Mesenchymal Stem Cells and Exosomes in Corneal Diseases

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The corneal functions (transparency, refractivity and mechanical strength) deteriorate in many corneal diseases but can be restored after corneal transplantation (penetrating and lamellar keratoplasties). Various studies have shown the differentiation of multipotent mesenchymal stem cells (MSCs) into various corneal cell types. With the unique properties of immunomodulation, anti-angiogenesis and anti-inflammation, they offer the advantages in corneal reconstruction. These effects are widely mediated by MSC differentiation and paracrine signaling via exosomes. Besides the cell-free nature of exosomes in circumventing the problems of cell-fate control and tumorigenesis, the vesicle content can be genetically modified for optimal therapeutic affinity.

mesenchymal stem cells exosomes corneal regeneration

1. Introduction

The human adult cornea is about 550 μm thick, comprising of five layers. The outermost layer is the corneal epithelium, followed by the Bowman's membrane, corneal stroma, Descemet's membrane and the innermost corneal endothelium (**Figure 1**) [1]. The cornea serves three functions: (1) as a mechanical and chemical barrier protecting inner ocular tissue, (2) as a transparent medium to allow light transmission and (3) light refraction (it provides about 70% of the eye's refractive power) [2]. Light passes through the cornea and reaches the retina for transduction into neural impulses. Hence the clarity of cornea enables uninterrupted passage of light to the retina.

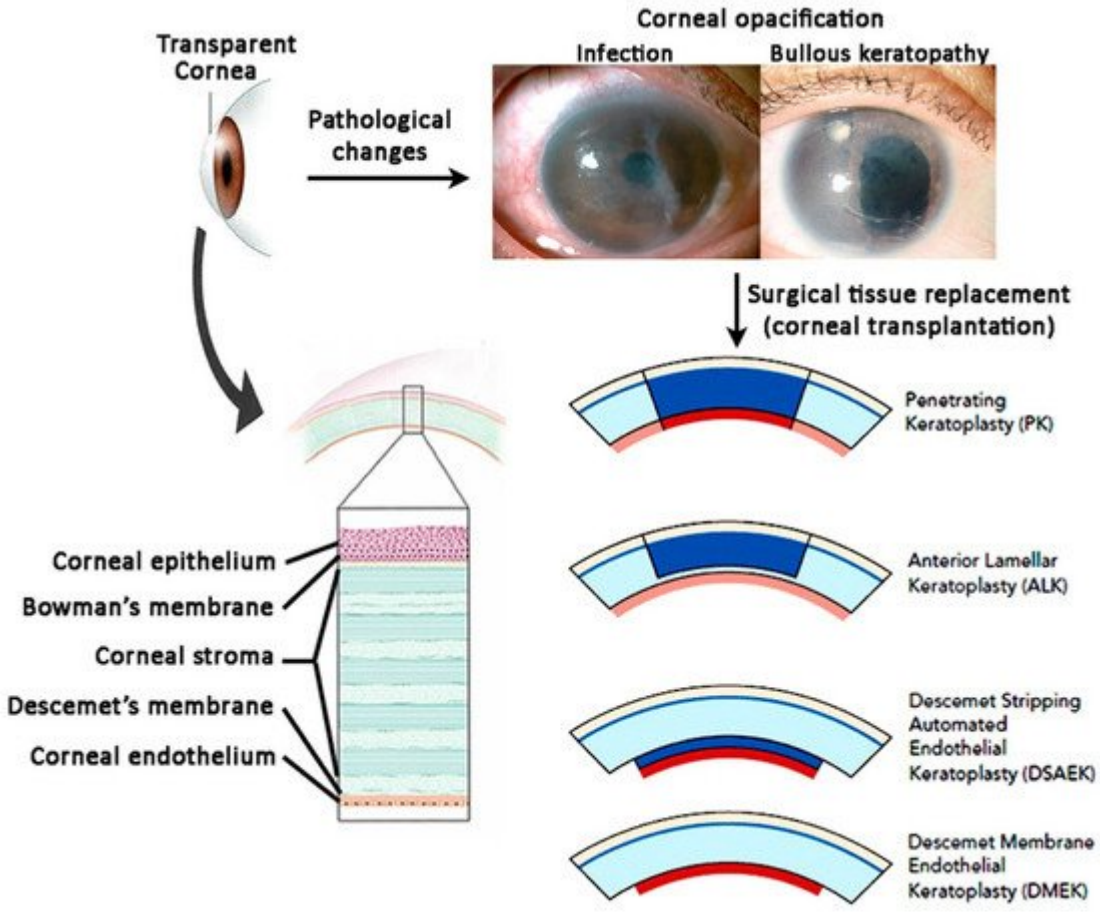

Figure 1. Overview of human cornea and its pathological opacification. Transparent cornea is composed of corneal epithelium, Bowman's membrane, corneal stroma, Descemet's membrane and corneal endothelium. Corneal pathologies (e.g., infection, ulcer, injuries) lead to corneal opacification, which is conventionally treated by surgical removal and donor corneal transplantation (penetrating and lamellar keratoplasties).

Loss of vision is a global burden. The number of visually impaired people of all ages is estimated to be 285 million worldwide, with 39 million blind (Global Data on Visual Impairment 2010, World Health Organization) [3]. These patients lose their independence and usually have a poor quality of life. Corneal diseases are a leading cause of visual loss, affecting more than 10 million people. This can be caused by several clinical conditions, including traumatic injury, chemical burns (acid and alkali injury), infections, iatrogenic causes, i.e., limbal stem cell deficiency, age-related degeneration, and corneal dystrophies (**Figure 1**). All of these conditions can cause defective changes to the cellular and structural components of the cornea [4]. The formation of corneal scars, haze and opacities, as well as corneal edema compromises corneal functions, causing visual deterioration. However, the majority of corneal blindness is preventable, if treated in a timely way. Many patients in under-developed and developing countries have poor access to healthcare and these diseases are often left untreated. The current treatment option of corneal blindness is corneal transplantation, to replace the damaged cornea with a healthy donor cornea (**Figure 1**). Despite the significant advances in corneal surgery over the past decade, there are issues related to the availability of donor tissue, limited allograft survival, long-term use of immunosuppressants and the need for surgical expertise [5][6]. Many patients do not have access to corneal transplantation due to high surgical and rehabilitation costs. These represent significant financial and logistic burdens, particularly in view of

our aging population. It has been estimated that the direct annual health cost due to corneal blindness is more than US$11,000 per person in 2010 in developed countries (data from Canadian Blood Service 2010 Cost Benefit Analysis: Corneal Transplantation; https://blood.ca/sites/default/files/otdt-indx-final-c2a.pdf). Unfortunately, no cost estimate can be made for the developing countries, but the socio-economic burden is expected to be higher.

Corneal transplantation is the most frequent type of transplantation worldwide and about 180,000 corneal transplants are performed annually [7]. Although the total number of donated eye globes/corneas has been increasing in recent years (there was a rise of 5.2% in 2013 compared to 2012, Eye Bank Association of America), the demand always outstrips the availability of transplantable donor tissue [6]. The global population is expected to rise by 113% in 2030 (and 122% by 2050) and life expectancy will increase at 0.07% annual rate (data from Department of Economic and Social Affairs, UN; https://www.who.int/blindness/data_maps/VIFACTSHEETGLODAT2010full.pdf). As the population lives longer, the demand for corneal transplants will undoubtedly increase, particularly if there is no disruptive treatment technology. This problem will be further exacerbated by the increased global prevalence of diabetes and systemic diseases, which can contribute to increased graft rejection and failure. Hence, alternative solutions, such as regenerative cell-based therapy, should be explored [8].

The cornea is an ideal organ for regenerative cell therapy, due to its immune-privilege and avascular nature [9]. The transplanted cells are not as likely to be rejected as in other tissues or organs. Mesenchymal stem cells (MSCs) with regenerative and differentiation capabilities have received much attention among ophthalmologists and visual scientists as an alternative modality in the management of corneal diseases. The paracrine effect of MSCs, mediated by exosomes, has also been suggested for their therapeutic effect. The cell-free nature of exosomes has gained particular interest with respect to its safety.

2. Mesenchymal Stem Cells (MSCs)

MSCs are a population of proliferative and multipotent stem cells present in various tissues throughout development. Human fetal MSCs have been found in different fetal tissues, including first-trimester blood and bone marrow [10], or from extraembryonic tissues (placenta, umbilical cord and amniotic fluid) [11]. In adult tissues, MSCs have been isolated from bone marrow, peripheral blood, adipose tissue, dermis, synovium, periosteum, cartilage, skeletal muscle, fallopian tubes, menstrual blood, gingiva and dental tissue, as well as in the eye (such as corneal stroma and trabecular meshwork) [8][12][13]. In general, fetal MSCs contain more primitive phenotypes than those from adult tissues, such as longer and more active telomeres and greater propagation capacity [14]. However, they often require vigorous ex vivo expansion in order to achieve sufficient numbers for therapeutic use and this can lead to replicative senescence and functional decline [15]. Hence, in vitro protocols have been developed to derive MSCs from human pluripotent stem cells, including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [16][17]. A summary of MSC sources is shown in **Figure 2**. According to the criteria proposed by The International Society for Cellular Therapy (ISCT), MSCs can be enriched via their plastic adherence, cell surface expression of CD73 (5'-nucleotidase), CD90 (Thy1) and CD105 (endoglin); and negative detection of CD34, CD45, HLA-DR, CD14 and CD11b (integrin αM chain) expression [18]. MSCs also exhibit differentiation potential into

various types of mesenchymal lineages, such as osteoblasts, adipocytes and chondrocytes, both in vitro under defined conditions and in vivo [19]. They possess the ability to proliferate and migrate to the injury sites, and promote wound healing by secreting anti-inflammatory and growth factors [20]. They also interact with innate and acquired immune cells and modulate immune response via paracrine action [21][22].

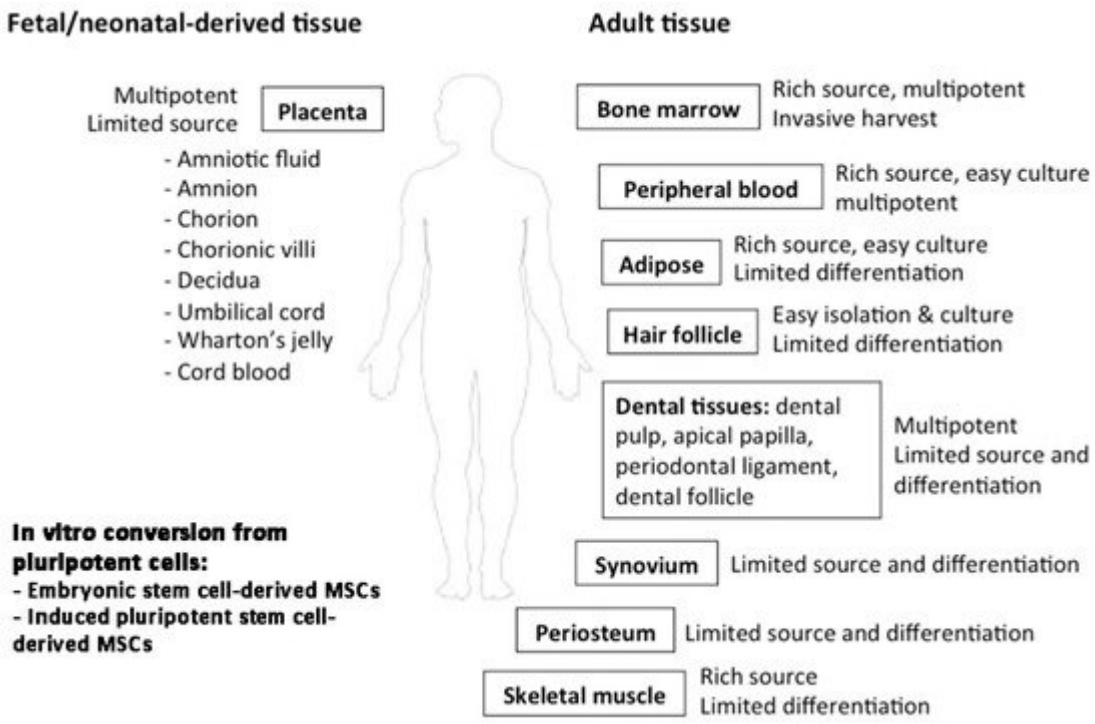

Figure 2. Major MSC sources in human tissues. MSCs can be harvested from (1) fetal/neonatal birth-associated tissues, including placenta (amnion, chorion, decidua), umbilical cord and cord blood; (2) adult tissues, including the rich source of bone marrow, peripheral blood, adipose tissue and limited source from hair follicle, dental tissue, skeletal muscle, etc.; and (3) in vitro conversion from pluripotent cells.

However, differences have been identified among MSCs from various tissues. In terms of phenotypic markers, bone marrow and adipose MSCs were found to express CD13, 73, 90, 105 and STRO-1, but had different expression of CD34, 49d, 54 and 106 [23][24]. Besides the different tissue origin, the phenotypic variation could also be attributed to the different isolation methods and propagation media and conditions. MSCs obtained from birth-related tissues, including placenta, amnion, umbilical cord and cord blood have greater proliferative and engraftment capacity as well as differentiation potential than MSCs from adult tissues, such as bone marrow [25][26]. Within the umbilical cord tissue, MSCs isolated from whole umbilical cord, from Wharton's jelly or from cord blood revealed different proteomic profiles, and the umbilical cord MSCs displayed better differentiation for musculoskeletal tissue engineering [27][28]. In addition, MSCs from umbilical cord exhibited higher proliferation capacity than from bone marrow [29]. Adult adipose MSCs also displayed variations with regards to different origins [30]. MSC from subcutaneous fat tissue proliferated faster than those from the omental region [31]. Early senescence of bone marrow MSCs was also detected while adipose MSCs displayed the associated signs at later passages [32].

3. MSC Mobilization, Migration and Homing in Corneal Changes

Stem cell mobilization, migration and colonization can be induced by injury and inflammation [20][33]. Upon corneal injury, such as trauma and infection, the endogenous bone marrow MSCs are triggered by specific chemo-attractants to mobilize into the peripheral blood. These circulating MSCs migrate to the injury site in the cornea and engraft to promote wound healing [34]. Ye et al. reported the migration and engraftment of intravenously administered bone marrow MSCs in the injured corneal tissue of a murine alkali-burn model [35]. Among chemokines, SDF-1 and substance P have been shown to regulate MSC mobilization and recruitment to the cornea [34]. Moreover, selectin and integrin-mediated leukocyte-like cell adhesion, transmigration and passive entrapment, are potent mechanisms through which MSCs home to tissues [36]. However, the efficiency of MSC homing and engraftment is generally low, owing to the first-pass retention in the lung, liver, kidneys and spleen after systemic administration [33][37]. Much effort has been made to improve MSC migration to target ocular tissues. Administering MSCs through sub-conjunctival injection and co-transplantation on amnion have been shown to improve the local concentrations of MSCs in injured corneal tissue [38][39][40][41]. This potentiates the MSC effect and reduces the application dosage. However, MSC migration and homing seem not necessary for the treatment to take effect. Roddy et al. demonstrated that systemically administered MSCs released TSG-6 (tumor necrosis factor-stimulated gene/protein 6) to reduce corneal inflammatory damage without MSC engraftment [42]. Similarly, the distal effects of MSC have been shown to decrease post-myocardial infarction inflammation and improve cardiac function, through TSG-6 secretion from MSCs embolized in the lung after intravenous delivery [43].

4. Application of MSC-Exo on Ocular Tissues

Several studies have evaluated the effect of MSC-Exo on ocular tissues. The periocular injection of human umbilical cord MSC-Exo to a rat experimental autoimmune uveitis (EAU) model decreased the infiltration and migration of leukocytes, macrophages and natural killer cells by downregulating MCP1/CCL21 and MYD88-dependent pathways [44]. These cells expressing Gr-1, CD68, CD161, CD4, IFNγ and IL17, and restored retinal function. Yu et al. also reported that intravitreal injection of exosomes from umbilical or adipose MSC cultures modified the pro-inflammatory milieu in laser-induced retinal injury, by inhibiting MCP1, ICAM-1 (intercellular adhesion molecule-1) and TNFα, hence improving visual function [45]. In a study by Zhang et al., intravitreal injection of human umbilical cord MSC-Exo improved hyperglycemia-induced retinal inflammation in diabetic rats, by transferring miR-126, which suppressed HMGB1 (high-mobility group box 1) signaling [46]. In another rat model of blue-light induced retinal damage, intravitreal umbilical cord MSC-Exo injection showed a dose-dependent suppression of choroidal neovascularization by downregulating VEGFA and inhibiting NFκB pathway, possibly through miR-16 transfer [47][48]. In addition, intravitreal injection of bone marrow MSC-Exo to a rat optic nerve crush model stimulated retinal ganglion cell growth through argonaute-2 signaling, which stabilized miR-16 activity from RNase digestion [49][50]. Since intravenous MSC administration caused similar restoration of retinal functions in EAU and laser-induced retinal injury models [51][52], it is conceivable that the therapeutic effects could be mediated through MSC-Exo action. In a recent clinical trial of five patients with refractory macular holes, the intravitreal

injection of human umbilical cord MSC-Exo promoted anatomical and functional recovery; however, one patient experienced an inflammatory reaction [53].

5. MSC-Exo Application on Corneal Tissues

Corneal wounds caused by chemical or thermal burns, traumatic injury, and/or immune and hereditary disorders are associated with inflammation, neovascularization, ulceration and scarring. Improper or delayed treatment may lead to blindness. Over the last decade, MSC therapy has been proposed as a possible treatment strategy for anti-inflammation, anti-angiogenesis and immunomodulatory activities. The paracrine action of MSCs has been shown to facilitate tissue wound repair and suppress inflammation and angiogenesis in several tissue models. This cell-free strategy may also exert a significant impact in promoting corneal wound repair, which involves the participation of different factors that modulate inflammation, angiogenesis and tissue regeneration. A few studies have demonstrated the therapeutic functions of soluble factors present in MSC-Exo on corneal wound models in vitro and in vivo. Rabbit corneal stromal cells, when cultured in the presence of rabbit adipose MSC-Exo, showed greater proliferation with less apoptosis, along with the deposition of new ECM proteins (including collagen) [54]. In a murine superficial stromal wound model, topical CSSC-derived exosomes suppressed corneal inflammation and corneal scarring by inhibiting neutrophil infiltration through TSG-6 dependent pathway and downregulated fibrotic markers, including tenascin-C, ACTA2, Col3A1 and SPARC [55]. Moreover, murine corneal epithelial wound healing was promoted by exosomes from human corneal mesenchymal stromal cells [56]. Umbilical cord MSC-Exo carrying β-glucuronidase reduced the accumulated glycosaminoglycans of a mouse mucopolysaccharidosis model, thereby reducing corneal haze [41]. These data have highlighted the potential of the therapeutic use of MSC-Exo in ocular surface diseases and congenital corneal metabolic disorders.

6. The Cargo of MSC-Exo and Potential Mechanisms for Therapeutic Effects

Several proteomic studies have shown that MSC-Exo contain several hundreds to thousands of proteins, depending on the parental MSC type [57][58]. The cell-type-associated abundance and composition of exosomal proteins have been demonstrated. Neprilysin, a β-amyloid-degrading enzyme, was expressed at four-fold higher levels in adipose MSC-Exo than in bone marrow MSC-Exo [59]. The application of exosomes from umbilical cord MSCs and bone marrow MSCs promoted U87MG glioblastoma cell apoptosis, while adipose MSC-Exo supported cell growth [60]. Hence, exosomes from different MSC types are not equivalent, and their activity must be fully identified before considering their therapeutic applications. Indeed, much is still unknown with regard to which components of the exosomal cargo are responsible for the various observed therapeutic effects.

More recently, the importance of miRNAs in MSC-Exo have been described. The exosomal cargo contains a variety of miRNAs, which regulate various events related to angiogenic, inflammatory and neurogenic processes [61][62]. The miRNA profiling work has suggested that several miRNAs in exosomes could serve as a tool to qualify cultured human corneal endothelial cells for cell injection therapy [63]. Reduced miRNA content in exosomes has

been described by the knockdown of Alix protein in CSSCs, without any changes in the levels of cellular miRNAs. When these exosomes with reduced miRNAs were topically applied to a murine superficial stromal wound model, they failed to suppress stromal scarring and inflammation, while clear corneas were observed with control exosomes without Alix knockdown [55]; Funderburgh et al. Association for Research in Vision and Ophthalmology (ARVO) 2019 Annual Meeting]. These results support the hypothesis that exosomal-related functions could be mediated through miRNAs. Whether any particular individual or groups of miRNAs are engaged in specific tissue functions need to be explored. A previous study has also shown that MSC-Exo carrying miR-16 downregulated VEGF and suppressed angiogenesis in a breast cancer model [47]. Recently, Fafian-Labora et al. reported the age-related reduced expression of miR-21-5p in bone marrow MSC-Exo (significantly higher in pre-pubertal group than in adults) and this could be associated to age-dependent differences in MSC immune profile through Toll-like receptor 4-mediated signaling [64].

7. Storage of Exosomes

There is currently no established protocol for the storage of exosomes. It is imperative to consider the effects of freeze-thaw, storage temperature and buffer on the stability of exosomes in terms of the lipid membrane integrity and therapeutic properties. There has been a mixed outcome regarding the stability of exosomes in sub-zero storage. One study has shown that −20 °C freeze-thaw cycles in PBS did not affect the size of MSC-Exo or impair the exosomal membrane integrity, but there was a significant reduction in vesicle size after two days of storage at 37 °C and after three days at 4 °C [65]. Webber et al. further added that the exosomes, frozen at −20 °C for as long as six months, did not alter the biochemical activity [66]. A study on neutrophil-derived exosomes, however, showed a reduction in vesicle size at −20 °C storage, but not at −80 °C for four weeks [67]. The addition of protease inhibitors before freezing of urinary exosomes at −20 °C did not prevent the loss of exosomal biochemical activity, while freezing at −80 °C resulted in near complete recovery of activity even after seven months of storage [68]. In a more recent study, Bosch et al. demonstrated that the addition of 25mM of trehalose could further improve the exosomal membrane and biochemical function stability [69]. These studies have demonstrated that exosomes can be stored long-term at an extremely low temperature with high recovery without compromising the bioactivity. The possibility to lyophilize exosomes in the presence of trehalose to allow storage stability of the vesicles at room temperature provides ease of handling and transport [70]. As a whole, unlike stem cells, which are sensitive to the storage conditions and can exhibit impaired therapeutic properties as a result of freeze-thaw cycles, improper storage temperature and absence of cryopreservative, exosomes are substantially more amenable to storage and transport, a key consideration from a translational point of view [71].

8. Sustained Delivery of Exosomes

The intended biological effects of exosomes can only be produced as a result of the vesicle uptake by target cells via an endocytosis pathway [72]; therefore, the ability to deliver exosomes locally in a sustained release manner is crucial to maximizing their therapeutic application. Studies have shown that direct intravenous, intraperitoneal or subcutaneous injection of exosomes, resulted in rapid clearance from the blood circulation and accumulation in the

liver, spleen, lung and gastrointestinal tract [73][74]. Regardless of the delivery route and cell source, the majority of systemically injected exosomes are rapidly taken up by macrophages in the reticuloendothelial system and ejected from the body [75]. Although the fate of exosomes following a subconjunctival injection or a topical application in the anterior portion of the eye has not been explored, we can assume that similar rapid clearance of these nanovesicles would also occur. Topical use of drugs on the ocular surface has always resulted in low drug bioavailability due to the presence of epithelial barrier and rapid tear turnover [76]. To compensate for this, it is often necessary to increase the dosage of drug, which requires further optimization. Alternatively, epithelial debridement to improve drug penetration could be carried out, but this increases the susceptibility of the ocular surface to external microbes.

Another problem that further advocates the need for a sustained delivery system of exosomes is the difficulty in producing the vesicles not only on a large scale but also in high purity and consistent quality. The yield of exosome is typically less than 1 µg of exosomal protein from 1 mL of culture medium, whereas the application dose of exosomes is 10–100 µg of protein [77]. In humans, the effective dose could be in an order of magnitude or more to compensate for the rapid clearance of exosomes from the body. A delivery vehicle, like a hydrogel implant, could be used to load exosomes and prolong the intended therapeutic effect over time (**Figure 5**). The implant will prevent the encapsulated exosomes from being cleared too rapidly, and also enable the possibility to administer a more localized and concentrated dosage by inserting the implant in the proximity of target sites.

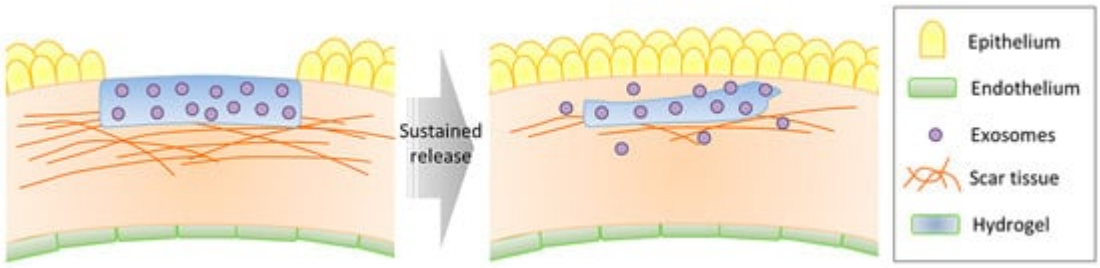

Figure 5. Illustration of sustained delivery of exosomes for the treatment of corneal scarring and neovascularization. The biodegradable hydrogel, placed on the bare stroma, provides first-line protection for the encapsulated exosomes from proteolytic degradation and allows sustained release of the exosomes as it gradually degrades over a period of time.

Research into encapsulating exosomes in a delivery vehicle is still in its infancy; hence, we have reviewed materials used to encapsulate exosomes that are derived not only from the MSC culture but also from blood plasma. The same methods or materials to encapsulate exosomes could be applicable regardless of the cell source. Qin et al. was the first to describe the idea of encapsulating exosomes in a hydrogel [78]. They used HyStem®-HP hydrogel, which is a thiol-crosslinked composite containing hyaluronic acid, heparin and gelatin, to load bone marrow MSC-Exo to promote bone regeneration. In addition, sodium alginate hydrogel loaded with exosomes isolated from platelet-rich plasma showed a sustained release of exosomes, resulting in a more efficient skin wound healing in the diabetic rat model, compared to the direct application of exosomes on the wound [79]. In a separate study of rat diabetic skin wound model, chitosan hydrogel loaded with exosomes derived from miR-125-

3p-overexpressing synovial MSCs improved the healing response [80]. Patching of exosome-loaded hydrogel over the skin wound revealed faster healing rate, compared to untreated and sham-treated with hydrogel only. For application on the cornea, a biodegradable film/hydrogel loaded with exosomes can be used as a patch over the bare stroma. These biomaterials can be tuned to degrade as the epithelial cells heal over the bare stroma so as not to impede vision for a prolonged period. If intact corneal epithelium has to be preserved from the onset of treatment, the exosomes-loaded film/hydrogel can be implanted intrastromally.

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