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Review

Corneal treatment with adipose derived stem cells

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***Purpose*: To evaluate the use of adipose tissue-derived stem cells (ADSC) from human lipoaspirate for the treatment of corneal damage in animal models. *Methods*: The study included data from experiments performed in rats and mice from 2009 to present in Italy and Spain, which are currently underway. Cornea lesion and histological experiments were performed to determine the best animal chemical burn. Rats underwent chemical burn lesions; and mouse eyes underwent laser-induced corneal lesions. Animals were randomly assigned to control or groups treated with stem cells, or blood plasma serum, or adipose tissue. Clinical and histological assessments were compared between groups to determine biosafety, re-epithelization, immunogenicity and efficacy of human derived ADSC in treating epithelial and stromal wounds in animal corneas. *Results*: Rat eyes treated with stem cells, or serum, or stem cells plus serum showed significantly smaller defect areas at each time point when compared with the control and adipose groups. The stem cell-treated eyes showed faster wound healing with smaller defect areas at each time point when compared to contralateral control eyes ($P<0.05$). Whole layer epithelium regeneration was observed in all stem cell-treated eyes. The epithelium of the stem cell-treated eyes closely resembled the native corneal epithelium. With regards to the mouse experiments, the fluorescein positive corneal lesion area was significantly smaller in the stem cell groups than the control eyes ($P<0.05$); on the first and the second day. Histological assessment indicated that epithelium from the stem cell-treated eyes was similar to uninjured epithelium, composed of 4 to 5 layers of uniform and perfectly structured epithelial cells. *Conclusion*: ADSC obtained from human lipoaspirate enhanced corneal wound healing in animal models.**

Keywords: corneal treatment, adipose derived stem cell; epithelization; lipoaspirate.

INTRODUCTION

The cornea is composed of three layers: epithelium, stroma and endothelium. The superficial layer is composed of non-keratinized stratified epithelium. The underlying stroma makes up most of the corneal thickness and is composed of keratocytes that produce the collagen fibrils organized in such a way to provide corneal transparency. The inner most portion is composed of a monolayer of endothelium cells, which is in contact with the aqueous humor.

Ocular surface damage can arise in patients with trauma, chemical or thermal burns, infection, contact lens use, autoimmune disease, etc. Transplantation involving one or more of the corneal layers is considered as the gold standard treatment; however, donor tissue is limiting, and surgical treatments tend to be invasive, expensive and sometimes lead to graft rejection. Other treatment options include conjunctival-limbal stem cell

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allografts from a donor or from the healthy fellow eye, amniotic membrane transplants and auto blood serum, however, success rates are limiting and complications can occur.

Cornea stromal cells have shown to be of mesenchymal stem cell (MSC) lineage (Arnalich-Montiel et al., 2008; Du et al., 2010; Du et al., 2009; De Miguel et al., 2010). Numerous studies have reported that adipose derived stem cells (ADSC) have multipotent MSC differentiation potential (Ambrose et al., 2010). These cells can be isolated from Processed Lipo Aspirate (PLA), which can be easily obtained from patient's abdomen in large quantities with minimal risks and easily processed (Schäffler and Büchler, 2007; Zuk et al., 2002). Arnalich-Montiel et al showed that transplanted human PLA cells into corneal stromal pockets can differentiate and act as functional corneal cells (Arnalich-Montiel et al., 2008). The anti-inflammatory and antiangiogenic role of MSC in corneal wound healing has been shown by Youn Ho et al (Oh et al., 2008).

Surgery can potentially be used in humans to deliver ADSC to the damaged corneal stroma, however, this may induce further iatrogenic damage to an already injured cornea, not to mention that it does not seem feasible or cost-effective. The purpose of our study was to assess the use of ADSC from human lipoaspirate applied topically to treat corneal damage in animal models.

MATERIAL AND METHODS

The study included data from experiments performed from 2009 to 2013 in Udine and Calabria (Italy) and Oviedo (Spain), which are still currently underway. The results from our preliminary study have been published (Zeppieri et al., 2013). In brief, cornea lesion and histological experiments were performed to determine the best animal chemical burn. With regards to the experiments performed in our Calabria labs, rats underwent chemical burn lesions and were randomly assigned to different treatment groups. Additional experiments were performed in Oviedo, Spain in mouse eyes using a laser induced corneal lesion model. Clinical and histological assessments were compared between groups to determine biosafety, re-epithelization, immunogenicity and efficacy of human derived ADSC in treating induced epithelial and stromal wounds in animal corneas.

Animals

Nineteen male Albino Wistar rats (280–330 g) and 40 black male mice C57BL/6 (30-40 g) were used in the experiments. Animal care and experiments were carried out in accordance with the European guidelines and approved by the Institutional Animal Care and Use Committee. Animals were anesthetized by intraperitoneal injection. Topical anesthesia was induced by 0.4% oxibuprocain eye drops. For histological studies, all animals were euthanized with an overdose of sodium pentobarbital and cervical dislocation. For the rat experiments, 6 animals were sacrificed after the chemical lesion for histology of corneal damage; the remaining 13 animals were sacrificed after 3 days to assess histology of the ocular surface lesion. For the mouse model, 16 of the 33 animals were sacrificed on day 3, while the remaining 17 on day 8. All rat and mice eyes were enucleated and fixed, then embedded in OCT compound.

Isolation and preparation of adipose derived stem cells

Adipose tissue was obtained from healthy patients (aged 27-62 years) undergoing elective lipoaspiration surgery. All patients gave informed oral and written consent, in accordance with the guidelines of the Tenets of the Declaration of Helsinki and approved by the Institutional Review Board (IRB) of the Hospital.

The ADSC were isolated from lipoaspirates and cultured in accordance to our previous studies (Beltrami et al., 2007; Ferro et al., 2011). Verification of the stemness nature was assessed on the basis of: mesenchymal surface immunophenotype; multipotency and the ability to differentiate into all three germ layers; and expression of the specific proteins Oct-4, Nanog and Sox2 (Beltrami et al., 2007; Ferro et al., 2011).

Blood serum

Human serum was prepared in accordance to protocol of our hospital in Udine. In brief, 500 ml of whole blood from one healthy young male donor (42 years old) was collected into sterile containers, which were left standing in an upright position to ensure clotting, then centrifuged. The supernatant serum was removed and the remaining serum was frozen until treatment use.

Chemical corneal wound in rats

After intraperitoneal and topical anesthesia, 19 rats were subject to corneal damage using a chemical burn model (Oh et al., 2008; Zeppieri et al., 2013; Ma et al., 2006). Based on histological results using different concentrations, a 3-mm diameter circular filter paper soaked in 0.2 NaOH was applied to the center of the cornea for 30 seconds (Figure 1) then quickly extensively irrigated with Hank's Balanced Salt Solution (HBSS).

Figure 1. A chemical corneal lesion was induced in rats prior to treatment. A 3mm diameter filter paper disk soaked in 0.2N NaOH was applied to the center of the cornea for 30 sec and then washed.

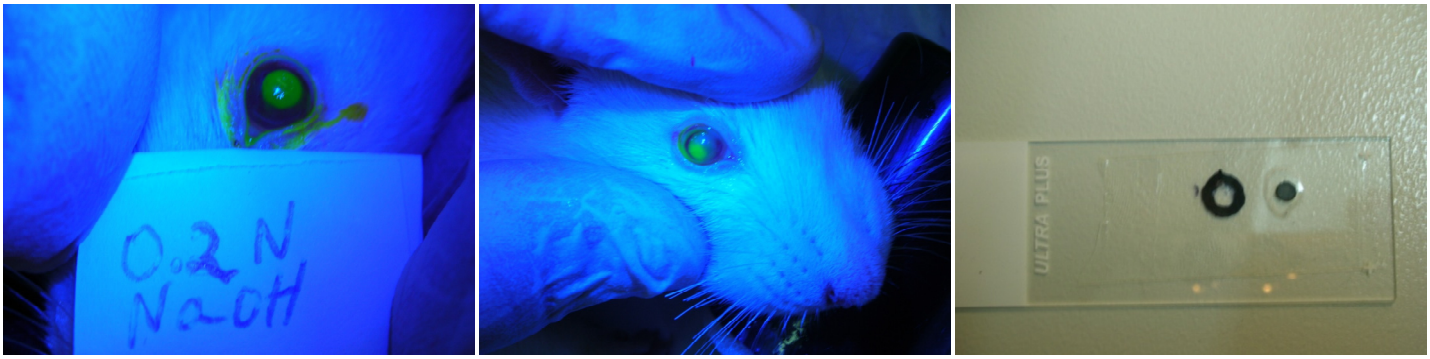

Figure 2. Masked graders determined the % fluorescein positive area of the lesion and time of complete re-epithelization at different time points after wound induction. The first figure shows a 100% fluorescein positive area at baseline. The middle figure shows a 70% fluorescein positive area after 20 hours. The last figure shows a transparent slide used for the comparison with the initial baseline lesion area.

Laser corneal wound in mice

After intraperitoneal and topical anesthesia, corneal lesions were performed on both eyes of 40 mice by laser induced photorefractive keratectomy (PRK) (Merayo-Lloves et al., 2010). The PRK ablation parameters included a diameter of 2 mm to the central optic zone and a total depth of 45 µm to induce a uniform lesion affecting both epithelium and stroma.

Treatment regimen

All animals were treated (prophylaxis) with topical eye drops of azythromycin 1.5% twice daily for 3 days after lesion (Merayo-Lloves et al., 2010). Rats were divided in five treatment groups: control, stem cells, serum, stem cells + serum, and adipose tissue. Control eyes received only antibiotic eye drops, while the other 4 groups also received topical treatment applied 3 times daily for 3 consecutive days. Stem cell topical eye drops were prepared daily with $1x10^5$ cells suspended in 25µL HBSS/treatment (Arnalich-Montiel et al., 2008; Zeppieri et al., 2013). The serum group received topical application of 25 µL human serum. For the studies in Spain, 80 eyes of 40 mice were divided in four treatment groups (n=20 eyes per group), which included: control, stem cells, basic serum, plasma rich in growth factor (PGRF, data not reported). Control eyes received only antibiotic eye drops, while the other groups also received topical treatment applied 3 times a day for 5 consecutive days.

Ocular surface evaluation in rat eyes

All eyes were examined with a stereo biomicroscope at 0, 20, 28, 45, 50 and 74 hours for the rat eyes; and at 0, 30, 54, 78, 100 and 172 hours after lesion for the mouse eyes. Topically applied fluorescein was used to evaluate the degree of the corneal epithelial defect. The defect size was determined by 2 masked graders, expressed as a semi-quantitative estimate of percentage of fluorescein positive area (Figure 2). The defect area was determined by the fluorescein positive remaining area under blue light (1mm=240 pixels) using ImageJ 1.45a software.

Statistical analysis

The Kolmogorov-Smirnov test was used to assess normality of the data distribution. Differences of the data amongst groups were analyzed using Kruskal-Wallis and Friedman test with the SPSS 20.0 program. Multiple

comparisons were performed with Dunnett's test. A P value of <0.05 was considered to be statistically significant.

RESULTS

The ADSC obtained from the human adipose tissue aspirates expressed the pluripotent state-specific transcription factors Oct-4, Nanog and Sox and were characterized by a mesenchymal stem cell immunophenotype (Zeppieri et al., 2013). The cultured cells displayed multipotency, being able to differentiate into mature cell types of all three germ layers (Zeppieri et al., 2013).

With regards to the chemical lesions in the rat eyes (Zeppieri et al., 2013), partial re-epithelization was seen in all rats at 20 hours, the first time point. Most eyes, with the exception to the 3 control eyes, were completely epithelized by 74 hours. The stem cell, serum, and stem cell+serum groups showed significantly smaller defect areas at each time point when compared with the control and adipose groups. Eyes treated with stem cells and/or serum showed complete re-epithelization within 50 hours. In animals #1-5, eyes were treated with stem cells on the right eye and control on the left. The stem cell treated eyes showed faster wound healing with smaller defect areas at each time point ($P<0.05$) (Zeppieri et al., 2013).

Qualitative histology assessment in the control eyes showed parakeratosis with epithelium discontinuation and ongoing epithelium regeneration, which was less apparent in the stem cell-treated eyes. Whole layer epithelium regeneration was observed in all stem cell-treated eyes. All control eyes showed mild inflammation with marked infiltration, while all other eyes showed no inflammation and little cellularity. The epithelium of the stem cell eyes closely resembled the native corneal epithelium (Zeppieri et al., 2013).

With regards to the mouse experiments, which are still ongoing, partial re-epithelization was seen in all mouse eyes at the first time point. All eyes were completely re-epithelized by 100 hours. After the first day, the fluorescein positive corneal lesion area was significantly smaller in the stem cell groups than the control eyes ($P<0.05$); on the second day, it was significantly larger in the controls, yet comparable between stem cell and serum treatment groups ($P<0.02$).

With regards to the epithelium histological assessment after 3 days, the stem cell group showed a slightly increased number of epithelial cell layers. The number of Ki67 cells in the peripheral epithelium was similar amongst groups, however, the stem cell-treated eyes showed less of these actively replicating cells in the epithelium, yet more in the stroma underlying the lesion when compared with the other groups. E-Cadherin protein accumulated in the basal cell layer of the epithelium and was present in the cytoplasm. The stem cell-treated eyes showed epithelium that was similar to uninjured epithelium, composed of 4 to 5 layers of uniform and perfectly structured epithelial.

DISCUSSION

Our preliminary study showed that ADSC obtained from human processed lipoaspirate enhanced corneal wound healing after chemical burn and laser ablation when compared to traditional topical therapy. The fluorescein positive areas in eyes treated with stem cells were significantly smaller at each time point in both the mice and rat models when compared to the control eyes. Stem cell treated eyes reached complete epithelium closure faster than the control and adipose treated eyes

A case report has been published a few years ago involving the topical application of autologous ADSC obtained by lipoaspiration in treating a persistent sterile corneal ulcer (Agorogiannis et al., 2011). Corneal healing was observed in less than 2 weeks, and corneal transplantation surgery was no longer needed. Our study adds to literature in this new field of research involving enhanced corneal healing with ADSC by providing promising preliminary results in animal models and histological assessments. Our study confirmed the biosafety, immunogenicity and efficacy of stem cells, which proved to be comparable to the serum treatment and better than traditional topical treatments currently used in clinics. Extensive preliminary studies are of utmost importance before nonconventional treatments can be applied in humans.

In conclusion, our preliminary study is limited by the brief period time for treatment and clinical assessment and small number of animals considered. The literature available regarding the use of ADSC in corneal wound healing is very limited, thus extensive prior studies are needed to provide the groundwork and experimental basis in this field. Studies are presently underway in our lab, which involve a greater number of eyes assessed over a longer time period, involving the comparison of epithelial recovery, inflammation, corneal haze, and quantitative histological assessments between the different treatment arms. Although the exact mechanisms underlying the beneficial effects of this cell therapy are unknown, our preliminary results suggest that the topical application of ADSC may enhance corneal epithelial wound healing. The clinical use of multipotent ADSC looks promising, especially considering the easy access and abundance of autologous tissue, relatively low health costs involved, and potentially enhanced corneal epithelium and stromal wound healing.

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