µg/ml. The cytoactivity of samples labeled with 28 µg/ml SPIONs was less than 80%. After 48 hours, samples labeled with various concentrations of SPIONs ≤14 µg/ml were >80% viable; samples labeled with >14 µg/ml SPIONs were <80% viable, using the SPION-labeled method. Samples that were not labeled with SPIONs were >80% viable at 24, 48, and 72 hours (Figure 1D).

**Alkali burn animal model.** Following the induction of the alkali burn, the injured central corneal epithelium was either markedly edematous or partly exfoliated, and the stroma was opaque and edematous. Parts of the conjunctiva and the limbus corneae appeared to be necrotic, secondary to ischemia, and these areas covered approximately 1/3 to 1/2 of the cornea. The proximal conjunctiva was pale. The iris and pupil could be seen but were obscured (Figure 2 A1).

Histopathological examination of the cornea indicated that the epithelium was edematous or broken, and there was obvious edema in parts of the wing cells and the basal cells coloboma, as well as the substantia propria layer. The order of the corneal tissue was lost (Figure 3A).
**Corneal opacity and neovascularization.** Figure 2 shows corneal epithelial wound healing 28 days after the burn induction in the control (PBS) group, the hAM-dMSCs transplantation group, the AM grafting group, and the hAM-dMSCs combined with AM grafting group. The results shown are representative of the experiments. In the control (PBS) group (Figure 2 A3), 42.9%±4.5% of the burn wound was recovered, whereas in the hAM-dMSCs transplantation (Figure 2 B3), AM grafting (Figure 2 C3), and association groups (Figure 2 D3) the wound healing rates were 75%±6.1%, 75%±5.3%, and 91.7%±5.8%, respectively. The control group exhibited a wound healing rate that was significantly lower than that observed in the other three groups (p<0.05), and the difference between the three groups was not statistically significant (χ² = 1.2; Figure 4).

The scores for corneal opacity seven days after the burn induction were 3.9 (1.75), 3.15 (0.80), 3.20 (0.38), and 2.45 (0.75) in the control (PBS), hAM-dMSCs transplantation, AM grafting, and association groups, respectively (Figure 4B). The control group exhibited corneal opacity scores that were significantly higher than those observed in the other three groups (p<0.05). The scores in the hAM-dMSC transplantation and AM grafting groups were significantly higher than those of the association group (p<0.01), but the difference between the hAM-dMSC transplantation and AM grafting groups was not statistically significant (U=71, p>0.05). In addition, the scores for neovascularization for all four groups are shown in Figure 4C.

**Histological documentation of inflammatory cell infiltration.** Light microscopic findings in the control, hAM-dMSCs transplantation, AM grafting, and hAM-dMSCs associated with AM grafting groups 28 days after the alkali burn are shown in Figure 3. In the control group, the corneal thickness was greatly increased and many polymorphonuclear leukocytes had infiltrated the corneal stroma with extensive neovascularization (Figure 3B). However, relatively few polymorphonuclear leukocytes had infiltrated, and less neovascularization had occurred in the cornea in the hAM-dMSCs transplantation, AM grafting, and association groups (Figure 3C,D,E). In addition, the corneal thickness was slightly increased in the hAM-dMSCs transplantation, AM grafting, and association groups, compared to the control group.

**Immigration of SPION-labeled hAM-dMSCs.** Using subconjunctival injections, 14 μg/ml SPIONs labeled hAM-dMSCs were transplanted. At day 28, the cornea was isolated for Prussian blue staining, and dark blue stained particles were detected in the limbus corneae, indicating that hAM-dMSCs had survived at this site (Figure 3F).

**TNF-α quantitation in the alkali-burned cornea using ELISA.** Concentrations of TNF-α in the aqueous humor of the control and study groups are shown in Figure 4D. The average TNF-α concentration in the study groups decreased with time, and the difference at different times was statistically significant. In the control group, the average TNF-α concentration also decreased with time, and the difference at day 1, day 3, day 7, and day 14 was statistically significant, but not statistically significant between day 14 and day 28. At corresponding times, the TNF-α concentration in the study group was significantly higher than in the control group.

**Ocular and systemic manifestations in the rabbits during the experiment.** There were no significant differences between the eyes in the experimental study group and those of the controls. The conjunctiva showed no hyperemia or edema and the cornea was transparent with no neovascularization. The iris and pupil were clear in all animals. All rabbits remained alive with normal dietary intakes during the experiment with no instance of diarrhea, bloody stools, excoriation, or other abnormalities.

**Discussion**

This study used MSCs that had been isolated from the human AM, and MSC characteristics were confirmed using flow cytometry. hAM-dMSCs cultured in CIM can present with neuron-like differentiation, which illustrates their multi-directional differentiation potency as adult stem cells, as has been shown by Fukuchi et al. [24].
In our study, the treated groups demonstrated a faster reconstruction of the corneal epithelium than the control group, and the association group revealed obvious benefits when compared to the hAM-dMSCs transplantation or AM grafting groups. The study groups showed a slight acute inflammatory reaction and surpassed the control group in terms of corneal opacity and the inhibition of neovascularization. Thus, we propose that hAM-dMSCs not only promoted epithelialization, but also an acute anti-inflammatory reaction. We did not find a significant difference between hAM-dMSCs and AM transplantation.

Chemical injuries to the cornea induce excessive activation of mononuclear macrophages, and generate mediators of inflammation such as IL-1, IL-6, and TNF-α, the most important inflammatory factor. To evaluate the therapeutic effect of hAM-dMSCs or AM transplantation in alkali burns of the cornea, we used TNF-α in the aqueous humor as our detection factor. The content of TNF-α clearly decreased post-treatment compared with the control group, which suggests that AM or hAM-dMSCs can inhibit delivery of pro-inflammatory factors such as TNF-α. This result is similar to that of Wang et al., and indicates that it is important that regular cytokine secretion occurs as quickly as possible following alkali burn [25].

Phinney and Prockop previously described how MSCs promote tissue repair through the secretion of a factor that regulates inflammation and angiogenesis, rather than through their differentiated characteristics [26]. Oh et al. also found that through a paracrine action, MSCs can relieve the corneal inflammatory reaction and neovascularization observed in mice following a chemical injury [27]. They co-cultured MSCs and burned the corneal epithelium, and concluded that MSCs can secrete a large quantity of inflammatory factors such as IL-6 when stimulated by chemical injury [28]. Thus, we suggest that MSCs can regulate the secretion of cytokines.

Bailot et al. transplanted MSCs extracted from fetal membranes to newly born pigs and rats...
by intraperitoneal injection, and detected human microchimera in the bone marrow, cerebrum, lung, thymus, kidney, liver, and spleen 14-90 days after transplantation. They conclude that MSCs derived from fetal membranes can be successfully transplanted into newly born pigs and rats [29]. Compared with stem cells derived from marrow or sebum, fetal membrane can be obtained from discarded placenta, without destroying embryonic and normal placental tissue. Thus, they favor MSCs derived from fetal membranes in cell therapy and transplantation [29].

To date, there has been little data on how MSCs survive in the transplanted site. In order to identify how hAM-dMSCs live and migrate in the eye, we labeled hAM-dMSCs before transplantation using SPIONs, the validity of which has been proven in our laboratory [20]. According to the MTT assay used in this study, we chose a labeled concentration of 14 μg/ml. Four weeks after transplantation, dark blue stained particles were still detected in the limbus corneae using Prussian blue staining, indicating that hAM-dMSCs can be successfully transplanted under the conjunctiva of the rabbit and can live in the transplanted site and migrate to the limbus corneae through the circulation of the eye. Our findings were similar to those of Bailot et al [29].

Zhou et al. reported that the survival of the AM epithelium on the surface of the eye depends on the existence of stem cells [30]. In our study, the therapeutic effect of AM grafting combined with hAM-dMSCs transplantation was better than AM

Figure 4. (A) Data is presented as the mean ± standard deviation (SD). The wound healing ratio is significantly higher in the hAM-dMSCs, AM and association groups than in the control group (p<0.05), and the difference between the three groups is not statistically significant ($t^2 =1.2$). (B) The scores for corneal opacity 7 days after the burn. Scores of control group were significantly higher than those of the other three groups (p<0.01), and scores of the hAM-dMSCs and AM groups were significantly higher than those of the association group (p<0.01), while the difference between the hAM-dMSCs and AM groups was not statistically significant ($t=0.374$, p>0.05). (C) Data is presented as the mean ± standard deviation (SD). The study groups shows significantly lower scores compared to the control group (p<0.05 or p<0.01), and the difference between the hAM-dMSCs, AM and association groups is statistically significant at days 14 and 28 (p<0.05). (D) Data is presented as the mean ± standard deviation (SD). The study groups have significantly lower scores than the control group (p<0.01), and the difference between the hAM-dMSCs, AM and association groups is statistically significant (p<0.01).
grafting or hAM-dMSCs transplantation alone. One possible reason for this is that AM possesses antibacterial, epithelialization, and anti-inflammatory properties, and can promote raw surface healing quickly when used as a sterilized dressing to cover the injured cornea. A further reason is that hAM-dMSCs have both immunogenicity and transferability characteristics, and this ensures their survival on the raw surface, as well as their migration. The cells’ survival not only supplies stem cells that promote repair in trauma through secretion of a factor that can regulate inflammation and angiogenesis, but also maintains the characteristics of the AM graft.

In the preliminary experiment, we found that the corneal epithelium had almost healed 2-4 weeks after the alkali burn. We established four weeks as the follow-up period for evaluating epithelialization and the anti-inflammatory effect, and found that the TNF-α concentration in the aqueous humor at week four was not significantly different, which demonstrates that the inflammation caused by the alkali burn had not changed. In order to evaluate the long-term effects of chemical injury, a longer follow-up period is necessary.

Allogenic transplantation often causes graft-versus-host disease or rejection episodes. Tissue damage caused by graft-versus-host disease results in clinical manifestations that most commonly involve the skin, gut, liver, and immune and hematopoietic systems, and can include rash, diarrhea, bloody stools, jaundice and severe infections [31]. In this study, there was no change in these three systems or in the ocular appearances in any of the rabbits, with no edema or angiogenesis in the cornea and no uveal reaction or other signs of immunological rejection. This study indicated that hAM-dMSCs did not lead to host-versus-graft rejection in the rabbits, and was a safe procedure with no instances of mortality in these animals.

In conclusion, the ability of hAM-dMSCs to enhance epithelial healing and to reduce corneal opacification and neovascularization in inflammatory corneal wounds was investigated. With its properties of non-immunogenicity and a relatively low risk of infection, hAM-dMSCs may be a useful approach for symptomatic treatment associated with intractable inflammatory ocular surface disease such as chemical burns.

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References


